Localization and Regulation of the N Terminal Splice Variant of PGC-1α in Adult Skeletal Muscle Fibers

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The transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) regulates expression of genes for metabolism and muscle fiber type. Recently, a novel splice variant of PGC-1α (NT-PGC-1α, amino acids 1–270) was cloned and found to be expressed in muscle. Here we use Flag-tagged NT-PGC-1α to examine the subcellular localization and regulation of NT-PGC-1α in skeletal muscle fibers. Flag-NT-PGC-1α is located predominantly in the myoplasm. Nuclear NT-PGC-1α can be increased by activation of protein kinase A. Activation of p38 MAPK by muscle activity or of AMPK had no effect on the subcellular distribution of NT-PGC-1α. Inhibition of CRM1-mediated export only caused relatively slow nuclear accumulation of NT-PGC-1α, indicating that nuclear export of NT-PGC-1α may be mediated by both CRM1-dependent and -independent pathways. Together these results suggest that the regulation of NT-PGC-1α in muscle fibers may be very different from that of the full-length PGC-1α, which is exclusively nuclear.

1. Introduction

The transcriptional coactivator peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) containing 797 amino acids (full-length) was first determined to be a cold inducible factor in a differentiated brown fat cell line [1]. Subsequent studies have shown that PGC-1α plays important roles in mitochondrial biogenesis via coactivating several transcription factors such as nuclear respiratory factors and peroxisome proliferator-activated receptors [1–3]. PGC-1α has also been found to mediate fast-to-slow skeletal muscle fiber-type transformation through coactivation of MEF2 [2, 4]. Exercise/muscle contraction has been found to increase PGC-1α mRNA and protein levels [5, 6]. Both the activity and expression of PGC-1α appear to be regulated by p38 MAPK, which phosphorylates PGC-1α on Thr-262, Ser-265, and Thr-298 and increases its stability [7–9]. In addition, PGC-1α expression is also modulated by AMPK-dependent phosphorylation at residues Thr-177 and Ser-570 [10, 11].

Recently, a novel biologically active 270-aa isoform of PGC-1α (NT-PGC-1α) was cloned by Zhang and his colleagues [12]. NT-PGC-1α is produced by alternative splicing of the full-length PGC-1α through a premature stop codon between exons 6 and 7, generating a truncated N-terminal form of PGC-1α containing the first 267 amino acids of full-length PGC-1α and 3 additional amino acids from the insertion [12]. Two of the three p38 MAPK phosphorylation sites (Thr-262, Ser-265) and one of the two AMPK phosphorylation sites (Thr-177) of full-length PGC-1α are present in NT-PGC-1α. Although lacking the nuclear localization signals present in the full-length PGC-1α, NT-PGC-1α has a predicted size only about 30 kDa which should allow it to be freely diffusible between nucleus and cytosol via the nuclear pores [12]. However, recent studies found that NT-PGC-1α is predominantly located in cytoplasm [12–14] and that the subcellular localization of NT-PGC-1α is dynamically regulated by protein kinase A (PKA), which phosphorylates NT-PGC-1α at residues Ser-194, Ser-241, and Thr-256 [13]. In adipocytes and in Chinese hamster ovary K1 (CHO-K1) cells, inhibition of CRM1 increased the nuclear content of NT-PGC-1α [12, 13]. Although NT-PGC-1α is found to be expressed in many tissues where PGC-1α plays important roles, including the skeletal muscle [12], no reports of the subcellular
distribution and regulation of NT-PGC-1α in skeletal muscle have been published. Therefore, in the present study, we use Flag-tagged NT-PGC-1α to examine the subcellular distribution of NT-PGC-1α in skeletal muscle fibers under control conditions and to demonstrate that the subcellular localization of NT-PGC-1α in muscle fibers is dynamically regulated by PKA and by CRM1-dependent nuclear export, but not by p38 MAPK, or AMPK.

2. Materials and Methods

2.1. Materials. dbcAMP (N6, 2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate sodium) and monoclonal anti-Flag antibody were from Sigma (Saint Louis, MO). AICAR (5-aminomimidazole-4-carboxamide-1-β-D-riboside), anti-p38 MAPK, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-AMPK, and anti-phospho-AMPK (Thr172) antibodies were purchased from Cell Signaling Technology (Boston, MA). Leptomycin B was obtained from LC Laboratories (Woburn, MA). The expression plasmid of GFP-PGC-1α cDNA was obtained from Addgene (Cambridge, MA).

