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Phosphorylation of Human TFAM in Mitochondria Impairs DNA Binding and Promotes Degradation by the AAA+ Lon Protease

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SUMMARY

Human mitochondrial transcription factor A (TFAM) is a high-mobility group (HMG) protein at the nexus of mitochondrial DNA (mtDNA) replication, transcription, and inheritance. Little is known about the mechanisms underlying its posttranslational regulation. Here, we demonstrate that TFAM is phosphorylated within its HMG box 1 (HMG1) by cAMP-dependent protein kinase in mitochondria. HMG1 phosphorylation impairs the ability of TFAM to bind DNA and to activate transcription. We show that only DNA-free TFAM is degraded by the Lon protease, which is inhibited by the anticancer drug bortezomib. In cells with normal mtDNA levels, HMG1-phosphorylated TFAM is degraded by Lon. However, in cells with severe mtDNA deficits, nonphosphorylated TFAM is also degraded, as it is DNA free. Depleting Lon in these cells increases levels of TFAM and upregulates mtDNA content, albeit transiently. Phosphorylation and proteolysis thus provide mechanisms for rapid fine-tuning of TFAM function and abundance in mitochondria, which are crucial for maintaining and expressing mtDNA.

INTRODUCTION

Human mitochondrial transcription factor A (TFAM) is essential for mitochondrial DNA (mtDNA) synthesis and expression as well as mtDNA packaging (Asin-Cayuela and Gustafsson, 2007; Ekstrand et al., 2004; Kaufman et al., 2007; Kukat et al., 2011; Larsson et al., 1998). In animal models, a *TFAM* knockout in mice severely depletes mtDNA, abolishes oxidative phosphorylation and leads to embryonic lethality (Larsson et al., 1998). A heart-specific knockout results in cardiomyopathy during embryogenesis and neonatal death (Li et al., 2000). By contrast, TFAM overproduction in transgenic mice increases mtDNA content (Ekstrand et al., 2004; Larsson et al., 1998) and also ameliorates cardiac failure (Ikeuchi et al., 2005), neurodegeneration, and age-dependent deficits in brain function (Hokari et al., 2010). TFAM is the most abundant component of mitochondrial nucleoids, which are protein complexes associated with mtDNA that orchestrate genome replication, expression, and inheritance (Bogenhagen et al., 2008, 2012; Kukat et al., 2011). The in vivo packaging of mtDNA by TFAM has been estimated at ~35–50 (Cotney et al., 2007; Maniura-Weber et al., 2004) to ~1,000–1,700 molecules per genome (Ekstrand et al., 2004; Kanki et al., 2004; Kaufman et al., 2007; Kukat et al., 2011; Pellegrini and Scorrano, 2007). Higher TFAM:mtDNA ratios are interpreted as resulting in tighter compaction of mtDNA and reduced accessibility to transcription, replication, or repair factors, whereas lower ratios are predicted to permit increased accessibility. Recently, a debate has emerged as to whether TFAM is required for basal transcription, and whether it functions as both an activator and a repressor of transcription (Asin-Cayuela and Gustafsson, 2007; Falkenberg et al., 2002; Litonin et al., 2010; Lodeiro et al., 2012; Shi et al., 2012; Shutt et al., 2010; Sologub et al., 2009; Zollo et al., 2012). Further experiments are required to resolve this debate. Another fundamental question that has yet to be addressed pertains to the regulatory processes that control the binding and release cycle of TFAM at the mitochondrial genome.

Mitochondrial Lon belongs to the AAA+ family of proteins (ATPases associated with various cellular activities) and requires ATP hydrolysis to degrade proteins (Venkatesh et al., 2012). As a quality-control protease, human Lon selectively eliminates certain abnormal proteins (Bota and Davies, 2002). However, Lon also degrades some folded (Ondrovicová et al., 2005) and regulatory proteins (Granot et al., 2007; Tian et al., 2011). Although the majority of Lon is soluble within the matrix, it is also present in mitochondrial nucleoids (Bogenhagen et al., 2008). Lon binds mtDNA in a sequence-specific and strand-specific manner, showing low-affinity binding to single-stranded sequences on the heavy-strand that forms parallel G-quartets

(Chen et al., 2008; Liu et al., 2004). In cultured mammalian cells, Lon preferentially binds to the control region of mtDNA (Lu et al., 2007), which contains the heavy-strand promoter (HSP) and light-strand promoter (LSP) (Bonawitz et al., 2006; Falkenberg et al., 2007), as well as an origin of replication. Lon is thus uniquely poised at the mitochondrial genome to regulate mtDNA metabolism or to remodel nucleoid composition. In *Drosophila* cells with normal mtDNA content, the knockdown of Lon increases the levels of both TFAM protein and mtDNA, whereas overexpression of Lon decreases these levels (Matsushima et al., 2010). By contrast, in human cells with normal mtDNA content, changes in Lon expression do not alter TFAM or mtDNA levels (Lu et al., 2007) (Figures S1A–S1C available online). Such differences between flies and humans may be linked to phylogenetic diversity in the structure and metabolism of mtDNA. Interestingly, in human tissue or cells that are depleted of mtDNA, the protein levels of TFAM are dramatically reduced, even though transcript levels are the same as in control cells with mtDNA (Larsson et al., 1994; Seidel-Rogol and Shadel, 2002). These findings implicate Lon in the proteolytic turnover of TFAM in humans as well as in flies.

