Metabolic control of G1–S transition: cyclin E degradation by p53-induced activation of the ubiquitin–proteasome system

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Cell cycle progression is precisely regulated by diverse extrinsic and intrinsic cellular factors. Previous genetic analysis in Drosophila melanogaster has shown that disruption of the mitochondrial electron transport chain activates a G1–S checkpoint as a result of a control of cyclin E by p53. This regulation does not involve activation of the p27 homologue dacapo in flies. We demonstrate that regulation of cyclin E is not at the level of transcription or translation. Rather, attenuated mitochondrial activity leads to transcriptional upregulation of the F-box protein archipelago, the Fbxw7 homologue in flies. We establish that archipelago and the proteasomal machinery contribute to degradation of cyclin E in response to mitochondrial dysfunction. Our work provides in vivo genetic evidence for p53-mediated integration of metabolic stress signals, which modulate the activity of the ubiquitin–proteasome system to degrade cyclin E protein and thereby impose cell cycle arrest.

Introduction

Molecular genetic analysis in Drosophila melanogaster has revealed that G1–S transition in mitosis can be modulated by the metabolic status of the cell (Mandal et al., 2005; Owusu-Ansah et al., 2008). Cells harboring a mutation in the gene encoding cytochrome C oxidase subunit Va (CoVa) of complex IV of the electron transport chain display a reduced level of cellular ATP and a consequent increase in the levels of cellular AMP. A signaling pathway that involves AMPK and p53 is then activated to reduce cyclin E protein level and cause G1–S arrest (Mandal et al., 2005). Unlike the results obtained in mammalian systems upon DNA damage induced by γ-irradiation (el-Deiry et al., 1993; Levine, 1997), this p53-mediated cyclin E effect in flies is not a consequence of Cdk inhibitor up-regulation, as the expression of dacapo, the p27 homologue in flies (de Nooij et al., 1996; Lane et al., 1996), is not affected in CoVa mutant cells (Mandal et al., 2005). The mechanism by which p53 controls cyclin E protein under conditions of metabolic stress was not well understood and forms the central focus of this study.

Progression from G1 to S phase of cell cycle is primarily regulated by the activity of the cyclin E–Cdk2 complex (Hwang and Clurman, 2005). An intricate balance between its timed synthesis and rapid degradation by the ubiquitin–proteasome system maintains the oscillating level of cyclin E during the cell cycle and ensures unidirectional and irreversible transition of a cell through the G1–S checkpoint (Reed, 2003). Processing by the ubiquitin–proteasome system involves the covalent attachment of ubiquitin molecules to the target protein followed by its degradation by the proteasome (Hershko, 2005). In flies, as well as in mammals, the ubiquitinylation of cyclin E is mediated by the Skp1–Cul1–F-box protein (SCF) complex (Nakayama and Nakayama, 2006). During normal cell cycle progression in mammals, recruitment of cyclin E to the SCF complex can be achieved by either of the two F-box proteins Fbxw7 or Skp2 (Nakayama et al., 2000; Koepp et al., 2001; Strohmaier et al., 2001), whereas in flies, only the Fbxw7

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homologue archipelago is involved in this process (Moberg et al., 2001). Further studies revealed the existence of a second cyclin E degradation mechanism involving Cullin 3, which occurs in an SCF-independent manner (Singer et al., 1999; Wimuttisuk and Singer, 2007). Together, these pathways are instrumental in achieving a rapid turnover of cyclin E during early S phase of the normal cell cycle.

In human and murine systems, p53 acts as a cellular hub for integrating diverse stress signals to generate different cellular responses that range from a block in cell cycle progression to the induction of apoptosis (Vousden and Lane, 2007; Jones and Thompson, 2009). In flies, the role of p53 has mainly been studied with relation to radiation-induced damage (Brodsky et al., 2000; Sogame et al., 2003). Based on those findings, coupled with the fact that there is no identified p21 homologue in flies and that dacapo (p27) is not responsive to irradiation (de Nooij et al., 1996), it was believed that in Drosophila, the p53 response to stress is primarily restricted to the activation of apoptosis and does not involve cell cycle control. However, our previous experiments indicated a role for p53 in promoting G1–S arrest in response to mitochondrial dysfunction (Mandal et al., 2005).

