PNUTS enhances *in vitro* chromosome decondensation in a PP1-dependent manner

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PP1 (protein phosphatase-1) is a serine/threonine phosphatase involved in mitosis exit and chromosome decondensation. In the present study, we characterize the subcellular and subnuclear localization of PNUTS (PP1 nuclear targeting subunit), a nuclear regulatory subunit of PP1, and report a stimulatory role of PNUTS in the decondensation of prometaphase chromosomes in two *in vitro* systems. In interphase, PNUTS co-fractionates, together with a fraction of nuclear PP1, primarily with micrococcal nuclease-soluble chromatin. Immunofluorescence analysis shows that PNUTS is targeted to the reforming nuclei in telophase following the assembly of nuclear membranes and concomitantly with chromatin decondensation. In interphase cytosolic extract, ATP-dependent decondensation of prometaphase chromosomes is blocked by PP1-specific inhibitors. In contrast, a recombinant PNUTS(309–691) fragment accelerates chromosome decondensation.

This decondensation-promoting activity requires the consensus RVXF PP1-binding motif of PNUTS, whereas a secondary, inhibitory PP1-binding site is dispensable. In a defined buffer system, PNUTS(309–691) also elicits decondensation in an exogenous PP1-dependent manner and, as in the cytosolic extract, a W401A (Thr⁴⁰¹ → Ala) mutation that destroys PP1 binding abolishes this activity. The results illustrate an involvement of the PNUTS:PP1 holoenzyme in chromosome decondensation *in vitro* and argue that PNUTS functions as a PP1-targeting subunit in this process. We hypothesize that targeting of PNUTS to reforming nuclei in telophase may be a part of a signalling event promoting chromatin decondensation as cells re-enter interphase.

Key words: chromosome, decondensation, mitosis, PNUTS, protein phosphatase-1, telophase.

INTRODUCTION

The serine/threonine phosphatase PP1 (protein phosphatase-1) is implicated in various aspects of cellular physiology including muscle contraction, glycogen metabolism and neurotransmission [1]. Several lines of evidence also argue that PP1 is involved in cell-cycle progression. Inhibition of PP1 activity arrests cells at the metaphase–anaphase transition in yeast [2,3], and genetic studies show that PP1 regulates mitotic events such as chromosome condensation, spindle formation, chromosome separation and cytokinesis [1]. In mammalian cells, PP1 associates with chromosomes at mitosis [4] and functional inactivation of PP1 results in mitotic arrest or deficient cytokinesis [5]. Upon exit from mitosis, PP1 dephosphorylates the retinoblastoma protein pRb, enabling the growth-suppressive effect of pRb [6]. Targeting of PP1 to the NE (nuclear envelope) by AKAP 149 (A-kinase anchoring protein 149) at the end of mitosis is required for dephosphorylation of B-type lamins and reformation of the nuclear lamina [7]. PP1 has also been proposed to contribute to vitamin D-induced cell-cycle arrest [8]. Moreover, PP1 is involved in nuclear processes such as transcription and mRNA processing [1].

Subcellular localization and activity of PP1 are regulated by association of any of the four PP1 catalytic subunits (PP1 α , PP1 γ 1, PP1 γ 2 and PP1 δ) with a regulatory subunit [9,10]. PP1 regulatory subunits provide targeting function, substrate specificity and susceptibility to regulation by signalling pathways. One of the nuclear targeting subunits of PP1 characterized to date is a cell-cycle phosphoprotein [I-2 (inhibitor-2)], a phosphoprotein involved in mitosis exit (Sds22), two splicing factors {PSF [PTB (pyrimidine tract-binding protein)-associated splicing factor] and

NIPP1 (nuclear inhibitor of PP1)} and R111/p99/PNUTS (where PNUTS stands for PP1 nuclear targeting subunit) [10].

PNUTS is a ubiquitously expressed regulatory subunit of PP1 that was shown to interact with PP1 α and PP1 γ in PC12 cell lysates [11,12]. Notably, PNUTS harbours a consensus PP1-binding 'RVXF motif' (L³⁹⁷VTW⁴⁰¹) and a second PP1-binding site of lower affinity that confers inhibition of PP1 phosphatase activity (E⁴⁴⁵TARRL⁴⁵⁰) [13]. Phosphorylation near the RVXF motif by cAMP-dependent protein kinase *in vitro* impairs association with PP1 [13]. PNUTS is exclusively nuclear in interphase and co-localizes with chromatin during telophase [11].

