Mitochondrial Function/Dysfunction in Health and Disease

Mre11 is expressed in mammalian mitochondria where it binds to mitochondrial DNA

Natalia I. Dmitrieva,1 Daniela Malide,2 and Maurice B. Burg1
1Laboratory of Kidney and Electrolyte Metabolism and 2Light Microscopy Core Facility, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland

Submitted 3 January 2011; accepted in final form 9 June 2011

Dmitrieva NI, Malide D, Burg MB. Mre11 is expressed in mammalian mitochondria where it binds to mitochondrial DNA. Am J Physiol Regul Integr Comp Physiol 301: R632–R640, 2011. First published June 15, 2011; doi:10.1152/ajpregu.00853.2010.—Mre11 is a critical participant in upkeep of nuclear DNA, its repair, replication, meiosis, and maintenance of telomeres. The upkeep of mitochondrial DNA (mtDNA) is less well characterized, and whether Mre11 participates has been unknown. We previously found that high NaCl causes some of the Mre11 to leave the nucleus, but we did not then attempt to localize it within the cytoplasm. In the present studies, we find Mre11 in mitochondria isolated from primary renal cells and show that the amount of Mre11 in mitochondria increases with elevation of extracellular NaCl. We confirm the presence of Mre11 in the mitochondria of cells by confocal microscopy and show that some of the Mre11 colocalizes with mtDNA. Furthermore, crosslinking of Mre11 to DNA followed by Mre11 immunoprecipitation directly demonstrates that some Mre11 binds to mtDNA. Abundant Mre11 is also present in tissue sections from normal mouse kidneys, colocalized with mitochondria of proximal tubule and thick ascending limb cells. To explore whether distribution of Mre11 changes with cell differentiation, we used an experimental model of tubule formation by culturing primary kidney cells in Matrigel matrix. In nondifferentiated cells, Mre11 is mostly in the nucleus, but it becomes mostly cytoplasmic upon cell differentiation. We conclude that Mre11 is present in mitochondria where it binds to mtDNA and that the amount in mitochondria varies depending on cellular stress and differentiation. Our results suggest a role for Mre11 in the maintenance of genome integrity in mitochondria in addition to its previously known role in maintenance of nuclear DNA.

mitochondrial DNA damage; osmotic stress; NaCl; bleomycin
incubated for 90 min at 37°C in a humidified incubator (5% CO₂-95%
indicated. Cells were incubated at 37°C and gassed with 5% CO₂-95%
prepared by adding NaCl, was substituted for the control medium, as
of control medium, was 300–320 mosmol/kg. Hypertonic medium,
bovine serum (HyClone, Logan, UT), 2 mML-glutamine, 10,000
and down 10 times. The resulting suspension was centrifuged at 160
10,000 U/ml penicillin G and 10,000 U/ml streptomycin sulfate. Osmolality
and its function in maintenance of nuclear DNA.

MATERIALS AND METHODS

Cell Cultures

mIMCD3 cells. Subconfluent cultures of mIMCD3 cells (33) were
used in passages 13–17 and were grown in medium containing 45%
DMEM low glucose (Invitrogen, Carlsbad, CA), 45% F12 Coon’s
modification (cat. no. F6636; Sigma, St. Louis, MO), 10% fetal
bovine serum (HyClone, Logan, UT), 2 mM l-glutamine, 10,000
U/ml penicillin G and 10,000 U/ml streptomyacin sulfate. Osmolality
of control medium, was 300–320 mosmol/kg. Hypertonic medium,
prepared by adding NaCl, was substituted for the control medium, as
indicated. Cells were incubated at 37°C and gassed with 5% CO₂-95%
Preparation of mouse kidney primary cells. Whole kidneys from
one 2- to 3-mo-old mouse were cut in 2 × 2-mm pieces and digested
in DMEM without phenol red (Invitrogen), containing 10% FBS
(HyClone), 2 mM l-glutamine, 10,000 U/ml penicillin G, and 10,000
U/ml streptomyacin sulfate, and supplemented with 2 mg/ml collagen-
nase B (Roche Applied Science). The pieces of renal tissue were
incubated for 90 min at 37°C in a humidified incubator (5% CO₂-95%
O₂). The tissue suspension was mixed every 15 min by pipetting up
and down 10 times. The resulting suspension was centrifuged at 160
g for 1 min and washed 3 times with DMEM containing 10% fetal
bovine serum, 2 mM l-glutamine, 10,000 U/ml penicillin G, and
10,000 U/ml streptomyacin sulfate. The cell suspension obtained from
two kidneys was plated in four 10-cm plastic dishes. After they
became confluent (3–4 days), cells were harvested by trypsinization
and used for experiments.