The following results unravel the mechanistic basis for this p53-induced G1–S block under conditions of attenuated mitochondrial function. Based on in vivo genetic analysis as well as in vitro studies involving CoVa mutants, we demonstrate that the activation of p53 under conditions of metabolic stress causes transcriptional up-regulation of the F-box protein archipelago. We establish a role for archipelago and the proteasomal machinery in the degradation of the cyclin E protein during mitochondrial dysfunction.

**Results and discussion**

The developing eye disc of Drosophila has been extensively used as a genetic model to understand mechanisms of intercellular signaling, pattern formation, and cell cycle control (Edgar and Lehner, 1996; Baker, 2001; Nagaraj and Banerjee, 2004). During the third larval instar, patterning of the retinal epithelium begins at the posterior end of the eye disc and progresses as a wave toward the anterior (Wolff and Ready, 1991). An indentation termed the morphogenetic furrow (MF) marks the leading edge of this wave (Fig. 1 A). Cells anterior to the furrow divide asynchronously but are arrested in the G1 phase at the MF. As the furrow progresses, a subset of these cells synchronously enters the S phase of a terminal round of cell division commonly termed the second mitotic wave (SMW; Fig. 1 A). These two distinct phases of cell division become apparent upon BrdU incorporation, a process that specifically marks cells in S phase (Fig. 1 B). This stereotyped pattern of incorporation is disrupted in eye discs bearing clones of CoVa mutations as the mutant tissue, either anterior or posterior to the MF, fail to incorporate BrdU (Fig. 1, compare B with C). However, a significant recovery in BrdU incorporation occurs in clones that are mutant for both CoVa and p53 (Fig. 1 D), suggesting that p53 functions downstream of CoVa in the pathway that is involved in the mitotic checkpoint control (Mandal et al., 2005).

Consistent with the synchronous entry of wild-type cells into S phase posterior to the MF, cyclin E is expressed in a narrow stripe of cells immediately posterior to the furrow (Fig. 1 E; Richardson et al., 1995). This expression of cyclin E is significantly reduced in CoVa clones (Fig. 1 F) but can be restored to normal levels if the cells are also mutant for p53 (Fig. 1 G). Loss of cyclin E in CoVa mutant cells is not at the level of transcription because wild-type amounts of cyclin E transcripts are expressed in CoVa-null mutant clones and in S2 cells in which CoVa is knocked down by RNAi (Fig. 1 L; Mandal et al., 2005). Consequently, in this study, we sought to describe a posttranscriptional mechanism that could account for the loss of cyclin E protein in CoVa mutant cells.

For a vast majority of known examples, regulation at the level of mRNA translation is achieved through the interaction of factors with untranslated sequences (5′ and 3′ untranslated regions [UTRs]) of the mRNA (de Moor et al., 2005; Pickering and Willis, 2005). We cloned the 5′ and 3′ UTRs of cyclin E mRNA and placed them upstream and downstream, respectively, of a reporter GFP coding sequence and expressed it in S2 cells under the control of an inducible metallothionein promoter (Fig. 1 H). These cells were transfected with either CoVa double-stranded RNA (dsRNA) or GST dsRNA (as control). No change in GFP expression was detected in CoVa dsRNA–treated cells compared with the control (Fig. 1, I–L). However, the CoVa-depleted cells showed a clear reduction in the level of endogenous cyclin E protein (Fig. 1 L). Consistent with earlier results, the expression of cyclin E transcript remains unchanged in CoVa dsRNA–treated cells (Fig. 1 M). These results reiterate that the control of cyclin E in the context of mitochondrial dysfunction is posttranscriptional, and based on these results, we consider it unlikely that the UTRs of the cyclin E transcript are involved in this regulation.

To consider whether the loss of cyclin E protein in the CoVa mutants resulted from its proteasomal degradation, we used a mutation in the gene l(3)73Ai, which encodes the β6 subunit of the 20S proteasome core complex (Saville and Belote, 1993). One copy loss of l(3)73Ai suppresses the CoVa mutant phenotype and causes the restoration of the normal pattern of BrdU incorporation (Fig. 1 N) and cyclin E expression (Fig. 1 O) in CoVa mutant clones. Interestingly, the glossy adult eye phenotype of CoVa mutant clones (Fig. 1, P–Q′, marked by the absence of red pigmentation) is also significantly suppressed (Fig. 1, R and R′). These results establish that cyclin E proteins can be restored to sufficiently high levels, allowing CoVa mutant cells to overcome the G1–S block upon attenuation of proteasomal function.