We show in the present study that PNUTS is targeted to the reforming nucleus in telophase after reassembly of nuclear membranes. In a buffer system and in a cytosolic extract supporting nuclear reformation, a recombinant PNUTS fragment promotes decondensation of purified mitotic chromosomes in a PP1-dependent manner. This activity requires the RVXF motif of PNUTS, whereas the secondary, inhibitory PP1-binding site is dispensable. Our results argue for a role of PNUTS distinct from PP1 inhibition during chromosome decondensation, at least that it occurs *in vitro*.

EXPERIMENTAL

Antibodies, DNA constructs and peptides

Affinity-purified rabbit polyclonal antibodies were raised against amino acids 384–407 of a human PNUTS fragment [14]. Human serum containing autoantibodies raised against the nucleoporin gp210 [15], human serum containing autoantibodies raised against B-type lamins [16] and affinity-purified antibodies

Abbreviations used: H3S10, histone H3 on Ser-10; I-2, inhibitor-2; IB, isolation buffer; LBR, lamin B receptor; mAb, monoclonal antibody; MNase, micrococcal nuclease; NE, nuclear envelope; PP1, protein phosphatase-1; NIPP1, nuclear inhibitor of PP1; pH3S10, phosphorylated H3S10; PNUTS, PP1 nuclear targeting subunit; TX-100, Triton X-100.

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raised against LBR (lamin B receptor) [17] were obtained from H. J. Worman (Department of Medicine, College of Physicians and Surgeons, Columbia University, NY, U.S.A.) and J.-C. Courvalin (Département de Biologie Cellulaire, Institut Jacques Monod, CNRS, Cedex, France). mAbs (monoclonal antibodies) raised against pH3S10 [phosphorylated H3S10 (histone H3 phosphorylated on Ser-10)] were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). Anti-PNUTS mAbs were obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Rabbit polyclonal antibodies raised against all PP1 isoforms (sc-433) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rabbit anti-pH3S10 antibodies were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Cy2-, Cy3- and horseradish peroxidase-conjugated antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). Plasmids encoding GST-PNUTS fusion proteins were obtained from P. Allen (Rockefeller University, New York, NY, U.S.A.) and Y.-G. Kwon (Department of Biochemistry, College of Natural Sciences, Kangwon National University, Kangwon, Korea) and proteins were expressed as described in [13]. Purified PP1 catalytic subunit and PP1 inhibitors NIPP1 and I-2 were described previously [18,19].

Cell fractionation

HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum and synchronized in prometaphase with 1 µg/ml nocodazole for 16–18 h. Mitotic extracts were prepared from nocodazole-synchronized cells. Briefly, cells were washed in cell lysis buffer (50 mM NaCl, 5 mM MgCl₂, 20 mM Hepes, pH 8.2, 10 mM EGTA, 1 mM dithiothreitol and a protease inhibitor cocktail) packed, resuspended in 1 vol. of lysis buffer and Dounce-homogenized on ice, and the lysate was sedimented at 15 000 g for 10 min at 4°C. The supernatant was cleared at 200 000 g for 2.5 h at 4°C, aliquoted and snap-frozen in liquid nitrogen. Interphase extracts were prepared similarly from unsynchronized confluent cells except that EDTA was omitted from the cell lysis buffer.

Mitotic chromosome clusters

Chromosome clusters were isolated from nocodazole-synchronized HeLa cells using a procedure modified from Paulson [20]. Mitotic cells were harvested by shake-off, washed twice in ice-cold PBS and resuspended in 10 vol. of ice-cold IB1 (isolation buffer 1; 10 mM Hepes, pH 7.2, 10 mM NaCl, 5 mM MgCl₂, 0.5 M sucrose, 0.01% Nonidet P40, 1 mM dithiothreitol and a protease inhibitor cocktail). Cells were Dounce-homogenized on ice (~50 strokes) until clusters were released. Clusters were sedimented through 1 vol. of IB2 (IB1 adjusted to 1.2 M sucrose) in a swing-out rotor at 250 g for 20 min at 4°C. The pellet was washed by suspension and sedimenting in 10 vol. of ice-cold IB3 (IB1 adjusted to 250 mM sucrose) at 250 g for 10 min at 4°C. Clusters were resuspended to 40 000 clusters/µl in ice-cold IB3 and used immediately.

