UBIQUITIN PROTEASOME DEPENDENT DEGRADATION OF THE TRANSCRIPTIONAL COACTIVATOR PGC-1α VIA THE N-TERMINAL PATHWAY

Julie Trausch-Azar¹²³, Teresa C. Leone⁴, Daniel P. Kelly⁴, Alan L. Schwartz¹²³
¹Departments of Pediatrics, ²Internal Medicine, and ³Developmental Biology
Washington University School of Medicine, St. Louis, MO
and ⁴Sanford-Burnham Medical Research Institute, Orlando, FL

Running head: Ubiquitin-mediated degradation of PGC-1α

Address correspondence to: Alan L. Schwartz, Ph.D., M.D., Department of Pediatrics, C.B. 8116, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110 U.S.A., Phone: 314-454-6005, Fax: 314-454-0537, E-mail: schwartz@wustl.edu

This study was supported by NIH grants to (GM067620 and DK045416) A.L.S. and D.P.K.

Abstract
PGC-1α is a potent, inducible transcriptional coactivator which exerts control on mitochondrial biogenesis and multiple cellular energy metabolic pathways. PGC-1α levels are controlled in a highly dynamic manner reflecting regulation at both transcriptional and post-transcriptional levels. Herein we demonstrate that PGC-1α is rapidly degraded in the nucleus (t½ 0.3h) via the ubiquitin proteasome system. An N-terminal deletion mutant of 182 residues, PGC182, as well as a lysine-less mutant form, is nuclear and rapidly degraded (t½ 0.5h) consistent with degradation via the N-terminus-dependent ubiquitin sub-pathway. Both PGC-1α and PGC182 degradation rates are increased in cells under low serum conditions. However, a naturally-occurring N-terminal splice variant of 270 residues, NT-PGC-1α, is cytoplasmic and stable (t½ >7h) providing additional evidence that PGC-1α is degraded in the nucleus. These results strongly suggest that the nuclear N-terminus-dependent ubiquitin proteasome pathway governs PGC-1α cellular degradation. In contrast, the cellular localization of NT-PCG-1α results in a longer-half-life and possible distinct temporal and potentially biological actions.

Introduction
Members of the PPAR-γ (peroxisome proliferator activated receptor-gamma) coactivator-1 (PGC-1) family of transcriptional coregulators serve as inducible coactivators of nuclear receptor and non-nuclear receptor transcription factors involved in the control of mitochondrial biogenesis and cellular energy metabolic pathways. Dysregulation of PGC-1α has been implicated in the pathogenesis of diabetes and insulin resistance (1,2). PGC-1α was identified through its functional interaction with the nuclear receptor PPAR-γ in brown adipose tissue (3). PGC-1α and its homologue, PGC-1β, are preferentially expressed in tissues with high oxidative capacity such as heart and skeletal muscle and serve critical roles in the regulation of mitochondrial function and cellular energy metabolism, as does a naturally occurring 270 amino acid 3’ splice variant (NT-PGC-1α) recently described by Zhang et al. (4). The docking of PGC-1 coactivators to specific transcription factors provides a platform for the recruitment of regulatory protein complexes that exert powerful effects on gene transcription triggering biological responses that equip the cell to meet the energy demands of the changing environment (5). Multiple PGC-1α coactivation targets have now been identified including PPAR-γ, PPAR-α, PPAR-β, RXRα, and glucocorticoid receptor. In addition, several non-nuclear receptor PGC-1 partners have been identified, including nuclear respiratory factors 1 and 2 (NRF-1 and 2) and Foxhead Box(01) (6,7). PGC-1α is inducible by a variety of physiological and dietary factors. For example, PGC-1α is stimulated by exercise and by fasting (1,5) and PGC-1α has recently been linked to regulation of angiogenesis (8). PGC-1α thus serves as an inducible regulator of metabolism and other key processes.
Ubiquitin-mediated degradation of PGC-1α

Cellular PGC-1α levels are controlled in a dynamic state dictated by the balance of its synthesis and degradation. Several studies have addressed determinants of PGC-1α synthesis. However, there is relatively little understanding of the mechanism(s) and regulation of PGC-1α degradation. Cytokines and phosphorylation have been implicated in the control of PGC-1α degradation rates (9). Puigserver’s group has shown that acetylation is involved in the post-transcriptional control of PGC-1α (10). Recently, Sano et al. (11) and Anderson et al. (12) proposed that PGC-1α degradation involves the ubiquitin proteasome pathway. In this pathway, the conjugation of ubiquitin to the protein substrate proceeds via a three-step cascade mechanism. Initially, the ubiquitin-activating enzyme, E1, activates ubiquitin. One of several E2 enzymes (ubiquitin-conjugating enzymes, Ubc’s) transfers the activated ubiquitin to the substrate that is specifically bound to a member of the ubiquitin-protein ligase, E3, family. Transfer of ubiquitin can be either directly to the E3-bound substrate, or via an additional E3-ubiquitin intermediate. E3s catalyze the last step in the conjugation process, covalent attachment of ubiquitin to the substrate. In successive reactions, a polyubiquitin chain is synthesized by progressive transfer of additional activated ubiquitin moieties to the previously conjugated ubiquitin molecule. The polyubiquitin chain serves as a recognition marker for the 26S proteasome. Following conjugation of ubiquitin, the target protein is degraded by the proteasome, and free ubiquitin is released and recycled (13).

Characterized by the initial site of ubiquitination, there are two sub-pathways of the ubiquitin system, the internal lysine-dependent pathway and the N-terminus-dependent pathway. In most cases, the first ubiquitin moiety is transferred to an ε-NH₂ group of an internal lysine residue of the target protein to generate an isopeptide bond. However, recent studies have suggested that the first ubiquitin may be covalently attached to the free and exposed N-terminal residue of the substrate (14). Substrates for N-terminus-dependent ubiquitination include MyoD (15), the human papillomavirus 16 (HPV 16) oncoprotein E7 (16), the Arf tumor suppressor (17), the Id1 and Id2 developmental regulators (18,19) as well as naturally occurring lysine-less proteins such as HPV-58 E7 and p16INK4α (20). The ubiquitination process may occur in the cytoplasm or in the nucleus (21).

