Roles for the Human ATP-dependent Lon Protease in Mitochondrial DNA Maintenance*

Bin Lu, Swati Yadav, Parul G. Shah, Tong Liu, Bin Tian, Sebastian Pukszta, Nerissa Villaluna, Eva Kutejová, Carol S. Newlon, Janine H. Santos, and Carolyn K. Suzuki

From the Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry of New Jersey (UMDNJ)-New Jersey Medical School, Newark, New Jersey 07101, the Department of Pharmacology and Physiology, UMDNJ-New Jersey Medical School, Newark, New Jersey 07101, the Slovak Academy of Sciences, Institute of Molecular Biology, 845 51 Bratislava, Slovak Republic, and the Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, Newark, New Jersey 07101

Human mitochondrial Lon is an ATP-powered proteolytic machine that specifically binds to single-stranded G-rich DNA and RNA in vitro. However, it is unknown whether Lon binds mitochondrial DNA (mtDNA) in living cells or functions in mtDNA integrity. Here, we demonstrate that Lon interacts with the mitochondrial genome in cultured cells using mtDNA immunoprecipitation (mIP). Lon associates with sites distributed primarily within one-half of the genome and preferentially with the control region for mtDNA replication and transcription. Bioinformatic analysis of mIP data revealed a G-rich consensus sequence. Consistent with these findings, in vitro experiments showed that the affinity of Lon for single-stranded DNA oligonucleotides correlates with conformity to this consensus. To examine the role of Lon in mtDNA maintenance, cells carrying an inducible short hairpin RNA for Lon depletion were used. In control and Lon-depleted cells, mtDNA copy number was essentially the same in the presence or absence of oxidative stress. However, when oxidatively stressed, control cells exhibited an increased frequency of mtDNA lesions, whereas Lon-depleted cells showed little if any mtDNA damage. This suggests that oxidative mtDNA damage is permitted when Lon is present and prevented when Lon is substantially depleted. Upon oxidative stress, mIP showed reduced Lon binding to mtDNA; however binding to the control region was unaffected. It is unlikely that oxidative modification of Lon blocks its ability to bind DNA in vivo as results show that oxidized purified Lon retains sequence-specific DNA binding. Taken together, these results demonstrate that mtDNA binding is a physiological function of Lon and that cellular levels of Lon influence sensitivity to mtDNA damage. These findings suggest roles for Lon in linking protein and mtDNA quality control.

DNA binding by the ATP-dependent Lon (La) protease is evolutionarily conserved from bacteria to man, suggesting that it is an essential property of the protein (1–8). Purified bacterial Lon has been shown in some experiments to bind double-stranded DNA without apparent sequence specificity when assayed with relatively large DNA molecules (e.g. chromosomal, bacteriophage or plasmid DNA) (1–3). However, other data demonstrate sequence-specific binding of bacterial Lon to short double-stranded DNA (dsDNA) oligonucleotides (9). In contrast to the bacterial protease, mammalian Lon interacts with single-stranded DNA (ssDNA) but not dsDNA; sequence-specific binding to G-rich DNA as well as RNA has been demonstrated (6–8). Previous work has shown that ATP and its nonhydrolyzable analogs block Lon binding to ssDNA or ssRNA and that the presence of a protein substrate increases the DNA binding affinity of Lon 2–6-fold (7). Furthermore, order-of-addition experiments demonstrate that once protein substrate stimulates DNA binding, added nucleotide leads to DNA release and vice versa. Thus, nucleotide inhibition and protein substrate stimulation coordinately regulate DNA binding. Whether nucleic acid binding by Lon is a physiological function of the protease has not been demonstrated.

In bacteria, Lon-mediated proteolysis negatively regulates proteins that are directly involved in DNA and RNA metabolism (10–17). In eukaryotes, Lon resides within the mitochondrial matrix. Yeast mutants lacking the Lon ortholog Pim1p exhibit large mtDNA deletions (18, 19), and Lon-mediated proteolysis is required for the expression of mitochondrial tran-

The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Fig. S1.

1 To whom correspondence should be sent: Dept. of Biochemistry and Molecular Biology, UMDNJ-New Jersey Medical School, 185 South Orange Ave., Newark, NJ 07101. Tel.: 973-972-1555; Fax: 973-972-5594; E-mail: suzukick@umdnj.edu.

2 The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; ATP6 and -8, ATP synthase subunits 6 and 8; ChlP, chlamydonin immunoprecipitation; COX1–3, cytochrome oxidase subunits 1–3; CR, control region; cytb, cytochrome b; D-loop, displacement loop; Dox, doxycycline; Tet, tetracycline; EMSA, electromorpho mobility shift assay; MAST, motif alignment and search tool; MEME, multiple expectation maximization motif elicitation; mIP, mitochondrial DNA immunoprecipitation; mtDNA, mitochondrial DNA; mtTFA, mitochondrial transcription factor A; ND1–6, NADH dehydrogenase subunits 1–6; QPCR, quantitative PCR; FITC, fluorescein isothiocyanate; RNAi, RNA interference; LSP, light strand promoter; HSP, heavy strand promoter.

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scripts that contain introns (20). However, in higher eukaryotes it is unknown whether Lon is involved in the maintenance and expression of the mitochondrial genome.