Nuclei and nuclear fractionation

Nuclei were isolated from unsynchronized HeLa cells by Dounce homogenization and NEs were purified as described in [21]. High-salt-extracted nuclear matrices were prepared by extraction of purified NEs with 0.5% TX-100 (Triton X-100) and 2 M NaCl. The resulting nuclear scaffolds were solubilized in 2 × SDS sample buffer (0.125 M Tris/HCl, pH 6.8, 20%, v/v, glycerol, 4%, w/v, SDS, 4%, v/v, 2-mercaptoethanol and Bromophenol Blue). Interphase chromatin was isolated by digestion of TX-

100-permeabilized nuclei with 1 unit/ml MNase (micrococcal nuclease). After sedimentation, the supernatant was collected and the pellet was dialysed against 50 mM Tris (pH 7.5), 50 mM NaCl, 5 mM EDTA and 0.1 mM PMSF. After sedimentation, the second supernatant was combined with the first to yield an MNase-soluble chromatin fraction. Soluble proteins were precipitated with 10% (w/v) trichloroacetic acid and dissolved in SDS sample buffer. For salt extraction, purified nuclei were suspended at 10⁶ nuclei/ml in a hypo-osmotic nuclear buffer (10 mM Hepes, pH 7.5, 2 mM MgCl₂, 25 mM KCl and protease inhibitors) containing 0.1% TX-100, and indicated NaCl concentrations were added dropwise under vortex-mixing. Nuclei were extracted on ice for 30 min and sedimented at 15 000 g for 10 min at 4°C. Sedimented material and methanol/chloroform-precipitated supernatant proteins were dissolved in SDS sample buffer.

In vitro chromatin decondensation

Decondensation in buffer

Chromosome clusters were diluted in 40 µl of IB3 to 4000 clusters/µl and incubated for 1 h at 37°C. The test tubes were placed on ice and decondensed clusters were immediately counted by phase-contrast microscopy. Clusters were protected from squashing with rubber cement placed between the slip and coverslip.

Decondensation in interphase extract

Mitotic chromosome clusters were incubated in interphase extract at 4000 clusters/ml under conditions described in the Results section. Extracts contained an ATP-regenerating system (2 mM ATP, 20 mM phosphocreatine and 50 mg/ml creatine kinase) to promote chromosome decondensation. The reaction mixture was incubated for 15 min at 37°C, chilled on ice and decondensed chromosome clusters were immediately counted. Each experiment was repeated at least three times with more than 100 clusters counted in each treatment and in each replicate.

Immunological procedures

SDS/PAGE and immunoblotting were performed with 200 000 cells/lane. Blots were hybridized with rabbit anti-PNUTS (1:1000 dilution) and anti-PP1 (1:500 dilution) antibodies. Immunofluorescence analysis was done as described in [7]. For DNase and RNase extractions, cells were plated overnight on poly (L-lysine) coated coverslips. Cells were incubated for 20 min at 37°C with either 1 mg/ml DNase I or 100 µg/ml RNase A in PBS containing 0.05% TX-100. Cells were fixed with 3% (w/v) paraformaldehyde and immunofluorescence analysis was carried out. In some instances, as indicated, cells were extracted with 1% TX-100 for 1 min before paraformaldehyde fixation. For analysis of chromosome clusters, clusters (160 000) were diluted in 500 µl of IB3 containing 3% paraformaldehyde and spun on to coverslips at 1900 g for 4 min before immunofluorescence analysis. The antibodies used for immunofluorescence were rabbit anti-PNUTS (1:500 dilution), anti-PNUTS mAb (1:50), anti-LBR (1:100), anti-B-type lamins (1:100), anti-gp210 (1:100), anti-pH3S10 mAb (1:50) and rabbit anti-pH3S10 (1:100). DNA was counterstained with 0.1 µg/ml Hoechst 33342. Images were processed using Adobe Photoshop.