Here we investigated the ubiquitin proteasome-mediated degradation of PGC-1α. We show that endogenous as well as exogenously expressed full-length PGC-1α are localized to the nucleus and very rapidly degraded (t½ < 0.5h) in a ubiquitin proteasome-dependent manner. Interestingly, a recently reported N-terminal splice variant of 270 residues, NT-PGC-1α, is cytoplasmic and stable (t½ > 7h). However, PGC182, (aa 1-182), a C-terminally truncated mutant of PGC-1α containing four lysines is nuclear and is very rapidly degraded (t½ < 0.5h) via the ubiquitin proteasome pathway, similar to full-length PGC-1α. A lysine-less mutant of PGC182 is also nuclear and rapidly degraded. Our findings thus suggest that PGC-1α is primarily degraded via the nuclear N-terminus-dependent ubiquitin proteasome pathway.

Experimental Procedures

Cell culture and chemical reagents

The C2C12 mouse myoblast and human HeLa and HepG2 cell lines were obtained from the American Type Culture Collection. The cells were propagated and differentiated as described earlier (18). Briefly, cells were propagated in growth medium (GM), Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G and 100 µg/ml streptomycin (Invitrogen), and maintained in a humidified chamber at 37°C with 5% CO₂. Myogenic differentiation of C2C12 cells was induced by changing the growth medium to differentiation medium (DM), Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G and 100 µg/ml streptomycin (Invitrogen), and maintained in a humidified chamber at 37°C with 5% CO₂. Myogenic differentiation of C2C12 cells was induced by changing the growth medium to differentiation medium (DM), Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum (HyClone), 100 units/ml penicillin G and 100 µg/ml streptomycin, when cells reached confluency. Cells were then maintained in differentiation medium for 6 days with medium being changed every 24 hours. HL1 mouse atrial cardiac myocytes were obtained from Claycomb and propagated as described by Claycomb et al. (22). Transient transfections of HeLa, HL1, HepG2 or C2C12 myoblasts were performed using the
Ubiquitin-mediated degradation of PGC-1α

Fugene 6 reagent (Roche Molecular Biochemicals) according to the manufacturer’s instruction. (23).

Plasmid Constructs
Full-length PGC-1α in the pSV-SPORT vector has been previously described (3). PGC182 was prepared by the addition of a stop codon following amino acid 182. Lysine and cysteine mutations were made using the QuikChange Site-Directed Mutagenesis Kit according to manufacturer’s instructions (Stratagene). N-terminal modifications (6-X-LL-myc, EGFP and p14ARF) were made using an adaptation of the QuikChange Site-Directed Mutagenesis Kit (Stratagene) in which PCR products were used as the homologous primer pair to either insert or delete sequence in lysine-less PGC182. FLAG-Ub was kindly provided by Aaron Ciechanover (Technion Israel Institute of Technology, Haifa, Israel).

NT-PGC-1α and NT-PGC-K3R-myc Plasmids
NT-PGC-1α was amplified by PCR from pcDNA3.1-PGC.797 (previously described, ref. 24) and cloned into the pCR2.1-TOPO vector using the TA Cloning Kit (Invitrogen, Carlsbad, CA). Insert and the pcDNA3.1 (-) vector were then digested with Xho1 and HindIII, gel purified and ligated.

NT-PGC-K3R-myc was amplified by PCR using a forward primer containing an Xho1 restriction site and a reverse primer containing the K3R-myc tag with NT-PGC-1α as a template. The 867 bp amplicon was subcloned into the pCR2.1-TOPO vector using the TA Cloning Kit (Invitrogen, Carlsbad, CA). Insert and the pcDNA3.1 (-) vector were then digested with Xho1 and HindIII, gel purified and ligated.

Immunofluorescence
Subcellular localization of PGC-1α, NT-PGC-1α, PGC182 and its lysine-less mutants was determined by indirect immunofluorescence in HeLa, HL1 and 2C12 cells. Briefly, cells were washed with PBSa, a phosphate-buffered saline solution (PBSa, Fisher Biotech) supplemented with 100mM CaCl2 and 50mM MgCl2, fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), quenched in 0.1M ethanolamine (pH 8.0) and permeabilized in 1% Triton X-100 (Sigma). After blocking in 1% BSA/PBSa/ 0.1% TW-80, subcellular localization was then determined using rabbit polyclonal anti-PGC-1α (1:1000 dilution (24), followed by incubation with AlexaFluor 488 goat anti-rabbit IgG (Molecular Probes). MG132 (20 μM, Peptides International) was added to cells 2 h prior to fixation where indicated. MG132 was prepared as a 10 mM stock solution in DMSO. Cells were observed using a Zeiss Axioscope microscope and images were taken using a Zeiss AxioCam digital camera.

Determination of Protein Half Life
As previously described (21,23), 16–20 h after transfection, HeLa, HL1, HepG2 or C2C12 cells were incubated with CHX (100 μg/ml, Sigma) to inhibit further protein synthesis. MG132 (20 μM) was added along with CHX as noted. The cells were lysed after 0, 0.5, 1, 2 and 3 h in PBS containing 5% Igepal, 1 mM EDTA, 1 mM DTT, 2 mM PMSF, 2.5 μg/ml leupeptin and 1 μM pepstatin for at least 30 min after which cells were sonicated, then centrifuged at 14,000 rpm for 10 min at 4°C in an Eppendorf microcentrifuge to remove cellular debris. The lysates were mixed with an equal amount of Laemmli sample buffer (Bio-Rad) and equal amounts of sample were run on a 10% Tris-HCl gel (Bio-Rad) followed by electroblotting onto nitrocellulose (Osmonics). For the detection of PGC182 and its mutants, the blots were probed with polyclonal anti-PGC-1α (1:1000 dilution; (24)) followed by incubation with a secondary horseradish peroxidase-conjugated antibody and detection by chemiluminescence (GE Healthcare). The resulting bands were quantitated using the Kodak EDAS system and the data was graphed using the Excel graphing program (Microsoft). Protein degradation rate is expressed as half-life (t½), the time for degradation of 50% of the protein. Half-life data reported was evaluated by ≥ 3 independent determinations.