To further investigate a possible role for the ubiquitin–proteasome degradation pathway in the loss of cyclin E, we analyzed the genetic interaction between CoVa and genes that encode members of the SCF complex. One copy loss of archipelago (ago) could significantly suppress the BrdU defect in CoVa mutant clones (Fig. 2 A) and restore the normal expression of cyclin E (Fig. 2 B). A significant suppression of the CoVa adult eye phenotype was also seen in a genetic background that is heterozygous for ago (Fig. 2, C and C′) in addition to being CoVa+/CoVa+. In contrast, loss of one copy of
Cullin 1 or Cullin 3, other potential members responsible for ubiquitinylation of cyclin E, did not rescue the defects in BrdU incorporation (Fig. 2, D and E) in CoVa mutant clones. These dosage-sensitive interactions with ago suggest that the archipelago protein functions upstream of cyclin E and is therefore a potential target for p53. Consistent with this observation,
our experiments of differential gene expression profiling using microarrays showed an increase in the level of ago expression in S2 cells expressing RNAi for CoVa compared with those expressing GFP RNAi as control (Fig. 2 F). This up-regulation in ago expression in CoVa-depleted S2 cells was subsequently validated by real-time RT-PCR (Fig. 2 G).

As an in vivo correlate of this result, we observed that even in eye discs having clones of CoVa mutant cells, ago is expressed at a higher level compared with those discs having clones of wild-type cells (Fig. 3 A). Interestingly, this up-regulation in ago expression is dependent on p53, as its expression is restored to normal levels in eye discs having clones that are double mutant for CoVa and p53 (Fig. 3 A). Together, these results clearly establish that up-regulation of ago induced by metabolic stress in CoVa mutant cells is mediated by p53.

To determine whether this up-regulation of ago is directly controlled by p53, we initiated a biochemical approach. The p53 protein functions as a sequence-specific DNA-binding factor and can activate genes whose promoters contain a p53 response element (el-Deiry et al., 1992; Farmer et al., 1992). Within 100 bp upstream of the transcriptional initiation site of ago, we identified a 21-bp sequence (Fig. 3 B) that strongly resembles the consensus for the p53 DNA-binding site (el-Deiry et al., 1992). Like those found upstream of the human target genes mdm-2 (Wu et al., 1993) and p21/WAF1 (el-Deiry et al., 1993) or that of Drosophila apoptotic gene reaper (Brody et al., 2000), this putative p53-binding site upstream of ago contains two tandemly arrayed 10 mers separated by a single nucleotide spacer (Fig. 3 C). The putative p53-binding site upstream of ago is highly conserved among the members of the melanogaster subgroup of Drosophila that diverged over 10 million years ago (Fig. 3 D). Binding of p53 to this response element was investigated by EMSA. As shown in Fig. 3 E, p53 binds to the potential binding sequence, and this binding can be efficiently competed with unlabeled oligonucleotides or eliminated when the binding sequence is mutated. To find a functional correlate, we cloned the archipelago promoter having either a normal or mutated p53-binding site upstream of the firefly luciferase reporter gene (Fig. 3 F) and transfected S2 cells. We observed that upon knocking down CoVa while cells with a normal p53-binding site in ago promoter lead to an increase in firefly luciferase expression, cells with a mutated p53-binding site in ago promoter showed luciferase expression comparable with control cells. S2 cells having normal ago promoter upstream of luciferase treated with GFP dsRNA were used as controls (Fig. 3 G). These results strongly suggest that ago can function as a direct downstream target of p53.

A schematic of the signaling pathway linking attenuated mitochondrial function to proteasomal degradation of cyclin E unraveled primarily based on loss of function genetic analysis is shown in Fig. 3 H. We conclude that AMPK is used as a metabolic signal for distressed mitochondrial function to initiate a cascade that involves activation of AMPK and p53 (Mandal et al., 2005) followed by the transcriptional up-regulation of ago. This leads to ubiquitinylization of cyclin E by the SCF complex and its subsequent degradation by the proteasome. As a consequence, cell cycle progression is blocked at the G1–S transition. Such mitochondrially regulated checkpoints could be useful for a temporary arrest in the cell cycle to tide over conditions of energy deficiency or hypoxia. By initiating a G1–S checkpoint, a cell lacking in adequate ATP levels ensures that it is not irreversibly damaged during the S phase but can resume proliferation upon restoration of normal conditions.