Human mtDNA is a small circular genome of ~16.6 kb that encodes 13 proteins involved in oxidative phosphorylation, two ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs) (Fig. 1A) (21, 22). MtDNA consists of a heavy and light strand; the heavy strand is distinguished by a higher percentage of guanine and thymine residues resulting in its greater mass. Both rRNAs and all but one structural protein are encoded by the GT-rich heavy strand. In addition, mtDNA contains a noncoding region, referred to as the control region (CR), that carries the light and heavy strand promoters (LSP and HSP, respectively), as well as replication elements and the displacement loop (D-loop), which is a stable three-stranded structure composed of the parental strands and a partially replicated daughter molecule (see Fig. 1A) (23, 24).

In this study, we have used a combination of cell biology, biochemistry, and bioinformatics to answer several fundamental questions pertaining to the function of human Lon in mtDNA maintenance. Does human Lon bind to the mitochondrial genome in living cells, what is the nature of DNA sequences bound, and does Lon play a role in mtDNA integrity? We show that in cultured cells, Lon binds to mtDNA sites, which are distributed within one-half of the mitochondrial genome and associates preferentially with the CR. When cells are oxidatively stressed, Lon binds to significantly fewer sites within mtDNA; however, binding to CR is unaffected. The binding affinity of purified Lon for DNA oligonucleotides correlates with their conformity to the G-rich consensus sequence identified by bioinformatics. Consistent with mIP, purified Lon binds with highest affinity to a mtDNA sequence overlapping LSP within the CR. Cellular levels of Lon influence the sensitivity to H$_2$O$_2$-induced mtDNA lesions such that increased damage is observed when Lon levels are normal as compared with when Lon is down-regulated. Taken together, these results provide new insight into the potential roles of Lon in mtDNA metabolism, damage, and repair.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa and LS174T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 110 mg/ml sodium pyruvate, 50 µg/ml uridine, and 100 units/ml of penicillin/streptomycin (complete DMEM).

**Inducible RNA Interference (RNAi)**—The pTER vector system (25) was used for integrating and expressing an inducible short hairpin RNA under a doxycycline (Dox)-regulated H1 promoter. A 68-base oligonucleotide 5’-gatctcgcttgcttcggcagggcttttcgaagagaagagttgccggagcaagttttgaggtgtt-3’ (starting at position 476 of the Lon coding sequence) was annealed to its complementary sequence and cloned into pTER$. The siRNA (short interfering RNA) construct and a control construct lacking an oligonucleotide insert were stably integrated into LS174T cells that expressed the tetracycline (Tet) repressor resulting in LonRNAi or control cell lines, respectively. Clones were selected in complete DMEM supplemented with uridine (50 µg/ml), blasticidin (4 µg/ml), and zeocin (500 µg/ml); inducible depletion of Lon was determined by immunoblotting protein extracts isolated from cells incubated with or without Dox (4.0 µg/ml). Expression of the short hairpin RNA leads to substantial but not complete Lon depletion, as determined by metabolic labeling with $[^{35}$S]methionine and immunoprecipitation with anti-Lon antibodies (data not shown).

**Mitochondrial DNA ImmunoPrecipitation (mIP)**—HeLa or LS174T cells were cultured to 90% confluency. Formaldehyde cross-linking solution (11% formaldehyde, 50 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA) was added to 1/10th final volume and incubated for 15 min at 37 °C. Cross-linking was terminated by adding glycerol (125 mM final concentration) for 10 min at room temperature. Cells were harvested and incubated in lysis buffer (0.5% Triton X-100, 300 mM NaCl, 50 mM Tris-HCl, pH 7.4, containing leupeptin, 100 µg/ml, and phenylmethylsulfonyl fluoride, 200 µM) for 15 min on ice. Cellular DNA was sheared by sonication using a Misonix Ultrasonic Processor XL fitted with a microtip (setting 3) for 5 s followed by a pause for 30 s and repeated four times. The size of DNA fragments produced was between 500 and 900 base pairs as determined by agarose gel electrophoresis and ethidium bromide staining. Cell extracts (1 mg) were precleared with protein A-agarose; a rabbit anti-Lon antibody (8) that was affinity-purified or a nonspecific affinity-purified rabbit anti-interleukin-8 antibody (PeproTech 500-P2850) was added and incubated overnight at 4 °C. Protein A-agarose was added for 2 h at 4 °C to isolate protein-DNA complexes. Samples were washed three times with RIPA buffer (10 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.1% SDS, 0.1% deoxycholate, 1 mM EDTA, 1 mM EGTA) containing 140 mM NaCl; three times with RIPA buffer containing 500 mM NaCl; three times with LiCl buffer (10 mM Tris-HCl, pH 8.0, 0.25 mM LiCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA); and two times with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). TE buffer containing 0.5% SDS (200 µl) was added to the samples and incubated for 6 h at 65 °C to reverse the cross-links. To each reaction, 20 µl of 0.1 M NaOAc (pH 5.2), 20 µg of glycogen, and 500 µl of absolute ethanol were added and placed overnight at ~80 °C. The samples were centrifuged at 13,000 × g for 15 min at room temperature, and the pelleted material was washed with 70% ethanol, resuspended in 200 µl of MilliQ water containing 50 µg/ml RNase, and incubated for 1 h at 37 °C. Proteinase K (100 µg/ml) and SDS (0.25% final concentration) were added, and the mixture was incubated overnight at 37 °C. The samples were subjected to phenol/chloroform extraction followed by chloroform/isoamylalcohol extraction and DNA ethanol precipitation. The mIP mtDNA was suspended in 50 µl of 10 mM Tris, pH 8.0. Control mtDNA from HeLa cells (p$^+$) was purified using a Wizard Plus Miniprep DNA purification kit (Promega) according to the manufacturer’s instructions.