Immunoprecipitation
HeLa cells were transfected with PGC182, its lysine-less mutants and FLAG-Ub in a 1:1 ratio. At 16-20 h after transfection, cells were lysed in 20mM Tris pH 7.6 and 0.25% TX-100, with 1 mM DTT added where indicated, for 30 min at 4°C. Cells were scraped into an Eppendorf tube, sonicated and spun at 14,000 rpm at 4°C for 10 min to remove cellular debris. Direct
immunoprecipitation was performed by incubation of PureProteome Protein A Magnetic Beads (Millipore) with polyclonal anti-PGC-1α antibody (1:400 dilution (24)) and monoclonal anti-FLAG antibody (1:200 dilution, Sigma) followed by addition of cell lysates. The beads were washed, and following the addition of reducing and non-reducing Laemmli sample buffer (BioRad) the immunoprecipitates were run on a 10% Tris-HCl gel and analysed via Western blot. The blots were probed with monoclonal anti-FLAG antibody (1:1000 dilution, Sigma) followed by incubation with a secondary horseradish peroxidase-conjugated antibody and detected by chemiluminescence (GE Healthcare).

**Results**

As an initial step towards defining the pathway of PGC-1α degradation, we examined the half-life of endogenous (full length, 1-797 aa) PGC-1α in HL1 cells. As seen in Figure 1A, endogenous PGC-1α is very rapidly degraded with t½ ~ 0.3h. Incubation of cells with MG132, a potent and selective inhibitor of the proteasome, markedly slowed the rate of PGC-1α degradation (Figure 1A). Exogenously expressed PGC-1α behaves similarly. Following transfection of full-length PGC-1α, the degradation rate was equally rapid (t½ ~ 0.4h), and was markedly slowed by proteasome inhibition (Figure 1B). These observations suggest that PGC-1α is degraded via the ubiquitin proteasome pathway. In addition, subcellular localization of endogenously expressed PGC-1α as well as transfected PGC-1α demonstrates predominantly nuclear staining (Figure 1C, see also Figure 3, and data not shown). These latter observations raise questions as to where PGC-1α is rapidly degraded; within the nucleus or following export to the cytoplasm and via which sub-pathway of the ubiquitin proteasome system, the internal lysine-dependent pathway or the N-terminus-dependent pathway.

Recent studies suggest that the N-terminus dependent pathway is predominant within the nucleus (21,25,26). In order to examine the role of these two sub-pathways in the degradation of PGC-1α, a lysine-less PGC-1α was used to specifically examine the N-terminus-dependent sub-pathway. As PGC-1α contains 50 lysine residues, we chose to examine a 182 amino acid carboxy-terminally truncated form of PGC-1α. This 182 residue N-terminal variant of PGC-1α preserves the activation domain as well as nuclear receptor recognition sites and contains only four lysine residues (at positions 54, 77, 143, 144). Similar to full-length PGC-1α, PGC182 is rapidly degraded (t½ ~ 0.7h) via the ubiquitin proteasome pathway in HL1 cells (Figure 2A). Furthermore, PGC182 is also rapidly degraded in HeLa cells (t½ ~ 1h) as well as in C2C12 myoblasts (t½ ~ 0.5h) and myotubes (t½ ~ 0.6h) (Figure 2B, C, D). Independent of cell type, PGC182 is rapidly degraded (0.7 ± 0.08h, mean ± SEM; 0.3-2.9h, range; n = 37). Localization of PGC182 is predominantly nuclear and is unaltered in the presence of MG132 (Figure 3).

In order to determine if PGC182 is degraded via the N-terminus dependent sub-pathway, we systemically mutated all four internal lysine residues to arginine. The cellular localization of lysine-less PGC182 is the same as that seen for PGC182, nuclear with some cytoplasmic staining both in the absence or presence of MG132 (Figure 3). As seen in Figure 4, PGC182 K143,K144R; PGC182 K77,K143,K144R; and PGC182 K54,K77,K143,K144R (i.e., lysine-less PGC182) are all rapidly degraded (t½ ~ 0.5h). Furthermore, the degradation of each of these species is markedly slowed by MG132 (Figure 4A-D). These results strongly suggest that PGC182 is degraded via the N-terminus-dependent sub-pathway. We also constructed mutants of lysine-less PGC182 with 6-lysine-less myc tags (6-X-LL-myc lysine-less PGC182), with EGFP or with p14ARF on the N-terminus as the addition of a bulky N-terminal tag to several substrates has been shown to force degradation through the lysine-dependent pathway by preventing ubiquitination at the N-terminal site. These constructs, however, were also rapidly degraded (data not shown).

In order to directly determine if lysine-less PGC182 is, as predicted, conjugated to ubiquitin, we transfected cells with PGC182, lysine-less PGC182 or 6-X-LL-myc lysine-less PGC182 with FLAG-ubiquitin in the presence or absence of MG132. High molecular weight ubiquitin conjugates of PGC182, lysine-less PGC182 and 6-X-LL-myc lysine-less PGC182 accumulate in the
Ubiquitin-mediated degradation of PGC-1α

In addition, the analyses (cell lysis, immunoprecipitation and SDS-PAGE) were performed in the absence or presence of reducing agents, since two recent reports demonstrate degradation substrates which carry ubiquitin moieties attached via thiol-ester conjugates (27,28). As seen in Figure 6A when samples were immunoprecipitated with anti-PGC-1α and blotted with anti-FLAG abundant high molecular weight ubiquitin conjugates are seen with PGC182 under both non-reducing and reducing conditions (lanes 1 and 2). A similar pattern of high molecular weight ubiquitin conjugates is also seen with lysine-less PGC182 and with 6-X-LL-myc lysine-less PGC182, under non-reducing and reducing conditions (lanes 3-6). Figure 6B demonstrates the high molecular weight ubiquitin conjugates of PGC182, lysine-less PGC182 and 6-X-LL-myc lysine-less PGC182 following immunoprecipitation with anti-FLAG followed by blotting with anti-PGC-1α. Furthermore, the ubiquitin-mediated degradation was unaltered in a lysine-less PGC182 mutant in which all five cysteine residues were mutated (i.e., lysine-less/cysteine-less PGC182) (t½ ~ 0.5h, Figure 7). Taken together, these results demonstrate the conjugation of ubiquitin to PGC182 as well as to lysine-less PGC182 and 6-X-LL-myc lysine-less PGC182 whose only free amino acceptor is at the N-terminus.