Here, we demonstrate that PKA-mediated phosphorylation of TFAM within high-mobility-group box 1 (HMG1) occurs inside the mitochondrion, resulting in rapid and selective degradation by the Lon protease. HMG1 phosphorylation of TFAM leads to DNA dissociation and reduced transcriptional activation. We propose that phosphorylation of TFAM within HMG1 causes electrostatic repulsion of the DNA phosphate backbone, thereby providing a mechanism for regulating mtDNA binding and release, which are essential for the maintenance and expression of the mitochondrial genome.

RESULTS

DNA-Bound TFAM Is Resistant to Lon-Mediated Proteolysis

We set out to test the hypothesis that Lon selectively degrades TFAM that is not bound to mtDNA. Purified TFAM and Lon (Figure S1D) were incubated with or without ATP/Mg^{2+}. TFAM was rapidly degraded by Lon only when ATP was present (Figure 1A). TFAM was not degraded by ClpXP, which is another AAA^+ protease in the mitochondrial matrix (Figures S1D–S1F). To determine whether DNA binding by TFAM affected proteolysis, single-stranded or double-stranded DNA (ssDNA or dsDNA, respectively) oligonucleotides were preincubated with TFAM prior to addition of Lon (Figure 1B); a higher Lon concentration was added to accelerate proteolysis and to accentuate the effect of DNA. We employed ssDNAs corresponding to heavy- and light-strand sequences upstream of LSP (LSP^{HS} and LSP^{LS}, respectively), as well as a dsDNA corresponding to the TFAM binding site ($dsDNA^{TFAM}$) (Figure 1B) (Dairaghi et al., 1995). DNAs with greater relative binding to TFAM conferred greater resistance to Lon (Figures 1B–1E). TFAM was strongly stabilized by LSP^{HS} and $dsDNA^{TFAM}$, but only marginally by LSP^{LS} (Figure 1B). Correspondingly, LSP^{HS} and $dsDNA^{TFAM}$ showed greater relative binding to TFAM compared to LSP^{LS} in gel shift and Southwestern assays (Figures 1C–1E). LSP^{HS} migrates as a fast-mobility linear ssDNA and as a slow-mobility G-quartet species on native gels (Figure 1C) (Chen et al., 2008; Liu et al., 2004).

Since Lon is a ssDNA-binding protein (Chen et al., 2008; Liu et al., 2004), its binding to LSP^{HS} or $dsDNA^{TFAM}$ may directly block its protease activity, resulting in TFAM stabilization. However, neither LSP^{HS} nor $dsDNA^{TFAM}$ inhibited Lon-mediated degradation of casein (Figure 1F). Although Lon shows the greatest relative affinity for LSP^{HS}, as compared to other mtDNA sequences (Chen et al., 2008; Liu et al., 2004), its binding to LSP^{HS} is substantially weaker than binding of TFAM to LSP^{HS} (unpublished results). Thus, it is not possible to test whether DNA-bound Lon degrades DNA-free TFAM.

Downregulation of Lon in Cells with Severe mtDNA Deficits Blocks TFAM Degradation and Increases mtDNA Content

The link between TFAM levels, mtDNA copy number, and Lon-mediated proteolysis was investigated in HeLa cells with either normal mtDNA content or severe mtDNA deficits. TFAM was strongly detected in cells with normal mtDNA levels (ρ^+ cells) (Figure 2A). By contrast, TFAM was barely detected in cells that were irreversibly depleted of mtDNA (ρ^0 cells) (Figure 2A). ρ^0 cells were generated by culturing ρ^+ cells with ethidium bromide for an extended period to deplete mtDNA, and then selecting the cells that were auxotrophic for pyruvate and uridine (King and Attardi, 1996). TFAM levels were also strikingly reduced in cells with low mtDNA content (ρ^{low} cells) (Figure 2A), which were generated by ethidium bromide incubation for a shorter time, resulting in cells that did not exhibit pyruvate and uridine auxotrophy. Although ρ^{low} cells had severely reduced mtDNA levels similar to ρ^0 cells (Figure S2), they still retained residual copies of the genome (see Figure 2C). To determine whether the reduced TFAM levels in ρ^{low} cells resulted from Lon-mediated proteolysis, we genetically knocked down Lon. A substantial increase in TFAM was observed upon transient siRNA knockdown of Lon, whereas TFAM levels remained unchanged in cells transfected with control siRNA (Figure 2B). By contrast, no upregulation of TFAM was observed when ClpP was knocked down (Figure 2B).

One potential consequence of upregulating TFAM by knocking down Lon in ρ^{low} cells is a coordinated increase in mtDNA. Lentiviral delivery of shRNAs targeting Lon was employed, as this approach showed higher efficiency, less toxicity, and lower background compared to transfections with siRNA or shRNA plasmids (data not shown). At 1 week, mtDNA copy number was essentially the same in control and Lon knockdown ρ^{low} cells. However, by ~2 weeks, the Lon depletion led to increased mtDNA copy number, which was reproducibly observed compared to the control (Figure 2C, 13 days). During this time period, Lon knockdown cells showed reduced levels of Lon and upregulated levels of TFAM (Figure 2D, 13, 17, and 27 days). By ~3 weeks, mtDNA copy number had declined in Lon knockdown cells (Figure 2C, 23 days), even though Lon was still depleted and TFAM remained elevated during this period (Figure 2D, 27 days). We speculate that the lentivirus-mediated knockdown of Lon in ρ^{low} cells only transiently increases mtDNA content even though TFAM remains elevated, because Lon is needed for mitochondrial homeostasis and genome