Growing mouse kidney primary cells on Matrigel matrix. Primary
kidney cells, prepared as described above, were grown in a 1-mm
layer of Matrigel basement membrane matrix (cat. no. 356234; BD
Biosciences, Bedford, MA). After 7 days, some cells formed tubules
and other cells remained undifferentiated. Cells and tubules were
recovered from the Matrigel matrix using cell recovery solution (cat.
no. 354253; BD Biosciences) and stained for Mre11 as described
below. Also, some cells were exposed to high-NaCl media for 1 h
before recovery from the Matrigel.

Mice

Mice were purchased at age of 2–3 mo (cat. nos. 129S6; Taconic
Farms, Hudson, NY) and housed in the National Heart, Lung, and
Blood Institute (NHLBI) animal facility. All mouse study protocols
were reviewed and approved by the NHLBI, and mice were housed in
an Association for Assessment and Accreditation of Laboratory An-
imal Care-accredited facility.

Immunohistochemical Detection of Mre11 in Paraffin Kidney
Sections

Mouse kidneys were fixed overnight in 4% paraformaldehyde at
4°C, and then embedded in paraffin. Sections were cut and mounted
on silanized slides by American Histolabs (Gaithersburg, MD). Sec-
tions were stained with anti-Mre11 (cat. no. 4895; Cell Signaling
Technology, Danvers, MA) as previously described (12). A Nikon
E800 Widefield Microscope was used for photography.

Immunofluorescent Detection of Mre11 and Mytochondia in Frozen
Kidney Sections

Mouse kidneys were embedded in cryoembedding medium by the
face down cryoembedding technique (32) (Pathology Innovations,
Wyckoff, NJ). Sections were cut using a Leica cryostat and put on
microscope slides. Sections were fixed with 2.5% formaldehyde (cat.
no. 18814; Polyscience, Warrington, PA) for 10 min, washed 3 times,
5 min each, with 0.1% Triton X-100 in PBS (PBST) and treated with
3% H₂O₂ in PBS for 10 min to block endogenous peroxidase. Sections
were blocked with 3% BSA in PBST for 1 h, then incubated overnight
at 4°C with anti-Mre11 (rabbit) (cat. no. 4895; Cell Signaling Tech-
ology) and human anti-mitochondrial antigen (cat. no. HMS-0100;
Immunovision) antibodies, followed by 1 h incubation with secondary
antibodies, anti-rabbit-horseradish peroxidase (HRP), and anti-human
Alexa Fluor 633 (Invitrogen). HRP was detected using tyramide
signal amplification kit (cat. no. T20924; Invitrogen) in which thyra-
mide Alexa Fluor 568 deposit is produced by reaction with HRP.
After two washes with PBS, cells were stained with DAPI (DNA
stain) (Invitrogen), mounted with ProLong Gold anti-fade reagent
(Invitrogen), and subjected to microscopy (Leica SP1 laser scanning
confocal microscope).