A 270 amino acid N-terminus splice variant of PGC-1α, termed NT-PGC-1α has been recently reported (4). In order to determine the degradation rate and localization of NT- PGC-1α we transfected HeLa cells with NT-PGC-1α. The half-life of NT- PGC-1α was >7h (Figure 8A) as was the half-life of NT-PGC-K3R-myc (not shown). As seen in Figure 8B, NT-PGC-1α and NT-PGC-K3R-myc were localized to the cytoplasm. These results demonstrate that, unlike full-length PGC-1α, the 270 amino acid N-terminal variant is both very slowly degraded and localized to the cytoplasm. Taken together these results further support the conclusion that PGC-1α is rapidly degraded in the nucleus via the N-terminal pathway.

In order to determine if PGC-1α and PGC182 respond to metabolic perturbations, we switched HeLa or HepG2 cells transfected with PGC-1α or PGC182 to low serum (0.2%) media. 18h in low serum media decreased the steady state concentration of PGC-1α by 40% (5 determinations) and of PGC182 by 29% (4 determinations). We then determined if the decreases in PGC-1α or PGC182 concentrations were a result of altered protein degradation rates. As seen in Figure 9, while at 10% serum PGC-1α is degraded rapidly (t½ = 0.4h) (A), the rate of degradation is increased (t½ = 0.3h) at 0.2% serum (B). Actin content did not vary. Similarly, PGC182 at 10% serum is degraded at t½ = 2h (C) and the rate of degradation is enhanced (t½ = 0.7h) at 0.2% serum (D). Again, actin content did not vary. The accelerated rate of PGC182 and PGC-1α degradation observed upon low serum exposure is ubiquitin proteasome-dependent. PGC182 is degraded rapidly in HepG2 cells in 10% serum (t½ = 1.3h) and this degradation is completely abrogated by MG132. In 0.2% serum, the rate of degradation is increased (t½ = 0.4h) and similarly is completely abrogated by MG132 (Figure 10). Similar results were seen with PGC-1α (data not shown). These observations demonstrate that PGC-1α and PGC182 are able to respond similarly to altered metabolic conditions. However, the molecular regulatory events and pathways involved are yet to be defined.

Discussion

PGC-1α is an inducible master regulator of cellular energy metabolism. As with most key regulatory molecules, cellular PGC-1α concentration is tightly controlled through a dynamic balance of its synthesis and degradation. Our investigation of the molecular mechanisms underlying PGC-1α degradation revealed a key role for the nuclear ubiquitin proteasomal pathway system. The following lines of evidence support this conclusion: i) steady-state, endogenous (or exogenous) PGC-1α is localized within the cell nucleus, but outside the nucleolus; ii) PGC-1α is a very short-lived protein with a half life of ~ 0.5h; iii) inhibition of the proteasome with MG132 markedly slowed PGC-1α degradation; and iv) an N-terminal variant of 270 amino acids (NT-PGC-1α) which is predominantly cytoplasmic, is stable (t½ > 7h). In order to determine the sub-pathway of the ubiquitin proteasome system involved in PGC-1α degradation we characterized the degradation of a C-terminally truncated PGC-1α,
Ubiquitin-mediated degradation of PGC-1α

PGC182 and its lysine → arginine mutants. PGC182 and lysine-less PGC182 are predominantly nuclear, and rapidly degraded by the ubiquitin proteasome system. Thus, the nuclear N-terminus-dependent pathway appears to play a dominant role in the dynamic balance of cellular PGC-1α levels.

PGC-1α functions as a master transcriptional regulator of cellular energy homeostasis via control of mitochondrial biogenic and energy metabolic networks, especially in brown fat and cardiac and skeletal muscle where it governs many aspects of fuel metabolism and ATP production. PGC-1α coactivates multiple transcription factors involved in mitochondrial biogenesis, oxidative phosphorylation, fatty acid oxidation, and glycogen and glucose flux (29). Given the potency of this coactivator, its activity must be tightly controlled in accordance with energy needs and substrate availability. Indeed, chronic overexpression of PGC-1α in heart results in toxicity leading to cardiomyopathy due to exuberant mitochondrial proliferation (24,30). It is well-established that the expression of PGC-1α is highly regulated at the gene transcriptional level accounting, at least in part, for its inducibility and tissue-specific expression (5,7). Little attention has been directed, however, to the mechanism(s) underlying or the regulation of PGC-1α degradation.

Puigserver et al. (9) suggested that PGC-1α phosphorylation between residues 262 and 298 served as p38 MAPK targets which slow PGC-1α degradation. More recently, Sano et al. (11) demonstrated nuclear localization of N-terminal-FLAG-tagged PGC-1α and suggested it was degraded via the ubiquitin-proteasome system although no half-life data was reported. Further, they noted two PEST-like regions (aa 80-144 and 255-270) and suggested that the ubiquitination and degradation of PGC-1α was dependent upon the integrity of the C-terminus containing arginine-serine rich domains and an RNA-recognition motif (aa 565-798). C-terminal deletion mutants showed that PGC-1α (aa 1-565) was both nuclear and cytoplasmic, whereas PGC-1α (1-292) was excluded from the nucleus. In addition, Zhang et al. (4) have also suggested that the C-terminal region is essential for targeting PGC-1α for ubiquitination and degradation based on their findings that an alternative 3′ splice variant (NT-PGC-1α) of amino acids 1-270 appears to be more stable than PGC-1α.