Control and mIP mtDNA were subjected to PCR analysis using 27 primer pairs; supplemental Table S1 shows the numerical assignment of primer pairs, the regions of mtDNA amplified, and the corresponding DNA sequences. PCR reactions were performed with 20 pmol of each primer in a 25-µl mixture (200 µM dNTPs, 1.5 mM MgCl$_2$; Applied Biosystems) using a Biometra TRIO 20 Thermoblock. Amplification conditions were: one cycle at 94 °C for 5 min followed by 35 cycles at 94 °C.
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for 1 min, 55 °C for 1 min, 72 °C for 1 min, and then finally one cycle at 72 °C for 5 min. Reactions were analyzed on an ethidium bromide-stained 1.3% agarose gel in TAE buffer (20 mM Tris acetate, 50 mM EDTA, pH 8.3).

Quantitative PCR (QPCR) of mtDNA—QPCR was performed with TaqMan primers and probes (supplemental Table S2) using 1 μl of the mIP DNA in a 50-μl mixture (1 × TaqMan Master Mix, each primer at 200 nM, 100 nM TaqMan probe). Thermal cycling was performed using the following parameters: AmpErase UNG activation was at 50 °C for 2 min, and AmpliTaq Gold activation was at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The amount of mtDNA amplified by QPCR from 1 μl of a mIP reaction was determined from standard curves using known concentrations of each target sequence (supplemental Fig. 1). This amount was multiplied by 50 to determine the total amount of mtDNA isolated by mIP/milligram of cell extract, as 1/50th of the mtDNA isolated by mIP was used for QPCR. The results of three independent mIP preparations were used to calculate the standard error of the mean presented in Fig. 1E.

Multiple Expectation Maximization Motif Elicitation (MIME) and Motif Alignment and Search Tool (MAST) Analysis—To identify a consensus sequence within the mIP amplified regions of mtDNA, computational analysis was performed using MIME (meme.sdsc.edu) (26). The training set included the 11 mtDNA regions amplified by mIP with the following parameter settings: MIME was set to find any number of nonoverlapping sites within each of the training set sequences; motif width setting was restricted to 15 nucleotides; both heavy and light strands of mtDNA were searched. To search sequences conforming to the MIME-derived motif within mtDNA (GenBank™ accession number NC_001807), analysis was performed using MAST (27).

H₂O₂ Treatment and Determination of mtDNA Copy Number and Integrity by Gene-specific QPCR—Cells were exposed to 100 μM H₂O₂ in serum-free medium for 1 h at 37 °C and then immediately washed in phosphate-buffered saline and harvested; QPCR was performed as described previously (28–30). Briefly, total DNA was isolated from control or LonRNAi cells with or without Dox and treated or untreated with H₂O₂. DNA was quantified using PicoGreen, and QPCR was performed with identical amounts of input DNA. Specific primers were used to amplify a fragment of the β-globin gene (13.5 kb) to determine nuclear DNA integrity; a large fragment of mtDNA (8.9 kb) to determine mtDNA integrity; and a small fragment (139 bp) of the mitochondrial genome to monitor changes in mtDNA copy number and to normalize the data obtained when amplifying the 8.9-kb fragment. Relative amplifications were calculated comparing H₂O₂ treated with respective controls; these values were used to estimate mathematically the number of lesions present in DNA, assuming a Poisson distribution (28–30). The data shown represent results from three independent experiments in which duplicate QPCR reactions for each target DNA region were performed; the standard error of the mean is indicated. Statistical significance was evaluated with Student’s unpaired t test.

Breakdown of H₂O₂—H₂O₂ concentrations in the culture medium were determined using Amplex Red reagent (Molecular Probes) according to the manufacturer’s protocol. Fluorescence was recorded in a microplate reader (Bio-Tek Synergy™ HT multi-detection microplate reader) with 528 nm excitation and 590 nm emission wavelengths. Standard curves were determined using known concentrations of H₂O₂ diluted in serum-free DMEM. Background fluorescence was measured using DMEM alone and subtracted from each value.

Oxidative Modification of Lon—Lon (4 μM) was incubated in the presence or absence of H₂O₂ for 60 min at 37 °C, after which 5 units of catalase was added and incubated for 15 min at room temperature. Carbonylation of Lon (20 pmol) was detected using the Oxyblot assay (Chemicon) according to the manufacturer’s specifications.