2.2. FDB Muscle Fiber Cultures. Experiments were carried out on skeletal muscle fibers obtained from 4–6-week-old CD-1 mice. Experimental protocols were approved by the University of Maryland Institutional Animal Care and Use Committee. Mice were euthanized and single muscle fibers were enzymatically dissociated from isolated flexor digitorum brevis (FDB) muscles. FDB muscle fibers were plated on laminin-coated glass coverslip floors glued over 1 cm holes in 35 mm plastic Petri dishes and cultured in MEM containing 10% fetal bovine serum and 25 μg/mL gentamicin sulfate in 5% CO2 at 37°C as previously described [15].

2.3. Adenovirus Infection. The recombinant adenovirus containing the Flag-tagged NT-PGC-1α (Flag-NT-PGC-1α) [13] was kindly provided by Dr. Thomas W. Gettys (Pennington Biomedical Research Center, LA). The constructions of recombinant adenoviruses of NFATc1-GFP and nuclear targeted Flag-NFATc1 were described in detail previously [16]. All viral constructs were confirmed by sequencing the viral DNA. Infection of muscle fiber cultures by recombinant adenoviruses was carried out about 20 h after the fibers were plated as previously described [16]. After 48 hours of viral infection, muscle fibers expressing Flag-NT-PGC-1α were either electrically stimulated or treated pharmacologically. Fiber electrical stimulation in culture was accomplished by mounting stainless steel electrodes in the floor of the culture dish. These electrodes were connected to a stimulator to give field stimulation. 28 V, 1 ms pulses produce visible fiber twitches and relaxation between stimuli without fiber detachment from the glass coverslip. After treatment, fibers were fixed immediately for immunostaining.

2.4. Fluorescence Immunocytochemistry. After electrical stimulation or pharmacological treatment, FDB muscle fibers were immediately fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton-X-100 in PBS for 20 min. Nonspecific binding sites were blocked by incubation with 5% normal serum. Immunostaining for Flag-tagged NT-PGC-1α was performed using monoclonal anti-Flag antibody (1:1000) followed by incubation with the fluorescent protein-conjugated secondary antibody. The fluorescence of the stained fibers was visualized using a confocal laser scanning imaging system (Olympus FLUOVIEW 500) using constant settings of laser intensity and photomultiplier gain. In each fiber image the mean pixel background fluorescence (off the fiber) was determined and subtracted from each pixel throughout the image. The average pixel fluorescence within user specified areas of interest (AOI) over the whole nucleus or the cytoplasm in each image was quantified by software custom-written in the IDL programming language (Research Systems, Boulder, CO), similar as we used in our previous study [17], and the
ratio of nuclear to cytoplasmic mean pixel fluorescence was then calculated for each nucleus, which should correct for fiber to fiber differences in level of expression of Flag-NT-PGC-1α or differences in overall staining level.

2.5. Western Blot Analysis. Cultured FDB muscle fibers were lysed with mammalian protein extraction reagent (Thermo Scientific, Rockford, IL) plus tablets of tissue protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and passed through a 21 gauge syringe 10 times. The lysates were subjected to centrifugation at 10,000 g for 10 min at 4°C. The supernatant was extracted and stored. Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA). 30 μg protein from each sample was fractionated by NuPAGE 3–8% Tris-Acetate Gel (Invitrogen, Carlsbad, CA) and transferred to PVDF membranes. Blots were probed with the appropriate primary and secondary antibodies and then visualized by Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL) and exposed to X ray film (Amersham Hyperfilm, GE Healthcare, Piscataway, NJ). Film was scanned and band intensity was quantified by Image J software (National Institutes of Health, Bethesda, MD) after subtracting neighboring background intensity.

2.6. Statistics. Student’s t-tests were used for comparisons of data obtained from two experimental conditions, and differences were considered significant if P < 0.05.