Our data herein differ somewhat from the previously published observations. First, we find both endogenous and exogenously expressed PGC-1α is rapidly degraded (t½ ~ 0.5h) in all cell types examined including HL1, HeLa, and C2C12. This degradation rate is very rapid and typical of many transcription factors and oncoproteins (31). Second, we find PGC182 is equally rapidly degraded (t½ ~ 0.5h) (Figures 2, 4) in all cells examined. Sano et al. (11) reported that N-terminal constructs of PGC-1α (e.g., aa 1-292) required the C-terminal region for ubiquitination and proteasomal degradation and Zhang et al. (4) reported that the N terminal (1-270) NT-PGC-1α was stable. Our data demonstrate C-terminal truncated PGC182 ubiquitination (Figure 5, 6) and rapid (t½ ~ 0.5h) proteasome-dependent degradation (Figures 2, 4, 7). One potential and significant difference between our studies and those of Sano et al. (11) is the use of N-terminal FLAG tags on all of Sano et al. (11) constructs. As we show herein using lysine-less mutants of PGC182, PGC182 can be rapidly ubiquitinated and degraded via its N-terminus. It is possible that in Santo’s et al. (11) studies the FLAG tag interfered with the physiological ubiquitination and degradation pathway. Third, we demonstrate that the C-terminal region(s) of PGC-1α are not required for the N-terminal region to be ubiquitinated and degraded. In addition, while PGC-1α appears to contain two PEST-rich regions, one of which is contained within PGC182 (i.e., aa 80-144), there is at present no data which supports this region’s role in PGC-1α degradation. Furthermore, while our data demonstrate similar half-lives of PGC-1α and PGC182, the 270 residue-N-terminal variant (NT-PGC-1α) is both remarkably stable (t½ > 7h) and localized to the cytoplasm. Finally, while the N-terminal constructs of PGC-1α of Sano et al. (11) and Zhang et al. (4) are relatively stable, they have not determined whether the limitation to degradation is the ubiquitination of the target protein or the inability of the ubiquitinated-substrate to be degraded by the proteasome. PGC182, a nuclear
protein, is however both ubiquitinated and degraded by the proteasome.

It has been well-recognized that single ubiquitin moieties are coupled to the ubiquitin activating enzyme (E1) and ubiquitin conjugating enzymes (E2s) via a thiol-ester bond (31). The recent reports that two proteins (Herpes virus MIR1 and Ube7) are conjugated to a multi-ubiquitin chain via thiol-ester linkage (27,28) deserves comment. This multi-ubiquitin chain thiol-ester linkage to a target protein is sensitive to cleavage upon reduction (e.g., β-mercaptoethanol). Our results in Figure 6, which demonstrate the identical pattern and abundance of ubiquitin conjugates of PGC182 and lysine-less PGC182 mutants under non-reducing and reducing conditions and results in Figure 7 of unaltered ubiquitin-mediated degradation of lysine-less/cysteine-less PGC182 eliminates the possibility of thiol-ester linked ubiquitin conjugates.

Our observation that PGC182 and its lysine-less derivatives are both nuclear and rapidly ubiquitinated and degraded via the proteasome and that NT-PGC-1α is both very stable and cytoplasmic also deserves comment. In terms of localization, full-length PGC-1α is predominantly nuclear as has been observed by others (3,11). A putative nuclear localization sequence (NLS) has been suggested within the C-terminal region (aa 651-668) (32). Our observation that PGC182 and its lysine-less derivatives are predominantly nuclear is of interest as it contains no apparent NLS, either classical or bipartite, as for example is found in MyoD (21). Thus, either PGC182 has a non-classical NLS or is chaperoned into the nucleus in association with one or more carriers. If the latter is the case, the chaperone abundance and/or efficiency must be remarkable as the observations with PGC182 were made following overexpression of the protein at a cellular level. Further studies will address these alternatives. Presumably NT-PGC-1α is unable to associate with chaperone carriers, interact with distinct cytoplasmic proteins, or has a dominant nuclear export sequence in residues 182-270.

Perhaps more important is the demonstration that PGC182 is degraded via the N-terminus-dependent pathway. The N-terminus-dependent pathway is relatively recently described. Herein, the initial ubiquitin moiety is covalently attached to the free and exposed alpha-amino group of the N-terminal residue of the substrate (20). Thereafter, a polyubiquitin chain is built upon the initial conjugated ubiquitin. Substrates for this pathway include at least a dozen proteins, several of which are transcription factors or other key regulatory molecules: e.g., MyoD (15,21), HPV16 oncoprotein E7 (16), the Id1 and Id2 developmental regulators (18,19), p16INK4a (20), p19ARF, p14ARF (17). The N-terminus-dependent pathway is the predominant degradation pathway for several of these substrates including Id1 and Id2.

Despite the recognition of the N-terminus as a site for ubiquitination (14), in only three instances (HPV58-E7, p21, and ERK3) has direct biochemical sequence evidence established ubiquitin covalently attached to the αNH2–terminus of the target protein (14,33). Most often the evidence is indirect and includes polyubiquitin-mediated proteasome-dependent rapid degradation of the lysine-less target protein, either naturally occurring lysine-less (e.g., p14ARF or HPV58-E7) or lysine-less mutants of the wild type protein (e.g., MyoD, LMP1). Many of these target proteins can be stabilized by addition of a bulky lysine-less peptide (e.g., 6xmyc or 6His-4HA). However, addition of HA tags to lysine-less p19ARF did not provide stability (17).