Studies in mouse and human cell lines have demonstrated that the expression of CDC4b, the gene that encodes the Fbxw7β isoform, is dependent on irradiation-induced p53 activation and have implicated this protein in p53-mediated cell cycle arrest as a result of radiation damage (Kimura et al., 2003; Mao et al., 2004;
Figure 3. **ago is a direct downstream target of p53.** (A) Real-Time RTPCR analysis of ago transcripts in third instar larval eye discs having somatic clones of wild-type cells, CoVa mutant cells (CoVa−/−), or that of CoVa and p53 double-mutant cells (CoVa−/−, p53−/−). The up-regulation in ago transcripts as seen in eye discs with CoVa mutant cells is restored to almost wild-type level in eye discs with double-mutant clones of CoVa and p53. (B) Genomic structure of ago highlighting the p53 response element in the 5′ regulatory region of ago-RB and ago-RC transcripts that share the same promoter region. Blue text denotes the putative p53-binding sequence and the mRNA, ago-RC, is shown in the figure. (C) Alignment of the consensus p53-binding sequence with the p53 response element found in the 5′ regulatory region of ago. The invariant core nucleotides of each 10-mer motif matches at seven of eight positions, whereas the other mismatches (shown in lower case) occur at the outer positions of the 20-bp element. (D) Conserved sequence in the 5′ regulatory region of ago across the members of the melanogaster subfamily of Drosophila with the p53 response element highlighted in blue. (E) EMSA demonstrating the binding of purified p53 to its putative binding sequence in ago. (F) The reporter constructs used to investigate p53-dependent activation of ago promoter in CoVa mutant cells. Construct i consisted of the 200-bp of the ago promoter with normal p53-binding site cloned upstream of the firefly luciferase reporter gene. Construct ii is similar to construct i except for the p53-binding site being mutated. (G) Relative folds of activation of the reporter firefly luciferase in GFP dsRNA or CoVa dsRNA–treated cells. The datasets were normalized to the expression of Renilla luciferase, and mean values with standard deviation of three independent experiments are displayed. Compared with GFP dsRNA–transfected cells, CoVa dsRNA–transfected cells show almost 1.7-fold increase in the expression levels of firefly luciferase. However, this increase is not seen when the p53 site in ago promoter is mutated. Asterisks indicate a mutation in the P53-binding site (p53BS). (H) A model for the p53-mediated pathway linking attenuated mitochondrial function in CoVa mutants to G1–S block caused as a result of degradation of cyclin (E). Error bars indicate SEM.
Tb stocks and genetics

Drosophila

Cellular ATP level modulates the process of ubiquitinylation. Another, as they regulate the activity of the ubiquitin–proteasome system, only a handful of reports have provided insight into the independent mechanism that links the functioning of oxidative phosphorylation to transcriptional regulation of the E3 ligase ago.

From that perspective, this work provides the evidence of an involvement of the ubiquitin–proteasome system in transcriptional regulation of a large number of genes associated with cell cycle progression and apoptosis (Bhaumik and Malik, 2008) has drawn significant attention in recent years to understand the mechanism by which the activity of this system is regulated. Although it is imperative that the metabolic condition of a cell would have a control over the activity of the proteasome system, only a handful of reports have provided insight into the processes by which glucose metabolism can modulate the proteasomal function in flies and mammals (Zhang et al., 2007).

Involvement of the ubiquitin–proteasome system at two different levels. Although the cellular glucose level directly regulates the function of the proteasome, the cellular ATP level modulates the process of ubiquitinylaton.

Materials and methods

Drosophila stocks and genetics

The following Drosophila strains were used: y, w, ey-flp; FRT82B, CoVa/TM6B, Tb; and y, w, ey-flp; FRT82B, CoVa, p53/TM6B, Tb (Mandal et al., 2005); I(S)37T3A/I/TM6B, Tb (Saville and Belote, 1993); ago1, FRTBOB/TM6B, Tb (Moberg et al., 2001); cul152/CoVa (Ou et al., 2002); gfh152/CoVa (Cullin3; Mistry et al., 2004). Adult eye clones were made by using flies of the genotype y, w, ey-flp; FRT82B P[w+]cl-R3/TM6B Tb, y and mutant clones were marked by the absence of pigmentation. Clones in third instar larval eye discs were generated by using flies of the genotype y, w, ey-flp; FRT82B, UAS-GFP, RpS3/TM6B Tb, y+, and the clones were marked by the absence of GFP.