3. Results and Discussion

3.1. Subcellular Distribution of Flag-NT-PGC-1α in Resting FDB Fibers. Using Flag-tagged NT-PGC-1α expressed by adenoviral infection in cultured adult FDB skeletal muscle fibers, we found that Flag-NT-PGC-1α is predominantly located in the myoplasm (Figure 1(a)), consistent with the recently described cytoplasmic localization of Flag-NT-PGC-1α in adipocytes and CHO-K1 cells [12, 13]. Quantification of the mean pixel fluorescence in nuclear and cytoplasmic AOIs of 120 randomly selected resting fibers stained with anti-Flag antibody showed that the average value of nuclear/cytoplasmic mean pixel fluorescence ratio (n/c fluorescence ratio) was 0.63 ± 0.01. This is in contrast to the distribution pattern of expressed full-length GFP-PGC-1α fusion protein, which is predominantly located in the muscle fiber nuclei (Figure 1(b); n/c fluorescence ratio: 9.53 ± 2.86) as previously seen in COS cells [1]. As negative controls, fibers either not exposed to Flag-NT-PGC-1α viral infection but stained with anti-Flag antibody or fibers with
Flag-NT-PGC-1α viral infection but without incubation with anti-Flag antibody show no evident fluorescence (data not shown), indicating the specificity of the anti-Flag antibody used. Finally, as a positive control for anti-Flag antibody accessibility to muscle fiber nuclei, we transfected muscle fibers with nuclear targeted Flag-tagged NFATc1 and found strong nuclear staining with anti-Flag antibody (Figure 1(c)), thus establishing nuclear penetration of the anti-Flag antibody.

3.2. Activation of PKA Increases Nuclear Flag-NT-PGC-1α. Recent studies in adipocytes and CHO-K1 cells have shown that activation of PKA can significantly increase the nuclear content of NT-PGC-1α [12, 13]. To test whether this regulation mechanism also exists in skeletal muscle fibers, we treated muscle fibers with 1 mM dbcAMP for 1 h in culture medium in the tissue culture incubator, as previously used to activate PKA in CHO-K1 cells [13]. Our results show that the Flag-NT-PGC-1α fluorescent staining was stronger in nuclei of fibers exposed to dbcAMP (Figure 2(a), bdcAMP) than in control fibers (Figure 2(a), control). The Flag-NT-PGC-1α n/c fluorescence ratio was significantly increased from \(0.71 \pm 0.03\) in control to \(0.98 \pm 0.07\) in dbcAMP-treated fibers (Figure 2(b); \(P < 0.05\)), a 1.4-fold increase. The nuclear increase of Flag-NT-PGC-1α after 1 h treatment of dbcAMP found here in skeletal muscle fibers is very close to what has been previously obtained in CHO-K1 cells (about 1.4 fold) and in differentiated adipocytes (about 1.5 fold) [13]. Together, these results support the notion that PKA regulates the subcellular distribution of NT-PGC-1α and is thereby involved in modulation of the NT-PGC-1α-dependent signaling pathway in muscle fibers. In brown adipocytes, it has been shown that PKA-dependent regulation of nuclear content of NT-PGC-1α plays a role in the transcriptional activation of UCP1 and CIDEA [13], but the downstream signaling pathway of NT-PGC-1α in skeletal muscle remains to be determined.

3.3. Muscle Activity Does Not Cause Nuclear Translocation of Flag-NT-PGC-1α. In previous studies from our laboratory, we found that repetitive muscle fiber activity elicited by electric field stimulation of isolated FDB fibers is effective for activating nuclear-cytoplasmic redistribution of expressed GFP fusion proteins of the transcriptional regulators NFATc1
Figure 4: Activation of AMPK does not alter the subcellular distribution of Flag-NT-PGC-1α in muscle fibers. (a) The n/c fluorescence ratio of Flag-NT-PGC-1α in muscle fibers with (AICAR) or without (control) 2 mM AICAR treatment for 5 h in culture medium in the tissue culture incubator. n/c values from 20 nuclei from 20 randomly selected different fibers were averaged to give the mean value for each group. (b) Representative Western blots showing the effect of AICAR treatment on AMPK activation. Protein expression was quantified and averaged from 5 independent treatments. Phos AMPK, phospho-AMPK. Asterisk indicates statistical significance between groups at \( P < 0.05 \).

(NFATc1-GFP) and HDAC4 (HDAC4-GFP) and that both of these effects are mediated by elevated Ca\(^{2+}\), NFATc1-GFP via Ca\(^{2+}\) dependent activation of calcineurin [16, 18], and HDAC4-GFP via Ca\(^{2+}\) dependent activation of CaM kinase [19]. Exercise/muscle contraction has also been found to increase the nuclear protein content of full-length PGC-1α [5, 6]. Since nuclear NT-PGC-1α has been shown to co-activate PPAR receptors as does the full-length PGC-1α [12], it is of interest to determine whether stimulation of muscle fibers can also induce the nuclear accumulation of NT-PGC-1α in FDB fibers. Our results show that the nuclear Flag-NT-PGC-1α fluorescence of fibers stimulated continuously for 4 h at 10 Hz (Figure 3(a), stimulation) was essentially the same as in fibers from a parallel culture kept under control conditions without stimulation (Figure 3(a), control). The mean Flag-NT-PGC-1α n/c fluorescence ratio was 0.62 ± 0.03 in control and 0.59 ± 0.03 in stimulated fibers (Figure 3(b); \( P > 0.05 \)). Thus, Flag-NT-PGC-1α does not translocate into the fiber nuclei in response to 4 h of continuous 10 Hz stimulation.