Recently, Anderson et al. (12) suggest PGC-1α is degraded by the proteasome within the nucleus. Using N-terminal GFP tagged PGC-1α they demonstrated increased nuclear accumulation of PGC-1α upon oxidative stress. However, they did not demonstrate nuclear degradation. We, however, have found rapid degradation of N-terminal EGFP, 6-X-LL-myc, and p14ARF tagged lysine-less PGC182 (data not shown). In addition, other N-terminally degraded proteins have not been examined following addition of bulky N-terminal peptides (e.g., HMGCoA reductase (34), neurogenin (35)). Our results with PGC182 demonstrate polyubiquitination and rapid proteasome-dependent degradation for wild type PGC182 and its lysine-less mutants, strongly supporting the N-terminus-dependent pathway.
The rapid ubiquitin proteasome-dependent degradation of PGC-1α and PGC182 raises the issue as to whether this regulatory mechanism is influenced by metabolic perturbations. Indeed, steady-state levels of PGC-1α and PGC182 decrease upon incubation in low serum media in HepG2 and HeLa cells. This decrease in PGC-1α and PGC182 levels occurred concomitantly with an increase in ubiquitin proteasome-dependent degradation rates (Figures 9, 10). These observations suggest that the degradation rates of PGC-1α are influenced by substrate availability (e.g., fatty acids) and/or serum regulatory factors. Future studies aimed at the physiological regulation of PGC-1α degradation are warranted.

Using nuclear localization sequence and nuclear export sequence mutants of MyoD, we demonstrated that the N-terminus-dependent pathway primarily functions within the nucleus (21). Thus, the results seen in Figures 4 and 5 strongly suggest that the N-terminus-dependent pathway is the predominant route of PGC182 degradation. In addition, it is likely that PGC182 is degraded via this pathway within the nucleus as is MyoD (25), however definitive proof awaits resolution of the nuclear-mediated uptake/export mechanism(s) as discussed above. Whether this is the case for the full-length molecule awaits further study. Consistent with this notion, NT-PGC-1α is both stable and cytoplasmic. It is tempting to speculate that NT-PGC-1α serves a specific transcriptional coregulatory role that requires longer action following the potent acute transcriptional coactivating effects of full-length PGC-1α. Taken together these data strongly suggest that a nuclear ubiquitin-proteasome system governs the rapid degradation of PGC-1α and its derivatives.

References

1. Lin, J., Handschin, C., Spiegelman, B.M. (2005) Cell. Metab. 1, 361-370
2. Handschin, C., Spiegelman, B.M. (2006) Endocr. Rev. 27, 728-735
3. Puigserver, P., Wu, Z., Park, C.W., Graves, R., Wright, M., Spiegelman, B.M. (1998) Cell 92, 829-839
4. Zhang, Y., Huypens, P., Adamson, A.W., Chang, J.S., Henagan, T.M., Lenard, N.R., Burk, D., Klein, J., Perwitz, N., Shin, J., Fasshauer, M., Kralli, A., Gettys, T.W. (2009) J. Biol. Chem. 284, 32813-32826
5. Finck, B.N., Kelly, D.P. (2006) J. Clin. Invest. 116, 615-622
6. Kelly, D.P., Scarpulla, R.C. (2004) Genes Dev. 18, 357-368
7. Puigserver, P., Spiegelman, B.M. (2003) Endocr. Rev. 24, 78-90
8. Arany, Z., Foo, S.Y., Ma, Y., Ruas, J.L., Bommi-Reddy, A., Girnun, G., Cooper, M., Laznik, D., Chinsomboon, J., Rangwala, S.M., Baek, K.H., Rosenzweig, A., Spiegelman, B.M. (2008) Nature 451, 1008-1012
9. Puigserver, P., Rhee, J., Lin, J.Z., Yoon, J.C., Zhang, C.-Y., Krauss, S., Mootha, V.K., Lowell, B.B., Spiegelman, B.M. Mol. Cell. (2001) 8, 971-982
10. Dominy, J.E. Jr, Lee, Y., Gerhart-Hines, Z., Puigserver, P. (2010) Biochim Biophys Acta (in press)
11. Sano, M., Tokudome, S., Shimizu, N., Yoshikawa, N., Ogawa, C., Shirakawa, K., Endo, J., Katayama, T., Yuasa, S., Ieda, M., Makino, S., Hattori, F., Tanaka, H., Fukuda, K. (2007) J. Biol. Chem. 282, 25970-25980
12. Anderson, R.M., Barger, J.L., Edwards, M.G., Braun, K.H., O’Connor, C.E., Prolla, T.A, Weindruch, R. (2008) Aging Cell 7,101-111
13. Schwartz, A.L., Ciechanover, A. (2009) Annu. Rev. Pharmacol. Toxicol. 48, 73-96
14. Ciechanover, A., Ben-Saadon, R. (2004) Trends Cell Biol. 14, 103-106
15. Breitschopf, K., Bengal, E., Ziv, T., Admon, A., Ciechanover, A. (1998) EMBO J. 17, 5964–5973
16. Reinstein, E., Scheffner, M., Oren, M., Ciechanover, A., Schwartz, A. (2000) Oncogene 19, 5944–5950
17. Kuo, M.L., den Besten, W., Bertwistle, D., Roussel, M.F., Sherr, C.J. (2004) Genes & Dev. 18. 1862-1874
18. Trausch-Azar, J.S., Lingbeck, J., Ciechanover, A., Schwartz, A.L. (2004) *J. Biol. Chem.* 279, 32614-32619
19. Fajerman, I., Schwartz, A.L., Ciechanover, A. (2004) *Biochim. Biophys. Res. Commun.* 314, 505-512
20. Ben-Saaden, R., Fajerman, I., Ziv, T., Hellman, U., Schwartz, A.L., Ciechanover, A. (2004) *J. Biol. Chem.* 279, 41414-41421
21. Lingbeck, J.M., Trausch-Azar, J.S., Ciechanover, A., Schwartz, A.L. (2003) *J. Biol. Chem.* 278, 1817-1823
22. Claycomb, W.C., Lanson, N.A Jr, Stallworth, B.S., Egeland, D.B., Delcarpio, J.B., Bahinski, A., Izzo, N.J. Jr. (1998) *Proc. Natl. Acad. Sci. USA* 95, 2979-2984
23. Sun, L., Trausch-Azar, J.S., Ciechanover, A., Schwartz, A.L. (2005) *J. Biol. Chem.* 280, 26448-26456
24. Lehman, J.J., Barger, P.M., Kovacs, A., Saffitz, J.E., Medeiros, D.M., Kelly, D.P. (2000) *J. Clin. Invest.* 106, 847-856
25. Lingbeck, J.M., Trausch-Azar, J.S., Ciechanover, A., Schwartz, A.L. (2005) *Oncogene* 24, 6376-6384
26. Sun, L., Trausch-Azar, J.S., Muglia, L.J., Schwartz, A.L. (2008) *Proc. Natl. Acad. Sci. USA* 105, 3339-3344
27. Cadwell, K., Coscoy, L. (2005) *Science* 309, 127-130
28. Ravid, T., Hochstrasser, M. (2007) *Nature Cell Biol.* 9, 422-427
29. Wende, A.R., Schaeffer, P.J., Parker, G.L., Zechner, C., Han, D.-H., Chen, M.M., Hancock, C.R., Lehman, J.J., Huss, J.M., McClain, D.A., Holloszy, J.O., Kelly, D.P. (2007) *J. Biol. Chem.* 282, 36642-36651
30. Russell, L.K., Mansfield, C.M., Lehman, J.J., Kovacs, A., Courtois, M., Saffitz, J.E., Medeiros, D.M., Valencik, M.L., McDonald, J.A., Kelly, D.P. (2004) *Circ. Res.* 94, 525-533
31. Hershko, A., Ciechanover, A. (1998) *Annu. Rev. Biochem.* 67, 235-279
32. Esterbauer, H., Oberkofler, H., Krempler, F., Patsch, W. (1999) *Genomics* 62, 98-102
33. Coulombe, P., Rodier, G., Bonneil, E., Thibault, P., Meloche, S. (2004) *Mol. Cell. Biol.* 24, 6140-6150
34. Doolman, R., Leichner, G.S., Avner, R., Roitelman, J. (2004) *J. Biol. Chem.* 279, 38184-38193
35. Vosper, J.M., McDowell, G.S., Hindley, C.J., Fiore-Heriche, C.S., Kucerova, R., Horan, I., Philipott, A. (2009) *J. Biol. Chem.* 284, 154588-15468
Figure Legends