Immunofluorescent Detection of Mre11 in Cells and Tubules
Recovered from Matrigel Matrix

Pelleted cells and tubules were fixed with 2.5% formaldehyde (cat.
no. 18814; Polyscience) for 10 min and then resuspended in 100%
methanol at −20°C. A drop of this methanol suspension was placed
on microscope slide and air dried. Slides were washed, blocked, and
stained with anti-Mre11 antibodies (cat. no. 4895; Cell Signaling
Technology) using Tyramide signal amplification kit (cat. no.
T20924; Invitrogen) and DAPI, as described for staining of frozen
kidney sections. Confocal microscopy images were taken with Leica
SP1 laser scanning confocal microscope (Leica Microsystems, Man-
heim, Germany). Series of images along the z-axis were collected
through the depth of the sample and reconstructed as three-dimen-
sional images using Imaris 7.2 (Bitplane, Zurich, Switzerland) soft-
ware. To assess changes in distribution over cytoplasm and nucleus,
the intensity line profile feature of Bitplane software was used:
confocal images were analyzed by drawing a line through cells in
transverse section, and fluorescence intensities along this line of each
channel were plotted as histograms. The profile of the DAPI channel
was used for nucleus positioning as previously described (43).

Immunofluorescent Detection of Mre11 in Primary Kidney Cells
and mIMCD3 Cells

Cells grown on eight chamber slides were fixed for 10 min in 2%
formaldehyde (cat. no. 18814; Polyscience) at room temperature,
washed with PBST, permeabilized with 0.1% Triton X-100 in PBST
and blocked with 3% bovine serum albumin for 1 h at room temperature.
Slides were incubated with primary antibodies for rabbit Mre11 (cat.
no. 4895; Cell Signaling Technology) and human mitochondrial antigen
(cat. no. HMS-0100; Immunovision, Springdale, AR) at 4°C
overnight, followed by secondary antibodies, anti-rabbit labeled with
Alexa Fluor 488 nm (green emission), and anti-human Alexa Fluor
630 (red emission; Invitrogen) at room temperature for 1 h. After two
washes with PBS, cells were stained with 2.5 μg/ml DAPI (DNA

AJP-Regul Integr Comp Physiol • VOL 301 • SEPTEMBER 2011 • www.ajpregu.org
Downloaded from http://ajpregu.physiology.org/ by 10.220.33.1 on June 19, 2017
stain; Invitrogen) and mounted with ProLong Gold antifade reagent (Invitrogen). Samples were examined by confocal microscopy using a Zeiss LSM 510 microscope (Carl Zeiss MicroImaging, Jena, Germany) with a ×63 NA 1.4 oil-immersion objective. Stacks of fluorescence images were captured sequentially to avoid bleed through, by using a 405-nm excitation and 385- to 470-nm emission for DAPI (2 channels were set: normal laser and detector gain to image nuclear DNA and higher laser and detector gain to image the weaker signal from mitochondrial DNA); 488-nm excitation and 505- to 550-nm emission for Alexa Fluor 488; and a 633-nm excitation and emission over 650 nm for Alexa Fluor 633. Images were deconvolved using Huygens software (Scientific Volume Imaging, Hilversum, Netherlands), prior to three-dimensional reconstruction and colocalization analyzes performed with Imaris 7.2 software (Bitplane). The degree of colocalization of Mre11 (green) and with mitochondria (red) was quantified in the three-dimensional data sets. In addition, voxels from the DNA channel were used to create a mask (white) excluding nuclear DNA. This mask was used as a region of interest to analyze colocalization of mtDNA (white) with Mre11 (green). Colocalized pixels (voxels in three-dimension) are displayed as a yellow (Mre11/mitochondria) or white (Mre11/mtDNA), respectively, overlapping the fluorescence channels over the images. The volume of Mre11 colocalized with mtDNA was compared between experimental conditions. Three series of images for each condition were analyzed in three separate experiments.

In Situ Fractionation Before Immunofluorescent Detection of Mre11

The cells, grown on eight well slides, were fixed with 2% formaldehyde and then treated for 10 min with either 0.1% Triton X-100 to permeabilize plasma membranes or with 1% Triton X-100 to also extract some soluble cytoplasmic and nuclear proteins. The cells were immunostained with rabbit anti-Mre11 (cat. no. 4895; Cell Signaling Technology) as described above.

Extraction and Quantification of Nuclear and Cytoplasmic Proteins

Nuclear and cytoplasmic proteins were extracted separately, using an NE-Per kit (cat. no. 78833; Pierce, Rockford, IL). Western blot analysis was performed by immunoblot of proteins separated by SDS-PAGE. ImmunobLOTS used primary antibodies against Mre11 (cat. no. 4895; Cell Signaling Technology) or white (Mre11/mRNA), respectively, overlapping the fluorescence channels over the images. The volume of Mre11 colocalized with mtDNA was compared between experimental conditions. Three series of images for each condition were analyzed in three separate experiments.