RESULTS

PNUTS is weakly associated with chromatin in interphase

We examined the subnuclear localization of PNUTS, a nuclear regulatory subunit of PP1, using several approaches. First, extraction of HeLa cell nuclei with 1% TX-100 showed that PNUTS

Figure 1 PNUTS is weakly associated with chromatin and components of the NE in interphase

(A) Purified HeLa cell nuclei were extracted with 1% TX-100 and soluble (S) and insoluble (P) fractions were analysed by Western blotting using anti-PNUTS and anti-PP1 polyclonal antibodies. (B) NE–nuclear matrix (NE/Mtx) preparations were extracted with 0.5% TX-100 and 2 M NaCl and soluble (NaCl wash) and particulate (Mtx) fractions were analysed by immunoblotting as in (A). (C) Chromatin was digested from purified nuclei with MNase, and soluble (S) and insoluble (P) materials were immunoblotted as in (A). Note that the faster migrating PNUTS product represents a degradation product. (D) Immunofluorescence analysis of PNUTS was performed using rabbit anti-PNUTS antibodies on cells extracted with 0.05% TX-100 alone or with either 1 mg/ml DNase I or 100 μ g/ml RNase A before fixation. DNA was stained with Hoechst 33342. Scale bar, 10 μ m. (E) Isolated nuclei were extracted with 0.1% TX-100 and increasing NaCl concentrations, and soluble (S) and insoluble (P) fractions were immunoblotted as in (A).

remained in a sedimentable pellet fraction, as did PP1, illustrating the insoluble nature of the PNUTS:PP1 holoenzyme (Figure 1A). Secondly, a fraction of PNUTS was found in an NE–nuclear matrix preparation from purified nuclei (Figure 1B, lane NE/Mtx); however, PNUTS was completely solubilized by a 2 M NaCl wash of NEs (Figure 1B), indicating that this PNUTS fraction is not a component of the nuclear matrix. Similarly, a proportion of nuclear PP1 was co-fractionated with PNUTS in NE–nuclear matrix and NaCl preparations (Figure 1B), illustrating a consistent recovery of a PNUTS:PP1 holoenzyme in these fractions. Thirdly, MNase digestion of interphase chromatin to primarily mononucleosomes (results not shown) solubilized PNUTS and PP1 (Figure 1C), indicative of a co-fractionation of PNUTS with a chromatin component. Fourthly, PNUTS was solubilized by extracting the cells adhering to glass coverslips with DNase I/TX-100, but not with RNase A/TX-100 or TX-100 alone, before fixation and immunofluorescence analysis (Figure 1D). Lastly, PNUTS was fully extracted from purified nuclei with 0.125 M NaCl and completely solubilized with 0.25 M NaCl in the presence of 0.1% TX-100 (Figure 1E). Notably, PP1 was only partially solubilized with 0.25 M NaCl (Figure 1E), illustrating its anchoring with other regulatory subunits insoluble at this salt concentration. Collectively, these results indicate that PNUTS is primarily loosely associated with the chromatin accessible to

MNase and that a minor proportion of PNUTS fractionates with a nuclease-insoluble but NaCl-extractable component.

PNUTS is imported into reforming nuclei at the end of mitosis

A detailed subcellular localization of PNUTS was examined throughout the HeLa cell cycle by immunofluorescence analysis using affinity-purified rabbit polyclonal anti-PNUTS antibodies (Figure 2A). In interphase, PNUTS was exclusively nuclear, colocalized with chromatin and was excluded from nucleoli (Figure 2A, 'Int'). In prometaphase, PNUTS was redistributed throughout the cytoplasm and displaced from chromosomes (Figure 2A, 'Promet'). From metaphase ('Met') to anaphase A and B ('Ana A' and 'Ana B'), PNUTS remained excluded from chromatin and was dispersed in the cytoplasm. At telophase, however, a clear association of PNUTS with the reforming nuclei was detected (Figure 2A, 'Tel'). Notably, brief extraction of HeLa cells with 1% TX-100 before fixation abolished all detectable PNUTS labelling in prometaphase and most of the labelling in late telophase (Figure 2B), illustrating the soluble nature of PNUTS at these stages of the cell cycle. Biochemical fractionation of interphase and mitotic cells confirmed the nuclear and cytoplasmic distribution of PNUTS at these stages of the cell cycle respectively (Figure 2C). Furthermore, immunoblot analysis

Figure 2 Subcellular distribution of PNUTS

(A) Immunofluorescence analysis of PNUTS distribution in HeLa cells using affinity-purified anti-PNUTS rabbit polyclonal antibodies. (B) HeLa cells were extracted with 1% TX-100 before fixation and analysed by immunofluorescence as in (A). DNA was labelled with Hoechst 33342. Scale bars, 10 μ m. (C) Interphase and nocodazole-synchronized mitotic HeLa cells (whole cells, WC) were fractionated into nuclei (Nuc), chromatin clusters (Chr) and cytoplasmic (Cyto) fractions. Each fraction was immunoblotted using anti-PNUTS polyclonal antibodies.

of extracts from HeLa cells synchronized in G₁, S, G₂ and M phases indicated that PNUTS levels remained similar throughout the cell cycle (results not shown).