1. Role of degradation (half-life) and localization of endogenous and exogenous PGC-1α. A. HL1 cells were treated with cycloheximide (CHX) or CHX plus MG132. Cells were lysed at 0, 0.5, 1, 2, and 3h and were evaluated via SDS-PAGE and Western blot for PGC-1α. The pixels for each band were measured and normalized so that the number of pixels at t = 0 was 100%. The log₁₀ of the percent of pixels was plotted versus time for each time point and the t½ calculated from the log of 50%. The t½ for PGC-1α was 0.3h and > 2h when MG132 was added. B. 18h after transfection with full-length PGC-1α, HL1 cells were treated with CHX +/- MG132 and degradation assessed as in A. Expression of exogenous PGC-1α was >200-fold that of endogenous PGC-1α. The t½ for exogenous PGC-1α was 0.4h. C. HL1 cells were fixed and localization of endogenous PGC-1α was visualized by immunofluorescence.

2. Rates of degradation of PGC182 in HL1, HeLa, C2C12 myoblasts and myotubes. 18h after transfection with PGC182 HL1 (A), HeLa (B), C2C12 myoblasts (C) or C2C12 myotubes (D) were treated with CHX +/- MG132 and half lives determined as described in Figure 1 legend. The t½ for PGC182 was 0.7h (HL1), 1.0h (HeLa), 0.5h (C2C12 myoblasts) and 0.6h (C2C12 myotubes).

3. Immunofluorescent localization of PGC-1α, PGC182 and lysine-less PGC182. 18h after transfection with PGC-1α, PGC182 or lysine-less PGC182, HeLa cells were incubated for 2h with or without MG132. Cells were thereafter fixed and localization of PGC was visualized with anti-PGC-1α via immunofluorescence.

4. Rates of degradation of PGC182 and its lysine mutants. 18h after transfection with PGC182 (A), PGC182 K143,144R (B); PGC182 K77,143,144R (C); or PGC182 K54,77,143,144R (lysine-less PGC182) (D), HL1 cells were treated with CHX +/- MG132 and half-lives determined as described in Figure 1 legend. The t½ for each of the four PGC182 species was 0.5h and extended to 3.8-5.5h with MG132.

5. Ubiquitin conjugates accumulate in the presence of MG132. HeLa cells were transfected with PGC182, lysine-less PGC182 or 6-X-LL-myc lysine-less PGC182 together with FLAG-ubiquitin. 18h later incubation was continued for 2h +/- MG132. Thereafter, cells were lysed and lysates immunoprecipitated with anti-PGC-1α, separated on SDS-PAGE and blotted with anti-FLAG (A). High molecular weight ubiquitin conjugates noted by bracket were quantitated (B).

6. Ubiquitin conjugate formation with PGC182 and lysine-less PGC182. HeLa cells were transfected with PGC182, lysine-less PGC182, or 6-X-LL-myc lysine-less PGC182 together with FLAG-ubiquitin. 18h later incubation was continued for 2h +/- MG132. Thereafter, either under non-reducing or reducing (350mM β-mercaptoethanol) conditions cells were lysed and lysates immunoprecipitated with anti-PGC-1α separated on SDS-PAGE and blotted with anti-FLAG (A), or immunoprecipitated with anti-FLAG and blotted with anti-PGC-1α (B). Lysates were also immunoprecipitated with anti-PGC-1α and blotted with anti-PGC-1α (C) to identify PGC182 and its lysine-less mutants. PGC182, lysine-less PGC182, and 6-X-LL-myc lysine-less PGC182 are noted by arrows. High molecular weight ubiquitin conjugates are noted by brackets.

7. Rates of degradation of PGC182, lysine-less PGC182 and lysine-less/cysteine-less PGC182. 18h after transfection with PGC182 (A), lysine-less PGC182 (B), or lysine-less/ cysteine-less PGC182 (C), HeLa cells were treated with CHX and the half-life determined as described in Figure 1.