Analysis of Mre11 Binding to mtDNA by mtDNA Immunoprecipitation

Analysis of Mre11 binding to mtDNA was by mtDNA immunoprecipitation (see also Fig. 7 for an overview of the method). mIMCD3 cells were grown on 15-cm dishes. Mitochondria were isolated from two dishes per condition with a mitochondria isolation kit according to the manufacturer’s instructions by using the reagent-based method (cat. no. 89874; Pierce). Isolated mitochondria were fixed with 1% formaldehyde for 5 min on ice, spun down at 12,000 g for 10 min at 4°C, washed 2 times with ice-cold PBS, and lysed for 10 min on ice with 50 μl of chromatin immunoprecipitation lysis buffer (1% SDS), 50 mM Tris-HCl (pH 8.0), 10 mM EDTA. The Mre11 mtDNA-immunoprecipitation was performed using reagents from the enzymatic chromatin immunoprecipitation kit (cat. no. 9003; Cell Signaling Technology). The volume of each lystate was adjusted to 500 μl by the addition of 450 μl of chromatin immunoprecipitation buffer. For each sample, 50 μl of the lysate was saved for analysis of input mtDNA. Then 200 μl of the mitochondrial lysate was immunoprecipitated for 2 h with 4 μg of anti-Mre11 antibody (cat. no. 4895; Cell Signaling Technology) or normal rabbit IgG (cat. no. 2729; Cell Signaling Technology), followed by 1-h incubation with 30 μl of protein G magnetic beads (cat. no. 9006; Cell Signaling Technology). Captured Mre11 complexes were eluted from the beads, the proteins and DNA in the samples were enzymatically digested, and the DNA was purified by phenol-chloroform extraction in 1.5-ml phase lock gel light tubes (cat. no. 2900306; 5 Prime, Gaithersburg, MD), followed by ethanol precipitation. mtDNA in input samples and in Mre11 and IgG IP samples was quantified by real-time PCR with SYBR-Green PCR Kit (cat. no. 204054; QIAGEN, Valencia, CA). Eight primer pairs specific for mtDNA were designed using Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Sequences of the primers (5′-3′): forward: CGAAATGGCCGGCCCTCTTGAC; reverse: CCAATGGCGCCGCTGGCTAT; forward: CCACACCCACCAGGGACTCAGC; reverse: GTATGACCAGGCTGGCTGAC; forward: CGAGTGCTAGCGGACGCA; reverse: TGGTGGTCCCTTCCTCAGC; forward: TAGTACCACCCACCGGACGAC; reverse: TCGTATGACCAGCGTGCTG; forward: TCGGAAGCCTCGCCCTCACA; reverse: GGCTCAGGGCTTGTTGGC; forward: CCCACCCAGGCACCTCGACAGC; reverse: TATGACCAGGGCTGGCTGAC; forward: ACCCCACCGGACTCAGCAG; reverse: CGCGGTTGCGTGCAACAAAT; forward: CGCGCAAACAGGACCCCCGGC; reverse: CACGGTCAGGATACCCGGGC. Specificity of the primers was verified by gel electrophoresis of the PCR products, each producing a single band.