Protease, ATPase, and DNA Binding Assays—Recombinant human Lon was purified as described previously (7). Proteolytic activity was determined using the QuantiCleave protease assay kit (Pierce). Reactions were carried out according to the manufacturer’s specifications, and 150 nM Lon, 4 μM FITC-casein, 2 mM ATP, and 10 mM MgCl₂ were added. The ATPase activity of Lon was determined with the Malachite Green phosphate assay kit (BioAssay Systems) using the manufacturer’s protocol with 50 nM Lon and 400 nM casein to stimulate ATP hydrolysis. Lon binding to DNA oligonucleotides corresponding to sequences within human mtDNA (supplemental Table S3) was determined by electrophoretic mobility shift assay (EMSA) as described previously (7, 8). Briefly, DNA binding reactions (30 μl) were performed using purified Lon (10 pmol of Lon monomer) incubated with radiolabeled probe (2–3 pmol) in the presence of 20 mM HEPES, pH 7.4, 2 mM MgCl₂, and 0.02 mM dithiothreitol for 15 min at room temperature. In competition experiments, unlabeled oligonucleotides were added at a 10-, 50-, and 100-fold excess. Binding to DNA by H₂O₂-treated Lon was determined by Southwestern analysis using 40 pmol of Lon that was separated electrophoretically on SDS-PAGE and then transferred to nitrocellulose. The membrane was incubated in renaturation buffer (20 mM Tris, pH 8.0, 50 mM KCl, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) overnight at 4 °C and then incubated with probe (1.6 × 10⁶ cpm/ml) for 2 h at room temperature and washed with buffer to remove unbound probe; the bound probe was visualized by phosphorimaging.

RESULTS

Mitochondrial DNA Immunoprecipitation—To establish whether Lon binds to mtDNA in living cells, we utilized the mIP assay, adapted from well-established protocols for chromatin immunoprecipitation (ChIP). ChIP has been used to identify protein-DNA interactions regulating DNA replication and chromosome stability and transcription (31–33). Both ChIP and mIP provide a spatial map of DNA binding that may change depending, for example, on the cell cycle, intra- or extracellular signals, or response to stress. These assays are not aimed at demonstrating sequence specificity and affinity per se. Briefly, HeLa cells were treated with formaldehyde to cross-link protein to DNA and then harvested and lysed, and cellular DNA was sheared by sonication. Lon was immunoprecipitated from cell extracts, and co-precipitated mtDNA was identified by PCR using primer pairs that amplified overlapping regions of
FIGURE 1. A, human mtDNA consists of a heavy strand (thick circle) and a light strand (thin circle) that encodes the 12 S and 16 S rRNA and 22 tRNA genes (gray dots) and subunits of oxidative phosphorylation enzymes: NADH dehydrogenase subunits ND1–6 and ND4L; cyt b, cytochrome oxidase subunits COX1–3; and ATP synthase subunits ATP6 and ATP8. The CR of mtDNA replication and transcription includes the HSP and LSP (thin arrows), the D-loop, the origin of heavy strand replication (OH), and the partially replicated heavy strand within the D-loop (thick arrow).

B, 27 primer pairs (1–27) were used to amplify overlapping regions of mtDNA (supplemental Table S1); regions bound by Lon in HeLa cells are circled. The gene products encoded within the mIP-amplified regions are indicated; single letters (D, H, K, L, and S) indicate the respective tRNAs for aspartate, histidine, lysine, leucine, and serine.

C and D, agarose gel of PCR reactions containing either mIP mtDNA co-precipitated with Lon (C) or purified mtDNA (D). The mIP results shown are representative of at least 10 experiments. E, quantitative PCR was performed with mIP mtDNA co-precipitated with Lon from HeLa cells. Primer-probe sets were used for amplifying mIP regions 27 (D-loop/CR), 12 (ATP6/8), 17 (ND4), and 24 (cyt b). S.E. values were 2.7, 0.9, 1.2, and 1.6, respectively.
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A

MEIME derived multi-level consensus

5'TGTGGTTTTAGGGGGGGGGGGGTTGACTG3'

Lon footprint in LSPas

B

Distribution of sequences conforming to the Lon binding consensus

| Number of sites per mtDNA region | p-value ≤ 10^-6 | p-value = 10^-5 | p-value = 10^-4 |
|----------------------------------|-----------------|-----------------|-----------------|
| 1                               |                 |                 |                 |
| 2                               |                 |                 |                 |
| 3                               |                 |                 |                 |
| 4                               |                 |                 |                 |
| 5                               |                 |                 |                 |
| 6                               |                 |                 |                 |
| 7                               |                 |                 |                 |
| 8                               |                 |                 |                 |
| 9                               |                 |                 |                 |
| 10                              |                 |                 |                 |
| 11                              |                 |                 |                 |
| 12                              |                 |                 |                 |
| 13                              |                 |                 |                 |
| 14                              |                 |                 |                 |
| 15                              |                 |                 |                 |
| 16                              |                 |                 |                 |
| 17                              |                 |                 |                 |
| 18                              |                 |                 |                 |
| 19                              |                 |                 |                 |
| 20                              |                 |                 |                 |
| 21                              |                 |                 |                 |
| 22                              |                 |                 |                 |
| 23                              |                 |                 |                 |
| 24                              |                 |                 |                 |
| 25                              |                 |                 |                 |
| 26                              |                 |                 |                 |
| 27                              |                 |                 |                 |

FIGURE 2. A, MEME analysis was performed using the sequences of mIP-amplified mtDNA. The size of the letters representing guanine (G), adenine (A), thymine (T), and cytosine (C) corresponds to the relative occurrence of the nucleotide at each position. Beneath the consensus sequence is shown the Lon footprint within the LSPas oligonucleotide (6). B, distribution of Lon consensus sites in mtDNA regions amplified by mIP. Bars indicate the number of sites within each region having p values of ≤10^-6 (black), 10^-5 (gray), and 10^-4 (white). mIP regions amplified in HeLa cells (*) and in untreated LS174T cells (●) (see Fig. 5A, left panel).