In order to verify the effectiveness of 10 Hz continuous stimulation on isolated FDB fibers, in separate experiments we infected fibers with adenovirus encoding NFATc1-GFP. Two days later, fibers were stimulated continuously for 4 h at 10 Hz, the same stimulation pattern used for the Flag-NT-PGC-1α expressing fibers above. As previously reported [16, 18], NFATc1-GFP fusion protein is present predominantly at the sarcomeric Z line in the cytoplasm of nonstimulated cultured adult FDB fibers (Figure 3(c), control). 4 h of 10 Hz continuous stimulation markedly increased the nuclear NFATc1-GFP fluorescence (Figure 3(c), stimulation), so FDB fibers clearly respond to this stimulus pattern. Thus, 10 Hz muscle fiber activity can cause nuclear accumulation of the transcription factor NFATc1-GFP, but not of Flag-NT-PGC-1α. However, it remains to be determined whether repetitive muscle activity results in modification of the expression level of endogenous NT-PGC-1α.

It was previously reported that activation of p38 MAPK, which phosphorylates PGC-1α on Thr-262, Ser-265, and Thr-298 [8], increases the activity and nuclear protein
level of full length PGC-1α in various tissues, including skeletal muscle [7–9]. Exercise/muscle contraction results in increased phosphorylation of p38 MAPK [9]. Using Western blot analysis, we found that 4 h of continuous stimulation can significantly increase the level of p38 MAPK phosphorylation in cultured FDB muscle fibers (Figure 3 (d)). However, despite this increase in MAPK activation, there was no evident nuclear accumulation of Flag-NT-PGC-1α as discussed above, even though NT-PGC-1α contains two of the three p38 MAPK phosphorylation sites (Thr-262, Ser-265). These results suggest that p38 MAPK is not involved in the regulation of the subcellular distribution of NT-PGC-1α in muscle fibers. Whether p38 MAPK plays a role or not in regulation of the protein stability and activity of endogenous NT-PGC-1α as it does for the full-length PGC-1α in muscle fibers will be an interesting question for future study.

3.4. Activation of AMPK Has No Effect on the Subcellular Distribution of Flag-NT-PGC-1α. It was previously reported that AMPK can directly phosphorylate full-length PGC-1α at residues Thr-177 and Ser-570 [11] and activate PGC-1α-dependent signaling pathways in skeletal muscle [10, 11]. As NT-PGC-1α contains one of the AMPK-phosphorylation sites, we next tested whether activation of AMPK can increase the nuclear accumulation of NT-PGC-1α in muscle fibers. Cultured FDB muscle fibers expressing Flag-NT-PGC-1α were treated for 5 h with 2 mM of AICAR, a powerful cell permeable AMP mimic for activating AMPK [20]. After 5 h of incubation with or without AICAR, fibers were fixed and stained with anti-Flag antibody. Fibers treated with AICAR exhibited similar nuclear Flag-NT-PGC-1α protein fluorescence as the control group. The mean Flag-NT-PGC-1α n/c fluorescence ratio was 0.61 ± 0.03 in control and 0.55 ± 0.03 in AICAR-treated fibers (Figure 4(a); P > 0.05). Figure 4(b) shows that 5 h of AICAR treatment did significantly increase the phosphorylation of AMPK. These results demonstrate that activation of AMPK has no effect on the subcellular distribution of Flag-NT-PGC-1α. One hour treatment with AICAR, or one hour of continuous electrical field stimulation at 10 Hz, caused no change in the nuclear/cytoplasmic distribution of Flag-NT-PGC-1α (Supplementary Figure 1 available online at doi: 10.1155/2012/989263).