RESULTS

Occurrence of Mre11 in Mitochondria

We previously found that high-NaCl treatment of cell lines, including mIMCD3 cells, mouse embryonic and dermal fibroblasts (9), Hela cells (11), Cos7 and HT116 cells (Dmitrieva NI, unpublished observations), causes some nuclear Mre11 to translocate into cytoplasm. In the present studies, to test whether Mre11 translocation into cytoplasm after treatment with high NaCl exists beyond immortalized cells in culture, we tested primary kidney cells. We find that Mre11 is translocated to the cytoplasm after treatment of primary mouse kidney cells with high NaCl (Fig. 1, A and B top), similar to the result in transformed and/or immortalized cells. In addition, even when NaCl is not high (i.e., at 300 mosmol/kg), some Mre11 already exists in the cytoplasm, arranged in a punctuate pattern (Fig. 1). After treatment with high NaCl, the amount of Mre11 in cytoplasm increases, leading to diffuse bright staining. To determine whether after treatment with high NaCl, Mre11 in...
cytoplasm is soluble or confined within cytoplasmic bodies, we performed in situ fractionation. We extracted soluble proteins by treatment of cells grown on slides with 1% Triton X-100. This leaves punctate staining of Mre11 in the cytoplasm (Fig. 1B, bottom). These results suggested that Mre11 exists in the cytoplasm within organelles, and that, after treatment with high NaCl, additional Mre11 translocates from nucleus to cytoplasm, where it exists both freely dissolved and confined within organelles. In the studies that follow we identify mitochondria as a cytoplasmic organelle containing Mre11.

We screened by Western blot analysis for Mre11 among proteins extracted from mitochondria isolated with a mitochondria isolation kit. Mitochondria from primary renal cells apparently contain Mre11, and its abundance apparently increases within 2 h after NaCl is increased (Fig. 2A). However, this result is of itself inconclusive because the mitochondrial preparation is contaminated with proteins from other organelles, including golgin-97 (from golgi) and histone H2A (from chromatin) (Fig. 2B). Therefore, we used confocal immunofluorescence microscopy to test further for Mre11 in mitochondria. Mre11 colocalizes with anti-mitochondrial antigen both at 300 and 500 mosmol/kg (Fig. 3), confirming that it is present in mitochondria. When NaCl is elevated, some Mre11 is also seen in cytoplasm outside of mitochondria (Fig. 3 bottom), consistent with the Western blot analysis result (Fig. 2A).

**Location of Mre11 Within Mouse Kidney Cells In Vivo**

Immunohistochemical analysis of Mre11 in paraffin-embedded sections from normal mouse kidney shows subcellular localization of Mre11 that differs between cell types (Fig. 4). Some cells have only nuclear staining, while others exhibit strong cytoplasmic staining (Fig. 4A). Double staining for Mre11 and mitochondria in frozen kidney sections shows that cytoplasmic localization of Mre11 occurs mostly in mitochondrial-rich cells and that the Mre11 colocalizes with mitochondria (Fig. 4, B and C).

Our observation that Mre11 is preferentially located in mitochondria of mitochondrial-rich cells of the kidney in vivo, but is mostly located in nuclei of proliferating cells in culture, suggested to us that the divergent distributions of Mre11 might depend on differentiation and metabolism. Cells tend to differentiate in continuous culture. Also, mtDNA damage could be greater in mitochondrial-rich cells of proximal tubules and thick ascending limbs. Elevated oxidative metabolism in those cells is associated with rapid production of ROS in their mitochondria.
mitochondria, and the ROS could damage their DNA (1, 3, 46). In our next experiments we tested those possibilities.

**Effect of Cellular Differentiation on Subcellular Location of Mre11**

The Mre11 complex is important for DNA replication because it rapidly repairs replication fork-associated lesions, preventing accumulation of DNA damage (reviewed in Refs. 4 and 42). This role might favor retention of Mre11 in the nuclei of actively replicating cells, but not in less-rapidly proliferating differentiated cells. To test for a possible role of cellular differentiation on subcellular localization of Mre11, we grew primary kidney cells in BD Matrigel matrix, which at room temperature polymerizes to produce biologically active matrix material resembling the mammalian cellular basement membrane. When primary kidney

**Fig. 3. Detection of Mre11 in mitochondria of primary renal cells by immunocytochemistry.** Primary kidney cells were exposed for 2 h to high NaCl (final osmolality 500 mosmol/kg) or kept at 300 mosmol/kg. The cells were immunostained for Mre11 (green) and mitochondrial antigen (red). DNA was stained with DAPI (blue). Mre11 is present in mitochondria (colocalization) both at 300 and 500 mosmol/kg. When NaCl is high, some of the Mre11 in cytoplasm is separate from mitochondria, consistent with the Western blot analysis result shown in Fig 2.

**Fig. 4. In mouse kidney, Mre11 is mostly cytoplasmic in metabolically active mitochondria-rich cells where it colocalizes with mitochondria.** Analysis of subcellular localization of Mre11 in normal mouse kidney. A: immunohistochemistry of Mre11 in paraffin sections of normal mouse kidney. The subcellular distribution of Mre11 differs between cell types. Mre11 is mostly in the cytoplasm of some cells, but mostly in the nucleus of other cells. B and C: immunocytochemistry of Mre11 in frozen sections from normal mouse kidney. Sections are immunostained with anti-Mre11 (green) and anti-mitochondrial antigen (red) antibodies. DNA is stained with DAPI (blue). B: images scanned sequentially at different wave lengths. C: merged image of B. Much of Mre11 colocalizes with mitochondria.
cells are grown in Matrigel matrix, some differentiate and form tubules, while others continue to proliferate. We analyzed the subcellular location of Mre11 at different stages of differentiation and at different levels of NaCl (Fig. 5). At 300 mosmol/kg Mre11 is mostly in the nucleus of nondifferentiated cells (Fig. 5B, left), but it is mostly in the cytoplasm of cells that have formed tubules (Fig. 5A, left). These results are consistent with the idea that active replication favors retention of Mre11 in the nucleus, but slowing of replication as a result of differentiation favors localization of Mre11 in mitochondria where active metabolic processes can damage mtDNA. When NaCl is increased, Mre11 occurs in the cytoplasm of both nondifferentiated and differentiated cells (Fig. 5, A and B, right). High NaCl increases ROS of mitochondrial origin (47, 48). We suggest that high NaCl causes Mre11 to move into mitochondria in response to the resulting mtDNA damage.

Effect of mtDNA Damage on Colocalization of Mre11 with Mitochondrial DNA

To test the possibility that Mre11 participates in the response to mitochondrial DNA damage, we visualized mitochondrial Mre11 and DNA by confocal microscopy in mIMCD3 cells (Fig. 6). Mre11 staining forms foci within mitochondria (Fig. 6A). mtDNA is organized into discrete protein-DNA complexes called nucleoids (21, 37), which appear in cytoplasm as relatively large particles (DAPI staining, Fig. 6B). Some Mre11 foci colocalize with mtDNA (Fig. 6B), consistent with participation of Mre11 in mtDNA processing. Bleomycin induces DSBs in mitochondrial DNA, which the mitochondria repair within several hours (29, 34). We determined the effect of bleomycin on the location of Mre11 within mitochondria and the degree of its colocalization with mtDNA (Fig. 6, C and D). Bleomycin decreases the fraction of mitochondria volume occupied by Mre11 compared with control conditions (Fig. 6D, left). Visually Mre11 staining appears in more distinct foci within mitochondria in bleomycin-treated cells compared with control (Fig. 6C). In addition, bleomycin treatment increased a fraction of mtDNA that is colocalized with Mre11 (Fig. 6D, right). Taken together, our analyses indicates that within mitochondria Mre11 partially colocalizes with nucleoids and bleomycin treatment causes Mre11 to translocate to mtDNA, where it presumably participates in repair of the damage induced by bleomycin. Nevertheless, colocalization of Mre11 with nucleoids does not prove physical binding of Mre11 to mtDNA, and redistribution of Mre11 within mitochondria could also be caused by changes in mitochondrial structure after bleomycin treatment. Therefore, we tested more directly for binding of Mre11 to mtDNA.

Fig. 5. Differentiation of primary kidney cells into tubules causes Mre11 to translocate from nucleus to cytoplasm. Primary kidney cells were grown in Matrigel matrix. After 7 days, some cells formed tubules, other cells stayed undifferentiated. Tubules and cells were recovered from the matrix using matrix solubilization solution and immunostained with anti-Mre11 (red). Some tubules and cells were treated with high-NaCl medium for 1 h before recovery from the matrix. Nuclei were stained with DAPI (blue), and confocal images were collected through the depth of the structures. Three-dimensional images reconstructed from a series of images along the z-axis (see MATERIALS AND METHODS for details) of tubule-like structures (A) and of nondifferentiated cells (B) are displayed (top). A semiquantitative analysis of Mre11 nuclear cytoplasmic redistribution was performed using line profile of Bitplane software (bottom). Fluorescence intensities along the white lines sectioning the cells through the cytoplasm and nucleus are plotted for both Mre11 (red) and DAPI (blue); DAPI locates the position of the nuclei, and red indicates corresponding nuclear Mre11. The plots show marked differences in the ratio of cytoplasmic/nuclear Mre11 with differentiation. A: in tubule-like structures, Mre11 is mostly in the cytoplasm both at 300 and 500 mosmol/kg. B: in nondifferentiated cells, Mre11 is mostly in nuclei at 300 mosmol/kg and translocates to cytoplasm after treatment with high NaCl.
mtDNA-Immunoprecipitation Confirms Binding of Mre11 to mtDNA

We analyzed binding of Mre11 to mtDNA by a method similar to chromatin immunoprecipitation but performed on mtDNA. Proteins in isolated mitochondria were crosslinked to mtDNA by formaldehyde, mitochondria were lysed, and Mre11-mtDNA complexes were immunoprecipitated with anti-Mre11. mtDNA in Mre11 immunoprecipitates was quantified by real-time PCR (see MATERIALS AND METHODS and Fig. 7A). We call the method “mtDNA-IP.” Some Mre11 is already bound to mtDNA at 300 mosmol/kg, and raising NaCl significantly increases binding of Mre11 to mtDNA (Fig. 7B). However, the increased binding following bleomycin is not statistically significant (Fig. 7B).

DISCUSSION

Mitochondria are known to repair many types of damage to their DNA (26), but it has not been clear what DNA damage-response proteins are involved. Recombinational repair mechanisms apparently exist since in vitro studies demonstrated homologous recombination and end-joining activities in mammalian mitochondrial extracts (6, 25, 41). When DSBs are induced in mitochondria by restriction endonucleases, both intramolecular and intermolecular recombination products with large deletions appear (2, 19). The deletions are most likely mediated by DNA repair involving homologous recombination and nonhomologous end joining. Thus, mitochondria apparently repair DNA DSBs. However, few of the molecular components involved in this repair are known. Possible candidates are retinoblastoma protein (18), BRCA1 (5), and p53 (17), all of which have been identified in mitochondria. Our identification of Mre11 adds another. These proteins presumably are involved in maintaining the stability not only of the nuclear genome, but of the mitochondrial genome, as well.

Fig. 6. Bleomycin changes distribution of Mre11 within mitochondria, increasing its overlap with mtDNA. mIMCD3 cells were treated for 30 min with bleomycin (10 μg/ml), known to induce double-strand breaks in mtDNA. The cells were immunostained for Mre11 (green) and mitochondrial antigen (red). mtDNA was stained, by using a high concentration of DAPI (white). Images through the depth of the cells (z-axis), obtained by confocal microscopy, were deconvolved with Hyugens software and quantitative volumetric colocalization was analyzed with Imaris software. A: representative merged image showing colocalization (yellow) of Mre11 (green) with mitochondria (red) in cells under control conditions. B: enlargements of the boxed-area in A. Merged images are top left: Mre11 (green), mitochondria (red) and mtDNA (white, high gain). Top right: Mre11 and mitochondria; bottom left: mitochondria and mtDNA; bottom right: Mre11 and mtDNA. C: representative images showing colocalization (yellow) of Mre11 with mitochondria (red) in the control condition and after treatment with bleomycin. D: quantitative analysis of % of mitochondrial volume (left) or mtDNA volume (right) colocalized with Mre11 (means ± SE, n = 6–9, *P < 0.05). Bleomycin decreases the volume of mitochondria occupied by Mre11 and increases the volume of mtDNA colocalized with Mre11.

Fig. 7. Mre11 binds to mtDNA. A: overview of the method used to detect binding of Mre11 to mtDNA (see also Analysis of Mre11 Binding to mtDNA by mtDNA Immunoprecipitation). B: osmolality bathing mIMCD cells was increased by adding NaCl or bleomycin (20 μg/ml), as shown. Mitochondria were isolated and mtDNA bound to Mre11 was immunoprecipitated, purified, and quantified by RT-PCR as described in A and MATERIALS AND METHODS. Data are presented as mtDNA bound to Mre11 relative to mtDNA in IgG control (3–4 experiments, 8 mtDNA primers in each: means ± SE, n = 24–32, *P < 0.05, t-test). Mre11 is bound to mtDNA in controls (300 mosmol/kg) and binding increases when NaCl increases.
We find Mre11 in mitochondrion of unstressed, as well stressed, mammalian cells. The presence even in unstressed cells is not surprising since mitochondria constantly produce ROS, an unavoidable byproduct of oxidative phosphorylation. Proximity to mtDNA could lead to the higher steady-state level of oxidative damage in mitochondrial, compared with nuclear DNA (1, 22, 46). Oxidized DNA bases can be a source of DSBs for which function of Mre11 is well studied, because DSBs are generated by the collapse of replication forks when the replication machinery encounters the single-strand breaks that are intermediates in repair of oxidative damage (7). In addition to the well-described role of Mre11 in repair of DSBs, it also participates in base excision repair (38). Base excision repair is a major repair pathway for 8-oxoguanine lesions, the form of base damage most frequently induced by hydrogen peroxide, which is produced in mitochondria. Therefore, repair of mtDNA damage is a likely candidate for function of Mre11 in mitochondria. This notion is supported by several observations from our study. First, Mre11 is highly expressed in mitochondria of mitochondrial-rich cells of proximal tubules and thick ascending limbs in normal mouse kidney (Fig. 4), where mtDNA damage could be greater due to the elevated oxidative metabolism in those cells that is associated with rapid production of ROS in their mitochondria. Second, Mre11 is mostly in the nucleus of nondifferentiated cells, but it is mostly in the cytoplasm of cells that have formed tubules (Fig. 5). These results are consistent with the idea that active replication, which increases DNA breaks in nuclear DNA, favors retention of Mre11 in the nucleus, but slowing of replication as a result of differentiation favors localization of Mre11 in mitochondria, where active metabolic processes still can damage mtDNA. Third, high NaCl increases Mre11 in mitochondria (Fig. 2). That also might be caused by increased mtDNA damage, since mitochondrial ROS production is increased by high NaCl (47, 48). Fourth, colocalization of Mre11 with nucleoids within mitochondria (Fig. 6), which bleomycin increases, supports the idea that Mre11 participates in mtDNA metabolism and repair (Fig. 6). Fifth, physical binding of Mre11 to mtDNA, demonstrated by mtDNA-IP (Fig. 7), strengthens the inference that Mre11 participates in repair of mtDNA.

We conclude that Mre11 is present in mitochondria where it binds to mtDNA and that the amount in mitochondria varies, depending on cellular stress and differentiation. Our results suggest a role for Mre11 in the maintenance of genome integrity in mitochondria, in addition to its previously known role in maintenance of nuclear DNA.

**Perspectives and Significance**

Mutations in mtDNA have been linked to a number of human diseases. Among them are such common pathological conditions as neurogenerative disorders, diabetes mellitus, and cancer (reviewed in Ref. 16). Also, accumulation of damage to the mitochondrial genome is central in the mitochondrial theory of aging (1, 20, 22). DNA repair serves to reduce accumulation of mutations in mtDNA, but the mechanisms of mtDNA repair are not nearly as well understood as those of nuclear DNA repair. Many questions remain to be answered. Identification of the components of mtDNA repair pathways and understanding the mechanisms of the repair of different types of mtDNA damage will help understand how such pathological states develop and how they can be prevented or delayed. Finding that Mre11, a major contributor to nuclear DNA repair, is associated with mitochondrial DNA is a step in this direction.

**ACKNOWLEDGMENTS**

We thank Matthew Gastinger (Biitplan, Zurich, Switzerland) for assistance with colocalization analysis.

**GRANTS**

This work was supported by the National Institutes of Health Intramural Research Program.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**


AJP-Regul Integr Comp Physiol • VOL 301 • SEPTEMBER 2011 • www.ajpregu.org