The results of the mIP performed with HeLa cells showed that 11 PCR products were amplified using the primer pairs 9–12, 16, 17, 19, 21, 22, 24, and 27 (Fig. 1, B and C). The amplified regions contained sequences encoding the D-loop/CR, cytochrome b (cyt b), cytochrome c oxidase subunits (COX1 and -2), F1F0-ATP synthase subunits (ATPase 6 and 8), and NADH dehydrogenase subunits (ND4, -4L, -5, and -6) as well as several tRNAs. Control PCR reactions using purified HeLa mtDNA resulted in products of the expected size (Fig. 1D and supplemental Table S1). Negative controls were performed by immunoprecipitating Lon from HeLa cells that completely lacked mtDNA (HeLa ρ0). As expected, no PCR products were amplified from contaminating nuclear DNA. Negative controls were also performed using an irrelevant rabbit antibody recognizing interleukin-8; no PCR products were amplified (data not shown).

In the mIP assay, the intensity of the DNA bands visualized on agarose gels cannot be used to quantify or even estimate the amount of mtDNA bound by Lon. This is because in standard PCR reactions, amplification efficiencies are not identical for all primer pairs because of the relatively large size of the DNA products and sequence content differences. Therefore, QPCR was employed to determine the amounts of mtDNA co-precipitated with Lon in mIP assays. In these QPCR reactions relatively small DNA products were amplified and quantified in real time using a “probe” that fluoresces upon release of a quencher. Primer probe sets were designed to amplify mIP regions 12, 17, 24, and 27 that contained sequences encoding cyt b, ND4, ATP6/8, and the D-loop/CR, respectively (supplemental Table S2). The amount of mtDNA associated with Lon was determined from standard curves using known amounts of target mtDNA (supplemental Fig. S1). The cyt b region is of particular interest, as it is the mIP-amplified region closest to the D-loop/CR. Results demonstrated that ~3 times more D-loop/CR associated with Lon than with the other regions examined (Fig. 1E).

Bioinformatic Analysis of Lon Consensus Sites—Potential Lon binding sites within mIP-amplified mtDNA were identified by computational analysis using MEME (26, 34). MEME searched mtDNA with a motif width setting restricted to 15 nucleotides (2 nucleotides longer than the Lon DNA footprint). Previous footprinting analysis demonstrated that Lon protected a 13-nucleotide region closest to the D-loop/CR. Previous foot-printing analysis demonstrated that Lon protected a 13-nucleotide region closest to the D-loop/CR.

Consensus sequences with p values ≤10^-6 were located in only 11 of the mIP regions. Consensus sequences with p values on the order of
10\(^{-5}\) were located in 21 of the mIP region, whereas those on order of 10\(^{-4}\) were located throughout the mitochondrial genome and in all mIP regions. In HeLa cells, mIP results showed that Lon was bound to mtDNA regions having consensus sequences with the lowest \(p\) values; 8 of the 11 mIP regions amplified contained such sites. The sequence protected by Lon in DNA footprinting experiments that is located within mIP region 27 was among the top “hits” identified by MAST. These bioinformatic data make predictions about the binding of Lon to DNA sequences in vitro.

**Lon Binding to Consensus Sites in Vitro**—The mIP technique does not demonstrate the sequence specificity or affinity of a protein for DNA, nor does it distinguish between protein binding to single- or double-stranded DNA. In addition, it has not been possible to specify Lon binding sites that are <300 bp apart within mtDNA, because the generation of such small mtDNA fragments by sonication is limiting. Therefore, in vitro experiments were performed to test the relative affinity of Lon for consensus sequences within mIP amplified regions. Twenty-four-base oligonucleotides corresponding to consensus sites within mIP region 27 was among the top “hits” identified by MAST. These bioinformatic data make predictions about the binding of Lon to DNA sequences in vitro.

**FIGURE 3.** A and B, Lon binding to consensus sequences either inside (A) or outside (B) of mIP-amplified regions. Lon binding to \(^{32}\)P-labeled DNA oligonucleotides was analyzed by EMSA. MAST \(p\) values measuring the strength of the sequence match to the consensus motif shown in Fig. 2A are indicated. *, indicates sequence-specific Lon binding. C, competition of Lon binding to \(^{32}\)P-labeled 27/LSPas using unlabeled oligonucleotides at 10-, 50-, and 100-fold excess (left panel) and 100-fold excess (right panel).
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mtDNA Copy Number and Integrity in Cells Inducibly Depleted of Lon by RNAi—We sought to examine whether Lon has a physiological role in mtDNA maintenance. A stable cell line was produced that permitted the inducible depletion of Lon using the RNAi pTER system described by Clevers and colleagues (25). A DNA oligonucleotide that corresponded to a Lon-derived sequence (469–487 of the human cDNA) was prepared with sequence specificity and binds preferentially to stranded G-rich sequences throughout the mitochondrial genome with sequence specificity and binds preferentially to stranded G-rich sequences throughout the mitochondrial genome.

mtDNA copy number and integrity were determined using the QPCR method established by Van Houten and colleagues (28–30). mtDNA copy number is determined by amplifying a short fragment of the mitochondrial genome (139 base pairs), as there is a low probability of lesions present in such a small segment. By contrast, mtDNA lesion frequency is determined by amplifying a large fragment of the mitochondrial genome (8.9 kb), as the occurrence of errors is greater. The presence of various types of DNA lesions, such as strand breaks, oxidative damage, and bulky DNA adducts, will slow down or block the progression of DNA polymerase. Thus, an increased frequency of DNA lesions corresponds to decreased DNA amplification.