3.5. Inhibition of CRM1 Induces Only Modest Nuclear Accumulation of Flag-NT-PGC-1α. NT-PGC-1α has a predicted size about 30 kDa which should allow it to be freely diffusible between nucleus and cytosol via the nuclear pores, a mechanism which is independent of CRM1. However, previous studies showed that treatment with the CRM1

Figure 5: Inhibition of CRIM1 increases nuclear accumulation of Flag-NT-PGC-1α. (a) Representative images of Flag-NT-PGC-1α in a control fiber (control) and that of another fiber treated with 40 nM CRM1 inhibitor leptomycin B (LMB) for 20 h in culture medium in the tissue culture incubator. Scale bar, 10 μm. (b) The n/c fluorescence ratio of Flag-NT-PGC-1α in muscle fibers with (LMB) or without (control) leptomycin B treatment. n/c values from 40 nuclei from 40 randomly selected different fibers were averaged to give the mean value for each group. Asterisk indicates statistically significant difference between groups at P < 0.05.
nuclear export inhibitor leptomycin B (LMB) increased the nuclear content of NT-PGC-1α in adipocytes and CHO-K1 cells [12, 13], suggesting that NT-PGC-1α shuttles from the nucleus to the cytoplasm in these cells at least partially via a CRM1-dependent efflux pathway. Whether nucleocytoplasmic shuttling of NT-PGC-1α proteins via a CRM1-dependent pathway also occurs in skeletal muscle is not known. To address this issue, we treated muscle fibers expressing Flag-NT-PGC-1α with 40 nM leptomycin B. Our results show that the Flag-NT-PGC-1α fluorescent staining was enhanced in nuclei of fibers exposed to leptomycin B (Figure 5(a), LMB) compared to control fibers (Figure 5(a), control). The Flag-NT-PGC-1α n/c fluorescence ratio was significantly increased from 0.61 ± 0.02 in control to 1.23 ± 0.06 in fibers treated with leptomycin B for 20 h (Figure 5(b); P < 0.05). However, the extent of nuclear accumulation of Flag-NT-PGC-1α in resting muscle fibers with blocked CRM1-dependent nuclear export is much lower than that previously observed for other transcriptional regulators such as NFATc1 and HDAC [18, 19], which are too large to pass passively through the nuclear pores.

The relatively low nuclear accumulation of Flag-NT-PGC-1α in the presence of leptomycin B (Figure 5) compared to other transcriptional regulators such as NFATc1 [18] or HDAC4 [19] in adult muscle fibers indicates that either the unidirectional rate of nuclear influx of Flag-NT-PGC-1α is extremely low so that there was little entry of Flag-NT-PGC-1α into the muscle fiber nuclei during the 20 h exposure to leptomycin B or, more likely, that there is a CRM1-independent pathway [21] for nuclear efflux of Flag-NT-PGC-1α. In this case, assuming that efflux is proportional to nuclear concentration and that the nuclear volume is negligible compared to the cytoplasm, the approximate doubling of n/c fluorescence ratio in exposure to leptomycin B indicates that nuclear efflux of Flag-NT-PGC-1α via the leptomycin B insensitive pathway would have doubled in the presence of leptomycin B, so about half of the nuclear efflux in resting fibers in the absence of leptomycin B would be via the CRM1-independent pathway. If the CRM1-independent pathway is passive efflux via nuclear pores, which is likely since Flag-NT-PGC-1α is below the cut off size for passive movement through the nuclear pores, Flag-NT-PGC-1α would be distributed passively between nucleus and cytoplasm in the steady state in the case of full blockade of the CRM1-dependent pathway by leptomycin B. In that case the free concentration of Flag-NT-PGC-1α would be the same in the nucleus and cytoplasm. The slightly higher nuclear than cytoplasmic concentration of total (i.e., free plus bound) Flag-NT-PGC-1α (1.23 fold; see above) would indicate that fraction of bound Flag-NT-PGC-1α would be slightly higher in the nucleus than in the cytoplasm. Whether the Flag tag on Flag-NT-PGC-1α might influence its nuclear/cytoplasmic distribution in muscle fibers remains to be determined. The possibility of rapid intranuclear ubiquitination followed by proteasomal degradation [14] of Flag-NT-PGC-1α within the nucleus as a mechanism for lowering nuclear Flag-NT-PGC-1α is unlikely since ubiquitination occurs at the N-terminal residue [14], which is Flag tagged in our Flag-NT-PGC-1α construct.

3.6. Other Considerations and Conclusion. Overexpression of full length PGC-1α, which is predominantly nuclear, gives rise to an increase in mitochondrial number and oxidative capacity in skeletal muscle [2, 3]. It will be interesting to determine in future studies whether overexpression of NT-PGC-1α, which is only partially nuclear, would also produce such effects on mitochondrial number and oxidative capacity.

In conclusion, our results suggest that in adult skeletal muscle fibers the nuclear-cytoplasmic distribution and regulation of NT-PGC-1α is very different from that of the full-length PGC-1α, which is exclusively nuclear.

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