8. Degradation rate (half-life) and localization of NT-PGC-1α. 18h after transfection with NT-PGC-1α, HeLa cells were (A) treated with CHX and the half-life determined as described in Figure 1(B) fixed.
and localization of NT-PGC-1α was visualized via immunofluorescence of antibody to PGC-1α as described in Figure 4. The t½ for NT-PGC-1α was >7h; two experiments are shown in ● circles in (A).

9. Effect of low serum on rates of degradation of PGC-1α and PGC182. HeLa cells were transfected with PGC-1α (A,B) or PGC182 (C,D) and either maintained in 10% fetal bovine serum (FBS), (A,C), or switched to 0.2% FBS (B,D). 18h later, cells were treated with CHX and half-lives determined as described in Figure 1. Actin content served as an internal control. Symbols represent mean ± SEM of six determinations. Representative Western blots are shown under all conditions.

10. Ubiquitin proteasome dependent degradation of PGC182 is accelerated by low serum. HepG2 cells were transfected with PGC182 and either maintained in 10% fetal bovine serum (FBS) (A,B) or switched to 0.2% FBS (C,D). 18h later, cells were treated with CHX or CHX plus MG132 and half-lives determined as described in Figure 1. Symbols represent mean ± SEM of three determinations. Representative Western blots are shown under all conditions.
Figure 1. Ubiquitin-mediated degradation of PGC-1alpha

**A**

Full Length Endogenous HL1 Cells

- CHX
- CHX + MG132

**B**

Full Length Exogenous HL1 Cells

- CHX
- CHX + MG132

**C**

Full Length Endogenous HL1 Cells

![Image of immunofluorescence staining](http://www.jbc.org/)

\[ t_{1/2} = 2.1 \text{h} \]
\[ t_{1/2} = 5.0 \text{h} \]
\[ t_{1/2} = 0.4 \text{h} \]
Figure 2.

Ubiquitin-mediated degradation of PGC-1alpha

A  PGC182
   HL1 Cells
   CHX

B  PGC182
   HeLa Cells
   CHX +
   MG132

C  PGC182
   C2C12 Myoblasts

D  PGC182
   C2C12 Myotubes

\[ t_1/2 = 0.7h \]
\[ t_1/2 = 3.8h \]
\[ t_1/2 = 3.7h \]
\[ t_1/2 = 1.0h \]
\[ t_1/2 = 5.1h \]
\[ t_1/2 = 0.5h \]
\[ t_1/2 = 9.7h \]
\[ t_1/2 = 0.6h \]
Figure 3.

Ubiquitin-mediated degradation of PGC-1α

|               | PGC-1α             | PGC182            | lysine-less PGC182 |
|---------------|--------------------|-------------------|--------------------|
| no treatment  | ![Image](image1)    | ![Image](image2)  | ![Image](image3)   |
| 20uM MG132    | ![Image](image4)    | ![Image](image5)  | ![Image](image6)   |
Figure 4.

A  
PGC182  
(KKKK)

CHX  
CHX + MG132

B  
PGC182 K143,144R  
(KKRR)

C  
PGC182 K77,143,144R  
(KRRR)

D  
PGC182 K54,77,143,144R  
(RRRR)

Ubiquitin-mediated degradation of PGC-1alpha

t1/2 = 3.8h  
t1/2 = 0.5h  
t1/2 = 5.2h  
t1/2 = 0.5h  
t1/2 = 4.7h  
t1/2 = 5.5h  
t1/2 = 0.5h  
t1/2 = 0.5h
Figure 5.

A  IP: anti-PGC  WB: anti-FLAG

|            | MG132 |       | lysine-less PGC182 |       | 6-X-LL-myc lysine-less PGC182 |
|------------|-------|-------|--------------------|-------|-------------------------------|
| PGC182     | -     | +     | -                  | +     | -                             |
| lysine-less PGC182 | -     | +     | -                  | +     | -                             |

B

% Ub-conjugates

| MG132 | -     | +     | -     | +     | -     | +     |
|-------|-------|-------|-------|-------|-------|-------|
|       | 100   | 300   | 100   | 300   | 100   | 300   |

Ubiquitin-mediated degradation of PGC-1alpha
Figure 6.

A. IP: anti-PGC WB: anti-FLAG

UBiquitin-mediated degradation of PGC-1alpha

B. IP: anti-FLAG WB: anti-PGC

C. IP: anti-PGC WB: anti-PGC
Figure 7.

A  
PGC182

B  
lysine-less PGC182

C  
lysine-less/cysteine-less PGC182

Log (% initial) vs. Time (h) for different treatments:
- A: PGC182 with CHX, t1/2=0.9 h
- B: lysine-less PGC182 with CHX, t1/2=0.4 h
- C: lysine-less/cysteine-less PGC182 with CHX, t1/2=0.5 h
Ubiquitin-mediated degradation of PGC-1α

Figure 8.

A

NT-PGC-1α

CHX

0 0.5 1 2 3 3h

Log (% initial)

2.5

2

1.5

1

0.5

0

Time (h)

B

NT-PGC-1α

0 1 2 3 4

t1/2 = 7.3 h
Figure 9. Ubiquitin-mediated degradation of PGC-alpha

A 10% FBS

PGC1-α

Log (% initial)

0 2h

0 2h

Actin

B 0.2% FBS

PGC1-α

Log (% initial)

0 2h

0 2h

Actin

C 10% FBS

PGC182

Log (% initial)

0 2h

0 2h

Actin

D 0.2% FBS

PGC182

Log (% initial)

0 2h

0 2h

Actin
Figure 10.

Ubiquitin-mediated degradation of PGC-1 alpha

A 10% FBS

CHX

CHX + MG132

0 2h

B

2.5

Log (% initial)

0 2h

t1/2=∞ MG132

t1/2=1.3h

C 0.2% FBS

0 2h

D

2.5

Log (% initial)

0 2h

t1/2=26h MG132

t1/2=0.4h
Ubiquitin proteasome dependent degradation of the transcriptional coactivator PGC-1alpha via the N-terminal pathway
Julie Trausch-Azar, Teresa C. Leone, Daniel P. Kelly and Alan L. Schwartz

J. Biol. Chem. published online August 15, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.131615

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts