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Dependence of the structure and mechanics of metaphase chromosomes on oxidized cysteines

Adrienne Eastland¹, Jessica Hornick¹, Ryo Kawamura^{1,2}, Dhaval Nanavati³, and John F. Marko^{1,2}

¹ Department of Molecular Biosciences, Northwestern University, Evanston, IL 60208, USA

² Department of Physics and Astronomy, Northwestern University, Evanston, IL 60208, USA

³ Proteomics Core, Chemistry of Life Processes Institute, Northwestern University, Evanston, IL 60208, USA

Abstract

We have found that reagents that reduce oxidized cysteines lead to destabilization of metaphase chromosome folding, suggesting that chemically linked cysteine residues may play a structural role in mitotic chromosome organization, in accord with classical studies (Dounce AL, Chanda SK, Townes PL (1973) *J Theor Biol* 42:275-285, Sumner AT (1984) *J Cell Sci* 70:177-188). Human chromosomes isolated into buffer unfold when exposed to DTT or TCEP. In micromanipulation experiments which allow us to examine the mechanics of individual metaphase chromosomes, we have found that the gel-like elastic stiffness of native metaphase chromosomes is dramatically suppressed by DTT and TCEP, even before the chromosomes become appreciably unfolded. We also report protein labeling experiments on human metaphase chromosomes which allow us to tag oxidized and reduction-sensitive cysteine residues. PAGE analysis using fluorescent labels shows a small number of labeled bands. Mass spectrometry analysis of similarly labeled proteins provides a list of candidates for proteins with oxidized cysteines involved in chromosome organization, notably including components of condensin I, cohesin, the nucleosome-interacting proteins RCC1 and RCC2, as well as the RNA/DNA-binding protein NONO/p54NRB.

Keywords

Metaphase chromosome; chromosome structure; disulfide; cysteine

Introduction

When eukaryotic cells divide, genomic DNA must be equally segregated into both daughter cells. To accomplish this, chromosomes become compacted into their cylindrical mitotic form. Despite extensive investigation, our understanding of the organizational scheme of

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metaphase chromosomes remains incomplete. While it is well-established that specific proteins, notably condensin SMC complexes (Hirano and Mitchison, 1994, Ono et al., 2003) and topoisomerase II α (Maeshima and Laemmli, 2003, Samejima et al., 2012, Shintomi et al., 2015) play major roles in the process by which chromatin fibers are folded into the distinctively rod-shaped metaphase chromatids seen during mitosis, exactly how those proteins cooperate to organize chromatin on long length scales remains quite obscure. The situation has prompted some researchers to postulate that there are other, as yet unknown factors involved in metaphase folding (Vagnarelli et al., 2006).

Prior experiments (Dounce et al., 1973, Sumner, 1984a, Sumner, 1984b, Jeppesen and Morten, 1985) have suggested that oxidized cysteines (Lewis and Laemmli, 1982) may play a role in organizing metaphase chromosomes, via observations of unfolding of isolated chromosomes after exposure to reducing reagents. Other researchers have also reported the dependence of the organization of chromosomes on reducing reagents (Struchkov and Strazhevskaya, 1989, Struchkov and Strazhevskaya, 1994, Struchkov et al., 1992, Struchkov et al., 1995). While the effects reported in these experiments were clear, the idea that oxidized cysteines might play a role in stabilization of metaphase chromosome organization has not been a focus of much research, despite a suggestion based on proteomic analysis that condensin subunits may be disulfide-linked *in vivo* (Kuga et al., 2007).

Here we report experiments that examine this question, based on three different approaches. First, we isolated chromosomes from mitotic cells in bulk to examine the sensitivity of the metaphase structure to reducing reagents, and to directly visualize the production of reduced cysteines along the chromosome via fluorescent labeling. Second, we isolated individual metaphase chromosomes to study how their native, gel-like elasticity (Sun et al., 2011) is modified by exposure to reducing reagents. Finally, we used our oxidized-cysteine-labeling procedure to extract and analyze reduction-sensitive proteins from metaphase chromosomes: we found that oxidized-cysteine-labeled proteins can be observed as distinct bands on an SDS-PAGE gel. We then used bottom-up proteomics (Ohta et al., 2010) to identify the labeled proteins, providing a list of candidate metaphase chromosome proteins with oxidized cysteines.

Materials and Methods

Human cell culture

Human HEK 293 cells were grown in culture medium consisting of 89% DMEM (DMEM with high glucose, L-glutamax and pyruvate, Cellgro), 10% Fetal Bovine Serum (FBS, Cellgro), and 1% Penicillin/Streptomycin (P/S) mix (Optimediate). Cells were cultured at 37°C in 5% CO₂, in 10 mL tissue culture flasks (Falcon).

Isolation of metaphase chromosomes

Metaphase chromosomes were isolated from HEK 293 cells using a protocol developed by (Uchiyama et al., 2005, Sone et al., 2002) with minor modifications. Metaphase chromosomes were isolated from HEK293 cells incubated with 0.1 mg/mL colchicine for 12 h. After 12 h incubation most of cells were arrested at metaphase. Metaphase cells were

collected from culture flasks and centrifuged at $500 \times g$ for 5 min. Culture medium was removed and the collected cells were incubated with 75 mM KCl for 30 min for hypotonic treatment.

After centrifugation at $800 \times g$ for 5 min, KCl solution was removed and cells were incubated in citric acid solution (0.1 mM citric acid, 0.1 mM sucrose, 0.5% Tween-20, pH 2.6, Sigma) and gently pipetted up and down several times to lyse cells. Cells were centrifuged $200 \times g$ for 3 min and supernatant was collected. The supernatant is rich in chromosomes, while the precipitant is rich in nuclei. Collected chromosomes were resuspended in citric acid solution or in phosphate-buffered saline solution (PBS, Fisher Scientific). Optical microscopy was used to verify that contamination by nuclei and cell debris was minimal.

Chromosome imaging

An inverted optical microscope with a 60x, 1.25 N.A. objective (IX-81, Olympus) was used for imaging cells and chromosomes. HEK293 cells and chromosomes were imaged using either phase contrast or fluorescence imaging using an EMCCD camera (Hamamatsu).

Chromosome spreads

Isolated metaphase chromosomes were pelleted at $500 \times g$ for 5 min. The supernatant was removed and then the pellet was resuspended and gently homogenized. 1 mL of 3:1 methanol:glacial acetic acid fixative solution was added dropwise; then 4 mL of fixative was added slowly while tapping the bottom of the tube to mix. Tube was then spun at $500 \times g$ for 5 min and the fixation procedure was repeated. Then, supernatant was removed leaving about 1 mL of slightly milky solution. For fluorescence visualization experiments, solution was exchanged with 60% PBS with 100 nM 4',6-diamidino-2-phenylindole (DAPI) dye and incubated for 10 minutes. Finally approximately 20 μ L of the suspension was pipetted (1 to 3 drops) onto an alcohol cleaned slide. Slide was allowed to dry thoroughly and then examined under oil immersion at 60x magnification.

Single-chromosome isolation and micromanipulation

Single-chromosome experiments were carried out following the method of (Sun et al., 2011). In brief, cells were initially cultured in flasks were transferred to and then grown in wells prepared using #1 microscope glass onto which 25 mm-diameter rubber O-rings were affixed using paraffin. HEK293 cells were cultured in the wells under the conditions described above. Experiments were performed on ~70% confluent samples. Metaphase cells were identified by phase-contrast imaging.

Pipette micromanipulation and imaging were done on the stage of an inverted microscope (IX70; Olympus) using a 60X 1.4 NA oil immersion objective heated to 30 °C using a temperature-controlled objective heater (B600, 20/20 Technology Inc.). Cell imaging was done using a CCD camera (Pelco, DSP B&W) with images acquired by a frame grabber (IMAQ PCI-1408, National instruments); or a CCD camera (Ixon3, Andor). In brief, prometaphase cells were identified by phase-contrast imaging, and a micropipette with a ~3 μ m opening, filled with 0.05% v/v Triton X-100 (Fisher Scientific) in PBS, positioned near

the cell using a manual manipulator (Taurus, World Precision Instruments) was used to destabilize the cell membrane by microspraying.

After the chromosomes were released from the cell, two micropipettes with smaller $\sim 1 \mu\text{m}$ openings, filled with PBS and controlled by MP-285 motorized manipulators (Sutter Instruments), were positioned into the sample dish near the opened cell. One of the two pipettes was pulled with a rather short taper so it was stiff (essentially unbendable for the roughly few hundred piconewton or pN forces encountered in this experiment), while the second one was pulled with a long taper so as to have a softer tip with spring constant of $\approx 50 \text{ pN}/\mu\text{m}$. The stiff pipette was used to catch one end of a single chromosome using aspiration. Then the floppy pipette was attached to the other chromosome end.

For micromanipulation, the stiff pipette was moved at a rate of $\sim 2 \mu\text{m}/\text{min}$, slow enough to avoid viscoelastic effects (Poirier et al., 2000). Bending of the force pipette was recorded to monitor the force applied on chromosomes. Each extension-relaxation measurement was repeated at least 3 times to ensure its reproducibility. Micromechanical data were collected using image analysis software written in Labview (National Instruments).

Chemical treatments of single micromanipulated chromosomes

For single-chromosome microspraying, reagents were diluted in PBS. Solutions were loaded into the spray pipettes, pulled and cut to have a $\sim 5 \mu\text{m}$ opening. The spray pipettes were mounted on a three-axis manual manipulator (Taurus, World Precision Instruments), and positioned manually towards the isolated chromosome. Spraying was carried out for 10 mins with applied pressure of 10-100 Pa, and stopped for several minutes to allow spray solution (total volume $\sim 10 \mu\text{L}$) to diffuse away into the 1.8 mL sample dish (Sun et al., 2011, Poirier and Marko, 2002, Pope et al., 2006, Kawamura et al., 2010).

Cysteine reduction of whole chromosomes for microscopy

Isolated metaphase chromosomes were pelleted at $500 \times g$ for 5 min, and then the supernatant was replaced with PBS with reducing reagent added (5 mM DTT and 5 mM TCEP) as indicated. Chromosomes were incubated at room temperature for variable times and then imaged.

Cysteine labeling of whole chromosomes for fluorescence microscopy

Isolated chromosomes were treated with 10 mM maleimide in PBS for 90 min to block unreacted cysteines. The chromosomes were then pelleted at $500 \times g$ for 5 min and the supernatant was replaced with 5 mM TCEP in H50 buffer (50 mM NaCl, 1 mM EDTA, 5 mM HEPES, pH 6.9) for 60 min to reduce oxidized cysteines. Following pelleting, the supernatant was replaced with 100 μM fluorescein-5-maleimide in molecular grade water for 30 min, to place a fluorescent label onto the previously reduced cysteines. Following this final labeling step, the chromosomes were pelleted again and the supernatant was replaced with PBS and the chromosomes were resuspended for further study.

Cysteine labeling of whole chromosomes for protein analysis

Isolated chromosomes were treated with 10 mM maleimide in PBS for 90 min to block unreacted cysteines. The chromosomes were then pelleted at $500 \times g$ for 5 min and the supernatant was replaced with 5 mM TCEP in H50 buffer for 60 min to reduce oxidized cysteines. Following pelleting, the supernatant was replaced with 100 μ M N-ethylmaleimide in molecular grade water for 30 min, to place a mass-distinct label onto the previously reduced cysteines. Following this final labeling step, the chromosomes were pelleted again and the supernatant was replaced with PBS and the chromosomes were resuspended for further study.

Extraction of proteins from isolated chromosomes

For one-dimensional SDS-PAGE or proteomic mass spectrometry analysis, purified or labeled chromosomal proteins were extracted from metaphase chromosomes and concentrated with trichloroacetic acid (TCA) protein precipitation. One volume of 10% (w/v) TCA was added to four volumes of protein sample and incubated for 5 min at 4 C, then heated to 65 C for 5 min, and then incubated for 5 min at 4 C. Chromosomes were then pelleted by microcentrifuge at 14,000 RPM for 10 min. The supernatant was aspirated, leaving the protein pellet intact. The pellet was washed with 200 μ L cold (4 C) acetone, and then centrifuged for 5 min at 14,000 RPM. Two additional acetone washes were then done, and then the pellet was dried at 95 C for 10 min.

Following this, for PAGE-SDS gel analysis, protein was dissolved in sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue) before loading into a 4-20% protein gradient gel (BioRad). Alternately, for mass spectrometry analysis, protein was dissolved in PBS to a final concentration of 1 mg/ml.

Mass spectrometry analysis

NEM- and maleimide- labeled mixtures of chromosomal proteins were denatured with 8 M urea by incubating at 50 C for 60 min. After denaturation, proteins were reduced by adding 10 mM DTT (final concentration 1mM) and incubating at 50 C for 15 min. After reduction, proteins were alkylated by adding 100 mM iodoacetamide (final concentration 10 mM) and incubating in darkness at room temperature for 15 min. Subsequently, the urea concentration was reduced from 8 M to 1 M urea by adding 100mM ammonium bicarbonate, followed by proteolytic digestion by sequencing grade trypsin. The sample was digested at 37 C overnight.

The trypsinized samples were desalted using reverse phase C18 spin columns (Thermo Fisher Scientific, Rockford IL). The desalted peptides were further fractionated by strong cation exchange spin columns (Nest group, Microspin columns, SEM HIL-SCX25) into seven fractions (10mM, 25mM, 50mM, 100mM, 150mM, 200mM and 350mM potassium chloride). Each fraction was further desalted using reverse phase C18 spin columns (Thermo Fisher Scientific, Rockford IL). This KCl fractionation procedure increases the number of peptides which are detectable.

After desalting, peptides were loaded directly onto a 15 cm long, 75 μ M reversed phase capillary column (ProteoPep™ II C18, 300 Å, 5 μ m size, New Objective, Woburn MA) and separated with a 200 minute gradient from 5% acetonitrile to 100% acetonitrile on a Proxeon Easy n-LC II (Thermo Scientific, San Jose, CA). The peptides were directly eluted into an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA) with electrospray ionization at 350 nl/minute flow rate. The mass spectrometer was operated in data dependent mode, and for each MS1 precursor ion scan the ten most intense ions were selected from fragmentation by collision induced dissociation. The other parameters for mass spectrometry analysis were MS1 resolution of 60,000; normalized collision energy 35%; activation time 10 ms; isolation width 1.5; and rejection of +4 and higher charge states.

Data were processed using Proteome Discoverer (Version 1.4, Thermo Scientific, San Jose, CA) and searched using the SEQUEST search engine embedded in Proteome Discoverer. The data were searched against the human reference proteome (UNIPROT, Uniprot.org). Other parameters were: (i) enzyme specificity: trypsin; (ii) modification: methionine oxidation, maleimide and N-ethyl maleimide modification on cysteine; (iii) precursor mass tolerance: ± 10 ppm; and (iv) fragment ion mass tolerance: ± 0.8 Da. Spectra were tested against target/decoy databases and a targeted false discovery rate of 1% was set to achieve high confidence peptide assignments. Protein grouping was enabled in Proteome Discoverer and proteins were grouped to satisfy the rule of parsimony. Final protein identifications were considered valid only if supported by at least one uniquely assigned peptide match.

Mass spectrometry analysis of proteins with oxidized cysteines: HSA disulfide control

To test our three-step (maleimide blocking, TCEP reduction, modified-maleimide labeling) oxidized-cysteine-detection approach, we carried out experiments on maleimide-labeled human serum albumin (HSA, Sigma, St Louis, MO) using the trypsinization, desalting, mass spectrometry and data analysis procedures described above, the only difference being omission of the KCl fractionation step which is not necessary for a single-protein-species sample.

HSA is a 66 kD monomeric protein (585 residues) with 35 cysteines (C58, C77, C86, C99, C114, C115, C125, C148, C192, C193, C201, C224, C269, C270, C277, C289, C302, C303, C313, C340, C384, C385, C393, C416, C461, C462, C472, C485, C500, C501, C511, C538, C582, C583, C591), which in its native state contains up to 17 disulfide bonds. We carried out three-step labeling experiments with fluorescein-maleimide (Supplementary Fig. 1). The results of varied treatments are as expected, with fluorescent labeling strongest when TCEP reduction is used (compare lanes 5 and 6 of Supplementary Fig. 1B). TCEP reduction leads to a slower-migrating band in PAGE (lanes 4 and 6 of Supplementary Fig. 1, consistent with the reduction of intramolecular cysteines which should increase the conformational size of the molecule. We also note that for HAS which is treated with blocking maleimide and then fluorescein-maleimide (lane 2, Supplementary Fig. 1) there is low fluorescence, indicating a high degree of efficiency of the blocking reaction.

We carried out labeling of HSA for mass spectrometry in the same way as for fluorescein-maleimide, apart from use of a non-fluorescent maleimide mass label, N-ethylmaleimide (NEM; this mass label is more compatible with mass spectrometry than fluorescein-

maleimide). Mass spectrometry runs were carried out with the initial maleimide labeling only (Supplementary Spreadsheet 1) and with the three reaction steps (maleimide, TCEP, NEM, Supplementary Spreadsheet 2). With maleimide treatment alone, 2474 peptides were identified, of which 2291 were from HSA ($93 \pm 2\%$), indicating a low level of contamination and misidentification of peptides. 19 of the 2291 HSA peptides ($0.8 \pm 0.1\%$; C58, C125, C201, C416, C511; Supplementary Spreadsheet 1) were observed to be maleimide-labeled, consistent with the native HSA having mainly oxidized disulfides. We also note that zero NEM-labeled peptides were identified, indicating a low level of false positive ID of NEM labeling ($< 0.04\%$).

The three-step labeling procedure led to identification of 3239 peptides, of which 3178 were identified as being from HSA ($98 \pm 2\%$). Of the 3178 peptides from HSA, 16 of those carried a maleimide ($0.5 \pm 0.1\%$; C58, C99, C201, C289, C485, C511), consistent with the number of maleimide labels seen in the maleimide-only run. In contrast, 1041 NEM labeled peptides were identified ($33 \pm 1\%$ of the total HSA peptides). Labeled residues were C58, C77, C86, C99, C125, C114, C115, C148, C192, C193, C201, C224, C269, C270, C289, C302, C303, C313, C340, C384, C385, C393, C416, C472, C485, C500, C501, C511, C538 and C591. Since this is the majority (30/34) of the known disulfide-bonding residues in HSA, we concluded that the three-step labeling procedure combined with mass spectrometry can determine disulfide bond locations reliably. Only 62 HSA peptides were observed which contained an unmodified cysteine site, compared to the 1057 maleimide- or NEM-labeled peptides, consistent with a high efficiency of cysteine labeling ($94.4 \pm 0.6\%$).

Results

Isolated metaphase chromosomes spread on slides and viewed in solution

Human HEK293 cells were grown in tissue culture flasks, stalled in metaphase, and then chromosomes were extracted, following the approach of (Sone et al., 2002, Uchiyama et al., 2005). We examined traditional spreads following methanol-acetic acid fixation which showed the expected flattened, dual-chromatid morphology expected (Fig. 1A). The same preparation of isolated chromosomes, but unfixed and suspended in solution (referred to below as unfixed isolated metaphase chromosomes) were similar in length, size, phase contrast, and DAPI fluorescence, but showed a less clearly X-shaped morphology (Fig. 1B).

Visualization of unfolding of isolated unfixed metaphase chromosomes by reducing agents

We isolated metaphase chromosomes from metaphase-stalled HEK293 cells (no fixation), and replaced the buffer in which they were suspended with PBS containing either no reducing reagent, 5 mM tris(2-carboxyethyl)phosphine (TCEP), or 5 mM dithiothreitol (DTT). Then the chromosomes were incubated at room temperature, and at times of 0, 4, 8 and 15 hours we examined the isolated chromosomes using phase contrast imaging of chromosomes suspended in solution (Fig. 2). The untreated chromosomes showed little or no morphology change over the time course examined, but the TCEP and DTT-treated chromosomes gradually unfolded (Fig. 2).

Reducing reagents greatly reduce metaphase chromosome elastic stiffness

We proceeded to carry out experiments on individual chromosomes removed from cells, following the approach of (Sun et al., 2011, Hornick et al., 2015). Metaphase chromosomes were removed from unsynchronized HEK293 cells using glass micropipettes; they were quickly (10 minutes) attached to a second pipette so as to allow measurement of their elasticity, using bending of one of the pipettes to detect force.

Untreated human chromosomes displayed reversible elasticity (Fig. 3, traces labeled N), with a spring constant of between 50 and 200 pN/ μ m (meaning that a force of between 50 and 200 pN was needed per μ m of stretching extension; the spring constant depends on the length of the chromosome segment between the pipettes which varies from chromosome to chromosome, but which is typically 5 μ m).

Following measurement of the untreated chromosome elasticity, a third pipette loaded with PBS and reducing reagent (either 5 mM TCEP or DTT) was used to spray the isolated metaphase chromosome for 20 minutes. Then the elasticity was measured again, with the result that it was dramatically reduced (Fig. 3, traces labeled T or D indicate results with TCEP and DTT, respectively). Quantification of the elasticity before and after exposure of reducing reagents indicated more than an 80% reduction of chromosome spring constant (slope of force-extension curves). We note that this dramatic reduction of spring constant occurred over times much shorter (20 min, Fig. 3) than those required for chromosome unfolding (8 hrs, Fig. 2C). This is most likely a result of the lack of any force being applied to separate chromatin fibers in the unfolding experiments, which leaves diffusion to cause the unfolding observed in Fig. 2. In contrast, the force-extension experiments force the chromosome to unfold and lead to immediate observation of reducing-agent-induced structural changes.

We carried out additional experiments of this type on newt (*Notophthalmus viridescens*) chromosomes following the methods of (Kawamura et al., 2010, Poirier et al., 2000, Poirier and Marko, 2002). We observed the same effect of a large reduction in chromosome spring constant following exposure of chromosomes to reducing reagents for ~20 min (Supplementary Fig. 2).

Fluorescent labeling of oxidized cysteines in mitotic chromosomes

A three-step procedure was used to label oxidized cysteines on chromosomes. First, initially reduced (-SH) cysteines were irreversibly blocked by reaction with nonfluorescent maleimide; then oxidized cysteines were reduced using TCEP (which is unable to remove the maleimide blocking reagent); finally, the chromosomes were treated with fluorescein 5-maleimide. The first step permanently eliminates the possibility of detection of unoxidized cysteine residues; the second step reduces oxidized cysteines (including breaking pre-existing disulfides); the third step puts a fluorescent label on the cysteines liberated during the second step. We note that TCEP has the property that it can be displaced from cysteines so as to allow the third labeling reaction to occur (Brinkley, 1992, Hermanson, 1996).

Fig. 4 shows phase contrast (A,C,E) and corresponding fluorescence (B,D,F) images for chromosomes treated with different combinations of blocking, reduction and fluorescent

labeling. First, isolated chromosomes without any treatment show no fluorescence (B). Metaphase chromosomes that were treated with all three steps showed appreciable fluorescence (D). If metaphase chromosomes were treated only with fluorescein-maleimide, fluorescence was observed (F), via the labeling of unreacted and accessible cysteines on the chromosome.

In a series of such experiments, the fluorescence in images of metaphase chromosomes was quantified. Fig. 5A shows that fluorescence is observed when all three reactions (cysteine blocking, S-S reduction, cysteine labeling) are used (3rd bar), but if the reduction step is left out, there is a low level fluorescence (1st bar) consistent with background (2nd bar, maleimide block only). This indicates that the maleimide blocking reaction is efficient, leaving few cysteines that are available for binding by the subsequent fluorescein-maleimide labeling step. The 4th bar in Fig. 5A shows the result when the chromosomes are exposed to only fluorescein-maleimide; this indicates that there are exposed, accessible free cysteines on the metaphase chromosomes.

The labeling reactions finish promptly; we show results for a series of block-reduce-label experiments where the final incubation with the fluorescein-maleimide was carried out for a time between 5 and 60 minutes before the chromosomes were resuspended in PBS for imaging. The results indicate that the reactions reach their conclusion after at most 5 minutes (Fig. 5B).

In separate experiments, similar effects were seen on single newt metaphase chromosomes. In a three-step blocking-reduction-labeling procedure, but with reagents microsprayed instead of introduced in bulk solution, led to fluorescent labeling only after TCEP reduction step (Supplementary Fig. 3). We note that in labeling experiments on the larger newt chromosomes, we observed rather homogeneous labeling along the length of the chromosome (Supplementary Fig. 3).

SDS-PAGE analysis indicates distinct proteins with oxidized cysteines

To ascertain the nature of the distribution of proteins which are labeled by the three-step oxidized-cysteine-labeling procedure, we carried out an SDS-PAGE analysis on proteins extracted from oxidized-cysteine-labeled metaphase chromosomes. We first isolated metaphase chromosomes, and then carried out the three-step labeling procedure (maleimide, TCEP, fluorescein-maleimide, see Materials and Methods). We then used trichloroacetic acid precipitation to extract and concentrate chromosomal proteins, which were then separated on SDS-PAGE on 4-20% protein gradient gels (BioRad). The gels were stained with Coomassie Blue dye and imaged in visible light and with a fluoroiager (Typhoon 9400). An example gel, with lanes loaded with equivalent amounts of protein treated with different combinations of the three reaction steps is shown in Fig. 6. Coomassie staining (Fig. 6A) shows that the dominant protein species are low-molecular weight (~10 kD) histone proteins, and that there are a set of distinct higher-molecular-weight bands, as expected for proteins isolated from metaphase chromosomes (Takata et al., 2007, Ohta et al., 2010). Supplementary Fig. 4 compares the Coomassie-stained gel of Fig. 6A to a replicate gel experiment loaded with more protein; note that similar band patterns are obtained with

and without TCEP treatment (compare Fig. S4 lanes 5, 6 and 7) indicative that there is not an appreciable loss of protein associated with cysteine reduction.

A fluorescence image of the same gel (Fig. 6B) first shows that without any treatment (lane 2), or after treatment by the maleimide block only (lane 3), there is no background fluorescence. Lane 6 shows the result if no blocking or reduction is carried out before labeling; a large number of proteins pick up the label, indicating the typically unoxidized cysteines expected for cytoplasmic proteins. We note that band indicated by the red arrow, which is the expected position of histone H3, which carries one unoxidized cysteine. Lane 4 indicates that if maleimide blocking is done, then only trace amounts of labeling occurs (the blob at the bottom of Lane 4 is unreacted dye).

Lane 7 shows the result if all three steps are taken, which is the main result of the PAGE analysis. The bands indicate proteins which had cysteines which were not blocked, but which were accessible after subsequent TCEP reduction; these bands correspond to proteins with initially oxidized cysteines. Little of the low-molecular weight proteins (10 kD to 30 kD) pick up labels; in particular no labeling at the expected position of histone H3 (lane 7, red arrow) was observed. However, there are a series of well-labeled, distinct bands observable at higher molecular weights (35 kD, 50 kD, 80 kD, 135 kD). The PAGE analysis indicates that there are specific oxidized-cysteine-carrying protein species in the metaphase chromosome in the size range of 35 to 150 kD.

Mass spectrometry analysis of metaphase chromosome proteins with oxidized cysteines

We collected metaphase chromosomes, and carried out a three-step labeling procedure (maleimide, TCEP, NEM, see Materials and Methods). Mass spectrometry identified 22,619 distinct peptides which mapped to 3804 proteins (Supplementary Spreadsheet 3, listed in order of number of observations of peptide sequence matches or PSMs, which is a semi-quantitative measure of protein abundances). Of the total of 69,869 peptides identified, 10511 of them contained at least one cysteine; only 113 of these ($1.1 \pm 0.1\%$) contained any unmodified cysteine(s), indicating a high level of efficiency ($\sim 99\%$) of the blocking (maleimide) and reduction-sensitive (NEM) labeling similar to that seen for our HAS study. Finally, out of the cysteine-containing peptides, 6716 carried at least one NEM label.

The overall number and distribution of distinct proteins was rather similar to the ~ 4000 protein identifications reported in a comprehensive mass spectrometry analysis of chicken metaphase chromosomal proteins (Ohta et al., 2010). A valuable result from that work was a classification of identified proteins into a series of groups, including a set of 724 metaphase chromosomal proteins, the group of interest to us. We were able to use this classification for our protein set (Supplementary Spreadsheet 3, columns B-E), in order to deal with the general problem of cytoplasmic contamination of metaphase chromosomes (Ohta et al., 2010). Of the 3804 proteins identified, 322 of those are chromosomal, following the classification of (Ohta et al., 2010) (Supplementary Spreadsheet 4). The distribution of chromosomal proteins is quite similar to that observed by (Ohta et al., 2010) (Supplementary Fig. 5). Also, the total number of chromosomal proteins we observed is quite similar to the 240 proteins reported by (Uchiyama et al., 2005, Takata et al., 2007) based on mass spectrometry analysis of human HeLa cells.

Out of the 322 chromosomal proteins identified, there are 69 proteins which carry NEM labels, which are candidates for chromosomal proteins carrying oxidized cysteines, including disulfide bonds (Supplementary Materials Spreadsheet 5). We sorted these in order of abundance as judged by numbers of peptides identified and sum of PSMs for all peptides identified for each protein. The highest-abundance proteins include major structural proteins, including cohesin subunits SMC1 (143 kD) and SMC3 (141 kD), and condensin-I-specific subunits hCAP-D2 (157 kD), hCAP-G (114 kD), hCAP-H (81 kD), plus nucleosome-binding proteins RCC1 (45 kD), and RCC2 (56 kD). Notably, none of the condensin-II-specific subunits (hCAP-D3, hCAP-G2 and hCAP-H2) were observed, suggesting that only condensin I has oxidized cysteines. Also, the nucleic-acid binding protein NONO/p54NRB (non-POU-domain-containing octamer-binding protein) was found to be NEM-labeled.

Discussion

We have reported experiments which suggest that the neatly folded structures of metaphase chromosomes from animal cells are sensitive to the cysteine-reducing reagents TCEP and DTT. In accord with older experiments (Dounce et al., 1973, Sumner, 1984a), we find that chromosomes isolated from cells by bulk techniques gradually unfold if exposed to reducing reagents (Fig. 2). Using single-chromosome isolation and micromanipulation (Marko, 2008), we find that individual chromosomes lose their characteristic elasticity (Sun et al., 2011) when exposed to the same reducing reagents (Fig. 3, Supplementary Fig. 2). This effect is consistent with removal of chromatin “cross-linking” constraints. The observation of these effects in both mammalian and amphibian species, and in quite different chromosome isolation experiments, suggests that sensitivity of structure to oxidized-cysteine reduction is a general feature of metaphase chromosomes.

We also have used a blocking-reducing-labeling scheme to replace oxidized cysteines with chemical tags on chromosome proteins, which we have used on bulk-isolated (Fig. 4) as well as on singly-isolated and micromanipulated (Supplementary Fig. 3) metaphase chromosomes. When labeled proteins were extracted from bulk-isolated chromosomes for PAGE analysis, we found that there is a relatively small set of protein species carrying labels indicating oxidized cysteines (Fig. 6).

Finally, we have used mass spectrometry to identify oxidized-cysteine-labeled protein species using bottom-up proteomics. This has led us to a list of labeled proteins which provides a candidate list for future studies. The most frequently observed proteins related to chromosome structure include cohesin (SMC1 and SMC3), condensin I subunits (CAP-G, CAP-H and CAP-D2), RCC1 and RCC2, and p54NRB (Supplementary Table 5). Interestingly, no condensin II subunits were observed to carry oxidized cysteines. We note that RCC1 is known to bind nucleosomes (Makde et al., 2010) and may have a structural role in chromatin folding as well as its well-known role in regulation of Ran GTPase.

We caution that our mass spectrometry assay can only qualitatively indicate abundances of proteins and oxidized cysteines on them. The coverage of the observed peptides ranges over 10% to 50% of the identified proteins’ sequences, and the efficiency with which each peptide is detected is a large unknown of this type of analysis. However, our results do

suggest that on a small subset of chromosomal proteins, oxidized cysteines may be quite common; for example, 6 of the 12 cysteine-containing peptides detected for the condensin I subunit hCAP-D2 carried an NEM label (Supplementary Table 5). We hope to develop more quantitative techniques for analyzing the occurrence of oxidized peptides in the near future.

One might argue that the oxidized cysteines observed in our experiments were not formed *in vivo*, but instead were due to oxidation that occurred after chromosome removal (the isolation buffers are necessarily devoid of reducing reagents). While this is possible, the result that oxidized-cysteine reducing reagent treatment leads to rather complete unfolding of chromosomes compared to their *in vivo* morphology [(Dounce et al., 1973, Sumner, 1984a) and Fig. 2] argues against this. Also, comparison of the robust elasticity of metaphase chromosomes consistent with that observed *in vivo* (Nicklas, 1963, Nicklas, 1983, Marko, 2008, Sun et al., 2011) with the much lower elastic response observed after oxidized-cysteine reduction (Fig. 3) suggests that there is not gross cysteine-cysteine crosslinking occurring in metaphase chromosomes immediately after their isolation.

Our results might also be considered to be conflict with the common assumption that cysteines are in a reduced state in the reducing conditions of the cytoplasm and that disulfides are formed enzymatically in the endoplasmic reticulum primarily in secreted proteins (Patil et al., 2015, Sevier and Kaiser, 2002, Bulleid and Ellgaard, 2011, Hudson et al., 2015). However, disulfide bonding of the condensin subunit CAP-G (Kuga et al., 2007), RCC1 (Chatterjee and Paschal, 2015), and NONO/p54NRB (Hwang et al., 2009) have been observed in other studies.

The notion that oxidized-cysteine-based bonds such as disulfides might form on metaphase chromosomes during mitosis may be related to observations of oxidative intracellular conditions during mitosis. The hypothesis that redox conditions vary with the cell cycle traces back to old papers tracking the cell-cycle dependence of intracellular thiols in sea urchin eggs (Rapkine, 1931), with a peak in thiol staining occurring during metaphase (Kawamura and Dan, 1958). More recent studies report that oxidizing conditions occur in the cell during mitosis (Menon and Goswami, 2007, Menon et al., 2003, Sarsour et al., 2009, Sarsour et al., 2014). Other studies have found that mitosis can be affected by changes in redox conditions (Olszewska et al., 1990), that topoisomerase II α translation is coupled to oxidative cellular conditions (Goswami et al., 2000), and that some cell-cycle-dependent proteins are disulfide-bonded during mitosis (Meikrantz et al., 1990).

Combining our results with these prior studies suggests that metaphase chromosomes may be stabilized by disulfide bonds or other chemical bonds involving oxidative reaction of cysteines (Lewis and Laemmli, 1982). Such chemical bonds may form intracellularly enzymatically, possibly aided by oxidizing cytoplasmic conditions that occur during mitosis. The appearance of linkages based on cysteine oxidation may be an adaptation which imparts the mechanical stability to chromosomes needed for them to survive the mechanical rigors of mitosis. Our proteomic data suggests that SMC complexes and other chromosome-organizing proteins may carry oxidized cysteines. Future advances in proteomics of oxidized proteins (Yang et al., 2016) may facilitate more precise determination of the role of cysteine modifications in metaphase chromosomes.

Finally, while in this paper we have focused on examining oxidized cysteines in metaphase chromosomes, the same methods could be applied to chromosomal proteins at other points in the cell cycle. It would be of interest to carry out the same analysis on G1 nuclei in order to determine whether cysteine oxidation shows similar patterns to those reported here during interphase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CAP	chromosome-associated protein
DAPI	2-(4-amidinophenyl)-1H-indole-6-carboxamide DTT dithiothreitol
FBS	fetal bovine serum
HEK	human embryonic kidney (cell)
HSA	human serum albumin
NEM	N-ethyl maleimide
PBS	phosphate-buffered saline
PAGE	polyacrylamide gel electrophoresis
pN	piconewton (10^{-12} Newton)
RCC	regulator of chromosome condensation
SDS	sodium dodecyl sulfate
SMC	structural maintenance of chromosome (complex)
TCEP	tris(2-carboxyethyl)phosphine

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Fig. 1.

Isolated human metaphase chromosomes. Human embryonic kidney (HEK) 293 cells were arrested at metaphase using colchicine (0.1 mg/mL, 12 hrs). Purified chromosomes are shown (A) in fixed onto a glass cover slip after resuspension in methanol/acetic acid fixative and (B) in solution phase, using phase contrast (left) and fluorescence after DAPI staining (center). Insets (right) show magnified views of fluorescence images.

Fig. 2.

Treatment of human metaphase chromosomes with 5mM reducing reagents induces unfolding. Phase contrast images of the unfolding pattern of isolated metaphase chromosomes are indicated after (A) 0 minutes in PBS, TCEP, and DTT, respectively and (B) 4 hours as dark condensed structures with no visible morphological changes. However, after 8 hours (C) unfolding can be seen for metaphase chromosomes treated with 5mM TCEP in the middle panel and DTT in the right panel to resemble nucleosomes and at 15 hours (D) to become totally unraveled. Metaphase chromosomes in PBS show no unfolding after 15 hours.

Fig. 3.

Mechanical experiments on human chromosomes. Human chromosomes were isolated from HEK 293 cells, their elasticity was measured, exposed to reducing reagents, and then their elasticity was measured again. Scale bars are 10 μm.

(A) 5 mM TCEP in 45 mM HEPES pH 6.9 was sprayed at 5 mM for 20 min, and elasticity was then measured (T), showing a large drop in elastic stiffness relative to the pre-spray result (N). Phase-contrast images show no large unfolding of chromosomes resulting from the TCEP treatment.

(B) 5 mM DTT in PBS was sprayed at 5 mM for 20 min, again a large drop in elastic stiffness was measured (D) relative to the pre-spray result (curve N). Phase-contrast images show no large unfolding of chromosomes resulting from the DTT treatment.

Fig. 4. Fluorescence labeling of oxidized cysteines in metaphase chromosomes. Isolated metaphase chromosomes (A) are non-fluorescent in the green (B). Metaphase chromosomes (C) blocked with 10 mM maleimide for 90 min, then reduced with 5 mM TCEP for in HEPES for 30, in, and then labeled with 100 μ M fluorescein-5-maleimide in HEPES for 10 min show green fluorescence (D). Metaphase chromosomes (E) treated with fluorescein-5-maleimide only also show green fluorescence (F).

Fig. 5. Quantification of fluorescence labeling of oxidized cysteines in chromosomes.
A. Fluorescence intensity per pixel (arbitrary units) plotted for maleimide blocking followed by fluorescent maleimide labeling (leftmost); maleimide blocking only (center left); blocking, reduction and fluorescent maleimide labeling (center right, strongest fluorescence); no blocking or reduction and fluorescent maleimide labeling (rightmost). Error bars indicate standard errors.
B. Average fluorescence intensity per unit area of labeled cysteines in metaphase chromosomes are plotted as a function of time.

Fig. 6.
One-dimensional gel electrophoresis patterns of oxidized-cysteine-labeled proteins.
A. Coomassie stained extracted metaphase chromosome proteins were separated by SDS-PAGE; the most prevalent proteins are histones (bottom of lanes). Different combinations of blocking, reduction, and fluorescent labeling show nearly the same Coomassie band patterns.
B. Green fluorescence intensity of oxidized-cysteine-labeled proteins (rightmost 7 lanes of A). No fluorescence is observed with no labeling (leftmost two lanes), with blocking only and no labeling (3rd lane), or with reduction only with no labeling (5th lane). With blocking and labeling (4th lane), only free dye is observed (diffuse blob at bottom of lane) with little or no labeling of bands. With no blocking or reduction and labeling (6th lane), strongly fluorescent bands are observed, indicating labeling level of free cysteines. Red bar indicates expected location for histone H3. When free cysteines are blocked, oxidized cysteines reduced, and resulting reduced cysteines labeled (7th lane), a subset of labeled bands are observed, with strong candidate bands observed near 30 kDa, 80 kDa and 135 kDa; no band at the expected position for histone H3 is observed.