The timing of association of PNUTS with reforming nuclei in telophase suggests that PNUTS is imported into nuclei rather than being targeted to chromosome itself. Indeed, in anaphase B and early telophase, both LBR, an integral protein of the inner nuclear membrane and an early marker of NE assembly [22], and B-type lamins, a major component of the nuclear lamina, labelled the nuclear periphery, while PNUTS remained cytoplasmic (Figures 3A and 3B). Furthermore, nuclear targeting of the nucleoporin gp210 to the nuclear periphery in late telophase coincided with co-localization of PNUTS with chromatin (Figure 3C). gp210 is a late marker of NE assembly whose targeting to nuclear membranes marks the sealing of the NE [22,23]. We concluded, therefore, that PNUTS is imported into daughter nuclei at the end of mitosis rather than being targeted to chromosomes.

Furthermore, TX-100 solubilization of PNUTS in telophase indicates that, despite its nuclear targeting, most of the PNUTS remains soluble at this stage of NE assembly.

H3S10 dephosphorylation precedes targeting of PNUTS to reforming nuclei

H3S10 phosphorylation correlates with chromosome condensation, and H3S10 dephosphorylation is mediated by PP1 in *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Xenopus laevis* [24,25]. To address the question whether PNUTS was involved in the regulation of PP1 activity towards pH3S10, cell-cycle distributions of PNUTS and pH3S10 were compared. As anticipated, pH3S10 decorated condensed chromatin in metaphase and anaphase A (Figure 4); however, pH3S10 labelling was essentially undetectable by anaphase B (Figure 4, 'Ana B'). Additionally, PNUTS was excluded from anti-pH3S10-labelled

Figure 3 PNUTS associates with the reforming nucleus following reassembly of nuclear membranes in telophase

HeLa cells were immunolabelled at indicated cell-cycle stages using anti-PNUTS mAbs and anti-LBR antibodies (A), anti-PNUTS mAbs and anti-B-type lamin human antibodies (B) and anti-PNUTS mAbs and anti-gp210 human antibodies (C). Scale bar, 10 μ m. DNA was labelled with Hoechst 33342.

chromatin from metaphase to anaphase B and reassociation of PNUTS with daughter nuclei in telophase clearly took place in the absence of detectable pH3S10 (Figure 4, 'Tel'). Therefore dephosphorylation of H3S10 precedes detectable nuclear targeting of PNUTS at mitosis exit, indicating that PNUTS is probably not involved in modulating the PP1 activity towards pH3S10.

PP1 is involved in chromosome decondensation in an interphase cell extract

Involvement of PP1 in the regulation of chromosome dynamics at mitosis and targeting of PNUTS to reforming nuclei in telophase raise the hypothesis that PNUTS plays a role in chromosome decondensation upon nuclear reassembly. To address this issue, we developed an *in vitro* assay relying on the decondensation of chromosome clusters isolated from prometaphase-arrested HeLa cells in a 200 000 g cytosolic extract derived from interphase HeLa cells. Isolated clusters were refringent by phase-contrast microscopy and were devoid of detectable PNUTS (Figure 5A). After 1 h at 37 °C in the interphase extract, > 80 % of the clusters decondensed, as judged by increased diameter of the clusters, reduced refringence and reduced Hoechst 33342 labelling intensity (Figures 5B and 5C). Addition of the PP1 inhibitors I-2 (500 nM) and NIPP1 (500 nM) to the extract blocked decondensation (Figure 5C). In contrast, 100 nM okadaic acid, a concentration

that inhibits PP2A but not PP1 *in vitro* [26], was not inhibitory (Figure 5C). Thus inhibition of PP1 activity with I-2 or NIPP1 abolishes chromosome decondensation in interphase cytosol.

The PP1-binding domain of PNUTS stimulates chromosome decondensation in an interphase extract and in a buffer system

To determine whether PNUTS had any function in modulating chromosome decondensation *in vitro*, we first investigated the effect of a GST-PNUTS(309–691) fragment (10 μ M), which contains the PP1-binding RVXF motif and the secondary, inhibitory PP1-binding site [13] in the assay described above. Surprisingly, in contrast with I-2 and NIPP1, PNUTS(309–691) did not inhibit decondensation during the 1 h incubation in interphase cytosol (Figure 5C).

Secondly, the effect of PNUTS(309–691) and deletion fragments thereof (each at 10 μ M) (Figure 5D) on the decondensation of chromosome clusters at a time point when decondensation is initiated in interphase cytosol was examined. By 15 min, only approx. 30 % of the clusters was decondensed and this proportion was not affected by addition of 500 nM I-2 (Figure 5E), consistent with our observations in Figure 5(C). In contrast, PNUTS(309–691) promoted decondensation of 72 % of the clusters within 15 min ($P < 0.001$; Figure 5E). Similar results were obtained with PNUTS(382–450) and PNUTS(382–486), which also harboured

Figure 4 H3S10 dephosphorylation precedes targeting of PNUTS to reforming nuclei

HeLa cells fixed at the indicated cell-cycle stages were immunofluorescently labelled with anti-PNUTS polyclonal antibodies and anti-pH3S10 mAbs. Insets: DNA labelled with Hoechst 33342. Scale bar, 10 μ m.

both PP1-binding motifs (Figure 5E). Notably, PNUTS(382–433), harbouring only the RVXF motif, also promoted decondensation to the same extent. However, a W401A (Trp⁴⁰¹ → Ala) mutation in the RVXF motif, which abolishes PP1 binding [13], did not potentiate decondensation ($P < 0.001$; Figure 5E). GST alone was ineffective (results not shown). These results indicate that the RVXF motif of PNUTS is required for triggering chromosome decondensation within 15 min in interphase cytosol, whereas a fragment of PNUTS that contains a secondary, inhibitory PP1-binding site is apparently of no importance. This suggests a targeting role of PNUTS, through the RVXF motif, in promoting decondensation in this system. This agrees with an active role for PP1 in the metaphase–anaphase transition [1]. Note also that chromosome clusters (results not shown) and interphase cytosol (see Figure 2C) contain some PP1, thus the system provides sufficient phosphatase to support decondensation.

To ascertain a role of PNUTS in chromosome decondensation *in vitro*, prometaphase chromosomes were induced to decondense in buffer (IB3) containing an ATP-regenerating system. This buffer system eliminated any cytosolic component that might influence cluster morphology. PP1 (50 nM) and 10 μ M of the indicated GST–PNUTS fragments were also added alone or in combination (Figure 6A). A basal ATP-dependent decondensation activity affecting approx. 40 % of the chromosome clusters was

detected by 1 h of incubation, which presumably was promoted by remaining chromatin-bound factors (Figure 6A). This proportion remained constant even after several hours of incubation (results not shown). Notably, addition of PP1 or PNUTS (309–691) alone did not enhance decondensation ($P > 0.1$; Figure 6A). In contrast, a significant increase in the percentage of decondensation occurred after addition of PP1 together with PNUTS(309–691) ($P < 0.001$; Figure 6A), illustrating a synergistic effect of PNUTS and PP1. The beneficial effect of PNUTS(309–691) on decondensation was enhanced by altering the stoichiometry of chromatin substrate and PNUTS protein (Figure 6B). Furthermore, 500 nM NIPP1 or 500 nM I-2 abolished PNUTS(609–391)-induced decondensation ($P < 0.001$; Figure 6A). PNUTS (382–433), containing only the RVXF motif, also promoted decondensation ($P < 0.001$), whereas the W401A mutation in the RVXF motif rendered the fragment ineffective (Figure 6A). This result confirms the importance of the RVXF motif in the decondensation-promoting activity of PNUTS identified in interphase cytosol (see Figure 5). Lastly, no decondensation beyond basal levels was observed when PNUTS(309–691) was replaced by 500 nM I-2 (Figure 6A). Collectively, the results indicate that in the buffer system, as in interphase cytosol, PNUTS(309–691) potentiates chromosome decondensation in a PP1-dependent manner. The beneficial effect of PNUTS requires the RVXF motif, whereas the secondary, inhibitory PP1-binding site is dispensable. Moreover, because NIPP1 or I-2 does not enhance decondensation, PNUTS may act as a targeting subunit, rather than as an inhibitor, of a subfraction of PP1 to promote chromosome decondensation.

DISCUSSION

We report a role of PNUTS, most probably as a PNUTS:PP1 holoenzyme, in chromosome decondensation in an *in vitro* system that mimics the decondensation at mitosis exit. Our *in vitro* data and immunological observations of mitotic cells argue that the earliest time point when the effect of PNUTS on chromosome dynamics is detected is in late telophase, when PNUTS is directed to reforming nuclei. However, our data do not address when at mitosis PNUTS may affect chromosome dynamics in a physiological context and do not preclude an effect of PNUTS on chromosome morphology at an earlier stage of mitosis.

Dual immunolabelling experiments show that PNUTS association with the reforming nucleus occurs only after LBR, an integral protein of the inner nuclear membrane, is assembled at the nuclear periphery and before, or concomitantly with, assembly of B-type lamins in the NE (Figure 3). PNUTS targeting also coincides with the first detectable NE targeting of the nucleoporin gp210, a late marker of NE reassembly whose nuclear targeting correlates with the sealing of the envelope [22,23]. Therefore PNUTS is targeted to daughter nuclei after reformation of nuclear membranes probably through a nuclear-import process, rather than being targeted to chromosomes itself a membrane-independent manner. The importance of nuclear import for chromatin decondensation at mitosis exit has been highlighted previously [27]. Nuclear import of PNUTS in late telophase may constitute a step to promote chromosome decondensation.

The ability of PNUTS to promote decondensation requires PP1 and a functional RVXF motif; however, the secondary, inhibitory PP1-binding site is dispensable. Thus PNUTS is unlikely to function as an inhibitor of PP1 activity during nuclear reconstitution. NIPP1, in contrast, inhibits PNUTS:PP1-mediated decondensation (the present study) and nuclear reconstitution *in vitro* (H. B. Landsverk, M. Kirkhus, M. Bollen, T. Kuntziger

Figure 5 PNUTS promotes chromosome decondensation in an interphase extract in a PP1-dependent manner

(A) Chromosome clusters purified from prometaphase HeLa cells and labelled with Hoechst 33342 and anti-PNUTS polyclonal antibodies. Insets show an interphase nucleus for labelling comparison. (B) Clusters after a 1 h incubation in interphase extract. Scale bars, 10 μ m. (C) Proportions (means $\pm$ S.D.) of decondensed chromosome clusters after a 1 h incubation in interphase extract containing either 500 nM I-2, 500 nM NIPP1, 100 nM okadaic acid (OA), 10 μ M GST-PNUTS(309–691) or buffer. (D) Upper panel: GST-PNUTS fragments used in the present study. The RVXF motif (residues 397–401) is shown in white and the secondary, inhibitory PP1-binding site (residues 445–450) is shown in black. The star represents the W401A mutation, which abolishes PP1 binding. Lower panel: Coomassie Blue-stained SDS/PAGE analysis of the GST-PNUTS fragments. (E) Chromosome clusters were incubated for 15 min in interphase extract containing 500 nM I-2 or 10 μ M of the indicated GST-PNUTS fragments. Decondensation was assessed by phase contrast microscopy. (C, E) * P < 0.001 (χ^2 tests).

and P. Collas, unpublished work). A number of regulatory subunits of PP1 act as PP1-specifiers, promoting or inhibiting the catalytic activity of PP1 in a substrate-dependent manner [28]. The differential effect of these subunits on PP1 activity has been explained by their association with distinct sets of binding sites on PP1 [10,28]. The localization of PP1 on mitotic chromosomes [4,5] before nuclear targeting of PNUTS in telophase argues that PNUTS does not target bulk cellular PP1 to chromatin itself. However, the involvement of PP1 in chromosome decondensation suggests that PNUTS may bring a subfraction of PP1 close to a chromosome- or spindle-associated substrate and conceivably stimulate PP1 activity towards this substrate. These possibilities remain to be addressed.

We observed that approx. 35 % of the chromosome clusters spontaneously decondense in interphase extract or in ATP-containing buffer, probably as a result of dissociation of chromosomes from mitotic condensation-promoting factors. Prometaphase chromosomes do not harbour any detectable PNUTS and exogenous PNUTS fragments do not bind chromosomes *in vitro*

(H. B. Landsverk, M. Kirkhus, M. Bollen, T. Kuntziger and P. Collas, unpublished work). Furthermore, in the buffer system, adding PNUTS alone to chromosomes does not enhance decondensation, suggesting that the amount of chromosome-bound PP1 is limiting. In contrast, > 70 % of chromatin masses decondense in the presence of both PNUTS and PP1. Thus PNUTS:PP1 holoenzyme activity on chromosome dynamics requires a threshold PNUTS concentration in the vicinity of chromosomes, but does not need to be continuously associated with chromosomes.

The nature of substrates susceptible of being modified by the PNUTS:PP1 holoenzyme and affecting chromosome dynamics at mitosis remains speculative. Studies in budding yeast, *C. elegans* and vertebrates point PP1 as the mitotic H3S10 phosphatase [29]. However, pH3S10 labelling is undetectable in telophase when PNUTS associates with reforming nuclei (the present study), and chromosome decondensation has been uncoupled from pH3S10 dephosphorylation in *Xenopus* egg extracts [25]. Furthermore, H3 phosphorylation does not directly promote

Figure 6 PNUTS and PP1 enhance chromosome decondensation in a buffer system

(A) Prometaphase chromosome clusters were incubated for 1 h at 37°C in ATP-containing buffer together with combinations of purified PP1 catalytic subunit (50 nM), indicated GST-PNUTS peptides (10 μM), NIPP-1 (500 nM) and I-2 (500 nM). Decondensation was assessed by phase-contrast microscopy and Hoechst labelling. * $P < 0.001$ (χ^2 tests). (B) Condensed clusters were incubated at a concentration of 2000 or 4000 clusters/μl in a buffer containing 0–20 μM GST-PNUTS(309–691) and an ATP-regenerating system at 37°C for 1 h. Decondensation was monitored as in (A).

chromosome condensation [25]. Thus pH3S10 dephosphorylation does not necessarily correlate with chromosome decondensation and PNUTS is probably not the PP1 regulatory subunit promoting pH3S10 dephosphorylation at mitosis exit. Nonetheless, H3S10 dephosphorylation may provide a signal for PNUTS activity on chromosome dynamics.

Candidate substrates for PNUTS:PP1 may include subunits of the condensin complex whose phosphorylation correlates with chromosomal targeting and stimulation of DNA supercoiling activity [30]. Another possibility is that PNUTS:PP1 promotes dephosphorylation of the RIIα subunit of cAMP-dependent protein kinase on Thr-54, shown to correlate with reassembly of nuclei in interphase extract [31]. Other potential substrates include topoisomerase II, whose mitotic phosphorylation has been proposed to activate and/or target the protein to chromatin [32], and pRb, which is dephosphorylated in late mitosis [33]. Conceivably, the PNUTS:PP1 holoenzyme may act on the building blocks of chromosomes or enzymes or proteins affecting chromosome dynamics.

In summary, our results show that PNUTS promotes chromosome decondensation *in vitro*, most probably as a PNUTS:PP1 holoenzyme. PNUTS does not seem to act on chromosome dynamics by inhibiting the phosphatase activity of PP1, but may target and stimulate PP1 activity towards an as yet unidentified substrate. We propose that targeting of PNUTS to reforming nuclei in telophase constitutes a signalling event promoting or enhancing chromosome decondensation in reforming nuclei.

We thank M. Beullens (Division of Biochemistry, Catholic University of Leuven, Campus Gasthuisberg, Leuven, Belgium), H. J. Worman and J.-C. Courvalin for reagents, P. Allen (Department of Psychiatry, Yale University School of Medicine, New Haven, CT, U.S.A.) and Y.-G. Kwon for PNUTS constructs and J. Ellenberg and F. Mora-Bermudez (EMBL, Heidelberg, Germany) for constructive comments. This work was supported by the Research Council of Norway, the Norwegian Cancer Society (H. B. L. and M. K.), the University of Oslo, EU Marie Curie Fellowship MCFI-2001-01266 (T. K.) and the Human Frontiers Science Program Organization. M. B. is supported by the Flemish Concerted Research Action and by the Prime Minister's Office (grant IAP/V-05).

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Received 26 April 2005/20 May 2005; accepted 23 May 2005

Published as BJ Immediate Publication 23 May 2005, doi:10.1042/BJ20050678