Total DNA was isolated from control and LonRNAi cells cultured with or without Dox for 15 days. In control and LonRNAi cells, no significant difference in mtDNA copy number was observed (data not shown). Parallel experiments showed that the frequency of mtDNA lesions was not significantly different in control or LonRNAi cells with or without Dox (Fig. 4B, left panel; compare bars 1, 3, 5, and 7).

Published work suggests that Lon is involved in the cellular response to oxidative stress; it has been reported that Lon degrades mildly oxidized aconitase and that Lon deficiency is correlated with the accumulation of carbonylated (i.e. oxidized) proteins (35–38). We examined the effect of oxidative stress on mtDNA copy number and lesion frequency in control and LonRNAi cells. The effects of reactive oxygen species have been routinely studied by exposing cells to H₂O₂. Within mitochondria, H₂O₂ is a normal byproduct of oxidative phosphorylation that can undergo Fenton chemistry, giving rise to highly reactive oxygen radicals (‘OH) that damage proteins, lipids, and nucleic acids. When cells expressing normal levels of Lon were treated with 0.1 mM H₂O₂ for 1 h at 37°C, a 6–8-fold increase in mtDNA lesions was observed as compared with untreated cells (Fig. 4B, left panel; compare bars 2, 4, and 6 with lanes 1, 3, and 5). The results obtained were comparable with published QPCR data demonstrating an increased frequency of mtDNA lesions induced by 0.1 mM H₂O₂ (39). To investigate whether Lon depletion influences mtDNA lesion frequency in cells subject to oxidative stress, LonRNAi cells were cultured with Dox for 15 days and then treated with 0.1 mM H₂O₂ for 1 h. In striking contrast to control cells, no significant increase in H₂O₂-induced mtDNA lesions was observed in Lon-depleted cells (Fig. 4B, compare lanes 7 and 8). Experiments performed in parallel showed that H₂O₂ had no effect on nuclear DNA integrity (Fig. 4B, right panel); analysis of lesion frequency within the β-globin gene showed no significant difference between control or LonRNAi cells cultured with or without Dox and treated with or without H₂O₂. In addition, no change in mtDNA copy number was observed in either control or LonRNAi cells with or without Dox and with or without H₂O₂ (data not shown).

mIP Analysis of Lon Binding to mtDNA in Oxidatively Stressed Cells—We investigated the effect of H₂O₂ treatment on mtDNA binding by Lon in the control LS174T parental line. H₂O₂ concentrations ranging from 0.05 to 0.4 mM were tested. A single dose of H₂O₂ (0.1 mM) for 60 min was determined to be
optimal, as higher concentrations led to visually detectable changes in cell morphology such as cell rounding or detachment. Published data demonstrate that a 60-min exposure to H$_2$O$_2$ between 0.1 and 0.4 mM has no detectable effect on the enzymatic activity of mitochondrial aconitase, whereas cytosolic aconitase is enzymatically compromised by such oxidative

Figure 4. A, control and Lon$_\text{RNAi}$ cells were cultured in the presence or absence of Dox (4 μg/ml) and treated with or without 100 μM H$_2$O$_2$. Cell extracts were immunoblotted for Lon and actin. B, control and Lon$_\text{RNAi}$ cells cultured with or without Dox for 15 days were treated with or without 100 μM H$_2$O$_2$ for 60 min at 37 °C. Results represent the average of three independent experiments ± S.E. Statistical significance was evaluated with unpaired Student’s t test. Left panel, total DNA was isolated and mtDNA integrity analyzed by QPCR; oxidative damage is expressed as mtDNA lesions/10 kb. S.E. values for samples 1–8 are 0.09, 0.18, 0.02, 0.02, 0.005, 0.24, 0.01, and 0.04, respectively; p values comparing samples 2 with 6, 4 with 8, and 7 with 8 are 0.85, 0.01, 0.04, and 0.15, respectively. Right panel, in parallel, total DNA was used to analyze nuclear DNA integrity; oxidative damage is expressed as nuclear DNA lesions/10 kb. S.E. values for samples 9–16 are 0.03, 0.06, 0.07, 0.07, 0.03, 0.12, 0.03, and 0.05, respectively; p values comparing samples 10 with 14, 12 with 16, 15 with 16, and 14 with 16 are 0.34, 0.17, 0.10, and 0.36, respectively. C, H$_2$O$_2$ concentrations in the medium of Lon$_\text{RNAi}$ cells cultured with or without Dox for 15 days and treated with 100 μM H$_2$O$_2$ for 60 min were determined using Amplex Red.
stress (60). Control cells were treated with or without 0.1 mM H$_2$O$_2$ for 1 h, and mIP was performed using anti-Lon antibodies to determine the regions of mtDNA bound by Lon. In the absence of H$_2$O$_2$, the regions of Lon binding to mtDNA in LS174T cells were largely overlapping with those observed in HeLa cells, in regions containing sites conforming to the consensus with low $p$ values of $<10^{-6}$ (Fig. 5A, left panel, and Fig. 2B). In LS174T cells additional Lon binding was also detected to mIP regions 1, 2, 4, 5, 8, 13, and 15; such differences were not unexpected, as discussed below. QPCR demonstrated that the D-loop/CR mIP 27 region was bound 3.4 times more than cyt b mIP 24 region (Fig. 5B), similar to the preferential binding observed in HeLa cells. Strikingly, exposure of cells to H$_2$O$_2$ significantly reduced Lon binding to mtDNA; the only mIP regions amplified were regions 3, 9, 12, 22, 24, and 27 (Fig. 5A right panel; see Fig. 1B). Reduced Lon binding to mtDNA was not attributable to an H$_2$O$_2$-induced reduction of Lon, as protein levels before and after exposure to H$_2$O$_2$ were essentially the same (see Fig. 4A). Thus, in oxidatively stressed cells, Lon binding to mtDNA is substantially reduced; however, binding to CR is unaffected.

**Oxidation of Purified Lon Blocks Enzymatic Activity but not DNA Binding**—In H$_2$O$_2$-treated cells, the interaction between Lon and mtDNA may be prevented by oxidative mtDNA damage resulting in the loss of available Lon binding sites that are occupied by repair proteins, or by the oxidative modification of Lon leading to its inability to bind mtDNA. To address whether oxidative modification affects Lon function, the purified protease was treated with H$_2$O$_2$ and assayed for its ability to (a) degrade protein substrate, (b) hydrolyze ATP, and (c) bind ssDNA. Lon was incubated in the absence or presence of increasing concentrations of H$_2$O$_2$ (0.1, 0.5, 1, and 4 mM) for 1 h at 37 °C after which catalase was added for 15 min at room temperature to remove the H$_2$O$_2$. Proteolytic activity was measured by Lon-mediated degradation of FITC-casein; casein has been routinely used to assay ATP-dependent proteolysis. Results showed that treatment of Lon with 0.1 mM H$_2$O$_2$ decreased proteolytic activity to 60%, and that at 0.5, 1, and 4 mM H$_2$O$_2$, protease activity was reduced to 18, 9, and 5%, respectively (Fig. 6A). ATP hydrolysis by Lon was determined by measuring the generation of orthophosphate. The presence of a protein substrate stimulates ATP hydrolysis, providing a robust signal, and thus casein was added in the ATPase assay. The data show that when Lon was treated with 0.1 mM H$_2$O$_2$, ATPase activity decreased to 30%, and no activity was detected after exposure to H$_2$O$_2$ 0.5 mM (Fig. 6B). Lastly, the effect of H$_2$O$_2$ treatment on Lon-dependent DNA binding to radiolabeled LSPas or LSPs was determined by EMSA. Results showed that H$_2$O$_2$ concentrations from 0.1 to 5 mM had no appreciable effect on sequence-specific binding to DNA by Lon (Fig. 6C).

We determined whether exposure of Lon to H$_2$O$_2$ resulted in carbonylation of amino acid side chains, which is the most general biomarker of oxidative damage. Carbonyl groups present on Lon were derivatized to 2, 4-dinitrophenylhydrazone and detected by immunoblotting using antibodies recognizing the dinitrophenyl moiety. Carbonylation of Lon was increased...
10-fold upon treatment with 0.1 mM H$_2$O$_2$; increasing carbonylation up to 40-fold was observed when Lon was treated with 0.5, 1, and 4 mM H$_2$O$_2$ (Fig. 6D). Taken together, these results demonstrate that Lon is sensitive to oxidation such that side chain carbonylation occurs at relatively low concentrations of H$_2$O$_2$. Although the ATPase and protease activities of Lon are oxidatively inactivated, DNA binding is largely resistant to such modification in vitro.

**DISCUSSION**

Little is understood about the regulation of mtDNA replication, transcription, and inheritance. However, the reconstitution of mtDNA replication and basal transcription in vitro (40, 41), the analysis of mtDNA using two-dimensional gel electrophoresis (42, 43), and assays for monitoring the morphological dynamics of mitochondria (44–46) and identifying protein components of mitochondrial nucleoids (47–51) have provided new information about the mechanisms underlying mtDNA stability and expression. The mIP assay described in this study will be useful as a general approach for analyzing the interaction of proteins with mtDNA under different cellular conditions.

The results of mIP show that Lon is associated primarily with mtDNA regions located between the D-loop/CR (bases 880-47) and COX1 (bases 15,593-6,790) (see Fig. 1B). Although the regions of mtDNA binding by Lon in HeLa and LS174T cells are largely overlapping at sites that conformed well to the consensus sequence ($p$ values of $\leq 10^{-6}$, see Fig. 2B, black bars), some differences were detected by mIP. mtDNA binding by Lon, as detected in the mIP assay, may vary depending on the state of the cell and between cell types as a result of the following four parameters: 1) the single-stranded state of mtDNA is a key determinant of whether a mtDNA region is bound by Lon; 2) the bioavailability of the mtDNA binding sites is critical, such as sites must not be masked by other proteins (e.g. mtSSB); 3) the affinity of Lon for a single-stranded mtDNA sequence will also contribute; 4) additionally, the total number of high and low affinity Lon binding sites present within a specific mIP region will contribute. The first two parameters will vary between different cell types because of differences in the levels of Lon, mtSSB, and other proteins involved in mtDNA replication and transcription. In addition, the mitochondrial environment of different cell types will likely influence the kinetics of the single-stranded versus double-stranded
mtDNA state and thus binding of Lon and other proteins. The last two parameters may also be affected by cell-specific polymorphisms in Lon or in mtDNA that lead to differences in Lon binding to the mitochondrial genome.

The strand displacement or asymmetric model of mtDNA replication holds that replication is initiated unidirectionally from the heavy strand origin within the CR such that the displaced parental G-rich heavy strand is progressively single-stranded (between cyt-b and COX1) until light strand synthesis is initiated further downstream (52, 53). The regions of mtDNA binding by Lon shown by mILP coincide with the displaced heavy strand during asymmetric mtDNA replication; it is possible that Lon has a role in modulating this process. Recent work, however, has questioned the strand displacement model and has supported instead a bidirectional strand-coupled mechanism (42, 43). Further experiments are required to determine the extent to which these alternate modes of mtDNA replication occur and whether Lon participates in either process.

Lon preferentially binds to a single-stranded sequence within the CR that overlaps not only the LSP sequence but also the binding site for transcription factor A of mitochondria (mtTFA or TFAM) (54). mtTFA is essential not only for transcription but also for mtDNA replication of the heavy strand, which requires short RNA transcripts generated from the LSP by mtTFA (53). It is possible that Lon and other proteins associate with the displaced G-rich heavy strand during transcription of the light strand and modulate the synthesis of both mtDNA and mtRNA. For example, our in vitro studies show that Lon selectively degrades both mtTFA as well as mtDNA polymerase γ catalytic subunit A (pol γA) only when these factors are not bound to DNA; when mtTFA or pol γA are bound to mtDNA they are resistant to Lon-mediated proteolysis. One can envisage that when mtTFA or pol γA dissociates from mtDNA as a result of protein interactions or environmental changes, Lon selectively degrades these proteins. Published data also show that Lon is associated with mitochondrial nucleoid proteins such as mtSSB and the recently identified PDIP38 (55). By binding to mtDNA, Lon would thus be poised to degrade and potentially regulate proteins involved in mtDNA replication and expression and to remodel protein components of mitochondrial nucleoids.

When cells are oxidatively stressed, the spatial map of Lon binding changes, and Lon is bound to fewer mtDNA regions. Reduced Lon binding may occur because DNA repair and replication proteins are recruited to the mitochondrial genome, effectively competing with Lon binding. Alternatively, Lon may be recruited away from the genome to degrade other proteins that become oxidatively damaged. Another possibility for reduced binding is that Lon is oxidatively modified and unable to bind mtDNA. This latter possibility is unlikely as Lon binding to D-loop/CR in vivo is unaffected by exposure of cells to H2O2. Furthermore, in vitro results show that oxidized/carbonylated Lon retains sequence-specific DNA binding (Fig. 6C).

Interestingly, Lon-depleted cells do not exhibit the increased number of H2O2-induced mtDNA lesions observed in controls (Fig. 4B). When Lon is present and bound to mtDNA, it may facilitate oxidative mtDNA damage by stabilizing ssDNA or by blocking lesion repair. By contrast, when Lon is absent, single-stranded mtDNA is perhaps less vulnerable to DNA damage and more efficiently repaired. Lon-depletion may also lead to higher steady state levels of substrate proteins that function as antioxidants (e.g. proteins with exposed thiol groups) (56, 57). In addition, reduction of Lon may lead to higher levels of repair proteins such as OGG1, NTH1, and NEIL1 (58, 59). We do not know whether mtDNA binding by Lon functions independently of its ATPase and/or protease activity. Further experiments are required to determine whether mtDNA binding by Lon facilitates oxidative damage to the genome and whether antioxidant proteins are stabilized or mtDNA repair processes up-regulated in Lon-depleted cells.

Studies show that mitochondrial Lon selectively degrades substrates such as aconitase, only when they are oxidized, suggesting that ATP-dependent proteolysis may be a key component in the defense against oxidative damage (35–37). However, the rate and function of the ATP-dependent proteases themselves that are exposed to reactive oxygen species and conditions of oxidative stress have not been examined. Indeed, published data show an age-dependent and organ-specific decline in ATP-dependent proteolysis within mitochondria that correlates with the accumulation of carbonylated proteins (35–38). The results presented here demonstrate that Lon is sensitive to oxidation and is perhaps even more susceptible to oxidation than substrates such as aconitase. When treated with 5 or 20 mM H2O2, aconitase retains 50 and 33% of its activity, respectively (35); at these concentrations of H2O2 Lon is almost enzymatically inactive. The effect of free radicals on Lon activity in mitochondria requires further investigation.

Future experiments will be aimed at determining the concomitant effects of oxidative stress on mitochondrial ATP-dependent proteolysis and substrate turnover and how the quality control systems that monitor protein and mtDNA integrity are integrated during metabolic changes such as oxidative stress and aging.

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