Immunohistochemistry

BrdU incorporation in the third instar larval eye imaginal disc was performed as described previously (de Nooj and Harinankar, 1995). In brief, the freshly dissected eye discs were incubated for 30 min in 400 μl 75 μg/ml BrdU in PBS followed by fixation in 4% formaldehyde. The tissues were incubated in primary antibody overnight at 4°C. Incubation in the corresponding secondary antibody was performed for 2 h at room temperature, and the tissues were rinsed in formaldehyde for 15 min before DNA denaturation by 2 N HCl. Subsequent incubations in anti-BrdU antibody and the corresponding secondary antibody were performed for overnight at 4°C and for 2 h at room temperature, respectively. The following antibodies were used: rat anti-BrdU (1:100; Abcam), mouse anti–cyclin E (1:50; H. Richardson, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia), guinea pig anti–cyclin E (1:150; T. Orr-Weaver, Massachusetts Institute of Technology, Cambridge, MA), mouse anti-GFP and rabbit anti-GFP (1:500; Invitrogen), and mouse anti-Armadillo (1:250; Iowab Hybriddoma Center). Cy3, FITC, or CY5-conjugated secondary antibodies (1:400; Jackson ImmunoResearch Laboratories, Inc.) were used. Acquisition of images was performed with a confocal microscope laser-scanning system (Radiance 2000; Bio-Rad Laboratories) and processed with a confocal assistant (Bio-Rad Laboratories).

Cell culture, RNAi, and microarray analysis

To prepare dsRNA, a coding sequence specific to CoVa, GST, or GFP was PCR amplified with primers carrying a 5′ T7 RNA polymerase-binding site. dsRNA was purified by using a Megascript RNAi kit (Ambion). S2 cells were transfected with 20 pg dsRNA as described previously (Clemens et al., 2000). Cells were harvested on 3, 5, and 7 d after transfection for protein analysis. Total RNA was isolated from the cells 3 d after transfection and was used to generate microarray probes that were hybridized to the Drosophila genome 2 arrays (Affymetrix). The Gene Chip Operating system (Affymetrix) and dCHIP program (Harvard University) were used to define present/absent calls and to generate pairwise comparisons between the transcription profile of GFP and CoVa dsRNA–treated S2 cells. The sequences of the primers to generate dsRNAs are provided in Table S1.

EMSA

This assay was performed using the Lightshift chemiluminescent EMSA kit (Thermo Fisher Scientific). Synthetic oligonucleotides containing the putative p53-binding sequence in ago, either wild type or mutated for both the core sequences, and the p53-binding sequence in mammalian p21 were biotinylated at their 5′ ends (IDT). The complementary oligonucleotides were then annealed. The probes were incubated for 20 min at room temperature with 20 ng of purified recombinant p53 protein (Active Motif) in the buffer supplied with the kit. This was followed by electrophoresis in 5% native gels and subsequent transfer onto a nylon membrane. Signals were detected using the standard protocol mentioned in the kit. The sequences of the different oligonucleotides used are provided in Table S2.

Real-time RT-PCR

RNA was extracted using the RNeasy Mini kit (Qiagen) and reverse transcribed with reverse transcription (Superscript II; Invitrogen). Real-Time PCR was performed on the cDNA according to the manufacturer’s protocol using iCycler (iQ; Bio-Rad Laboratories) with SYBR green as the fluorophore.

Firefly luciferase reporter assay

The proximal 200 bp up to and including the TATA box of the ago promoter having the p53-binding site either normal or mutated were cloned into the pGL3 basic vector containing firefly luciferase (Promega). S2 cells were transfected with either of these reporter constructs in conjunction with dsRNA for GFP (control) or CoVa using the calcium phosphate transfection method (Clemens et al., 2000). Transfection also included a luciferase reporter containing a minimal promoter linked to Renilla luciferase for normalization purposes. 7 d after transfection, the cells were assayed for firefly and Renilla luciferase activities in triplicate per the manufacturer’s protocol (Glomax Bioluminescence system; Promega).

Online supplemental material

Table S1 shows the list of PCR primers used for synthesis of dsRNA. Table S2 shows sequences of the oligonucleotides used for EMSA. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200912024/DC1.

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