Subnuclear localization and mitotic phosphorylation of HIRA, the human homologue of *Saccharomyces cerevisiae* transcriptional regulators Hir1p/Hir2p

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The *HIRA* gene encodes a nuclear protein with histone-binding properties that have been conserved from yeast to humans. Hir1p and Hir2p, the two *HIRA* homologues in *Saccharomyces cerevisiae*, are transcriptional co-repressors whose action resides at the chromatin level and occurs in a cell-cycle-regulated fashion. In mammals, *HIRA* is an essential gene early during development, possibly through the control of specific gene-transcription programmes, but its exact function remains to be deciphered. Here we report on the subnuclear distribution and cell-cycle behaviour of the *HIRA* protein. Using both biochemical and immunofluorescence techniques, a minor fraction of *HIRA* was found tightly associated with the nuclear matrix, the material that remains after nuclease treatment and high-salt extraction. However, most *HIRA* molecules proved extractable. In non-synchronized cell populations, extraction from chromatin

necessitated 300 mM NaCl whereas 150 mM was sufficient in mitotic cells. Immunofluorescence staining and microscopic examination of mitotic cells revealed *HIRA* as excluded from condensed chromosomes, confirming a lack of association with chromatin during mitosis. Western-blot analysis indicated that *HIRA* molecules were hyper-phosphorylated at this point in the cell cycle. Metabolic labelling and pulse-chase experiments characterized *HIRA* as a stable protein with a half-life of approx. 12 h. The mitotic phosphorylation of *HIRA* could provide the dividing cell with a way to retarget *HIRA*-containing multi-protein complexes to different chromatin regions in daughter compared with parental cells.

Key words: histone-binding protein, nuclear matrix, protein half-life.

INTRODUCTION

The *HIRA* protein has now been identified in several eukaryotic species [1–5]. Its structure has been strikingly conserved during evolution. In the N-terminal third, seven reiterations of a module, called the WD repeat, define the most constant region, which is predicted to assemble into a characteristic β -propeller structure [6]. As reviewed recently, β -propellers offer convenient surfaces for inter-molecular contacts between constituents of macromolecular complexes [7]. In mammals, *HIRA* is essential for proper development since *HIRA*-null mice are grossly abnormal and die early in embryogenesis [8]. The human *HIRA* gene lies within a gene-rich region of human chromosome 22q11.2 that is reduced to hemizyosity in most patients with the developmental conditions known as DiGeorge syndrome or velocardiofacial syndrome [9,10]. In such patients, organs and tissues derived from or affected by neural crest cells in the pharyngeal region of the developing embryo can be affected [11]. Two recent reports have demonstrated that haploinsufficiency of *Tbx1*, another gene within the large 22q11 deletion, is sufficient to generate in mice most of the cardiovascular defects observed in DiGeorge syndrome/velocardiofacial syndrome patients [12,13]. *HIRA* may play an additional role, albeit partial, in pathogenic processes, as in both mouse and chick it is also expressed in the affected embryological region at the proper time in development

[3,14]; in addition, oligonucleotide-induced inhibition of *HIRA* expression in chick neural crest cells results in some of the malformations of the cono-truncal region of the heart that are observed in syndromic patients [15].

Most functional studies on the *HIRA* protein family have been performed in *Saccharomyces cerevisiae*, which provides an exception to the rule of a single *HIRA*, as it contains two structural homologues, Hir1p and Hir2p, produced from separate genes that probably evolved divergently after an ancient gene-duplication event [1]. The two *HIR* genes were originally identified as important for the periodic control of histone gene transcription [16,17]. In the absence of *HIR* gene products, transcription becomes constitutive at the *HTA1-HTB1* histone *H2A/H2B* gene locus, which is normally repressed outside the late G_1 and S phases of the cell cycle, indicating that Hir1p and Hir2p participate in cell-cycle-dependent transcriptional repression [18]. The mechanism of this repression has not been elucidated totally, but the current view implies a role for these proteins in local chromatin structure. Indeed, maximal transcription at this locus is reached when Hir1p/Hir2p-dependent repression is relieved following recruitment of the Swi/Snf multi-protein complex (or a Swi/Snf subcomplex) [19]. Swi/Snf is a major ATPase-dependent chromatin-remodelling machinery [20–22] which has long been known to antagonize chromatin-mediated transcriptional repression in this yeast [23]. How Hir1p and Hir2p

Abbreviations used: DAPI, 4',6-diamidino-2-phenylindole; CIP, calf intestinal phosphatase; PARP, poly(ADP-ribose)polymerase; CSK, cytoskeletal buffer; GST, glutathione S-transferase; FCS, fetal calf serum.

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alternate between co-repressor and co-activator states at precise times during the cell cycle remains to be determined. Because it is structurally similar to Hir1p and Hir2p and physically interacts with both core histones [24] and DNA-binding transcription factors [25], HIRA is likely to also function in transcriptional regulation. Having recently been identified as a novel cyclin/cyclin-dependent kinase 2 substrate, the transcriptional role of the human HIRA protein may well relate to cell-cycle progression [26], similar to its yeast counterparts.

We have previously reported that, in exponentially growing cells, the human HIRA protein was largely nuclear; in interphase, it is seen as numerous foci dispersed throughout the nucleus, with the exception of the nucleoli [24]. Using a biochemical approach, we have found that human HIRA is part of a high-molecular-mass multi-protein complex present in nuclear extracts from human tumour cells (J. Machold and M. Lipinski, unpublished work). To progress our understanding of the HIRA protein in higher eukaryotes and to get further clues as to its possible function(s), we have examined its turnover and details of its intra-nuclear distribution and biochemical properties during the cell cycle. HIRA was characterized as a stable protein with a half-life of approx. 12 h, a fraction of which was associated tightly with the nuclear matrix, the insoluble material that remains after high-salt extraction of nuclease-treated nuclei [27]. Those HIRA molecules that were soluble were phosphorylated at the time of mitosis and excluded from mitotic chromosomes. This may provide a way to retarget HIRA-containing multi-protein complexes in the following cell cycle.

EXPERIMENTAL

Cell culture and synchronization

K562 cells were grown at 37 °C in 5% CO₂ in RPMI (Life Technologies) supplemented with 5% fetal calf serum (FCS; Seromed). HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS.

For cell-cycle synchronization in mitosis, exponentially growing HeLa cells were treated for 16 h, usually with 0.4 µg/ml nocodazole (Sigma). Some experiments were carried out with a shorter incubation period, in the presence of lower drug concentrations or in the presence of 0.25 µM demecolcine (Sigma) with equivalent results. Mitotically enriched floating cells were harvested away from adherent cells, analysed for DNA content by propidium iodide staining and flow cytometry using a FACScalibur (Becton-Dickinson) and for the proportion of cells in mitosis after 4',6-diamidino-2-phenylindole (DAPI) staining (0.4 µg/ml, 5 min) and observation under a fluorescence microscope (Olympus). This protocol reproducibly yields greater than 80% of cells with a DNA content corresponding to the G₂/M phases, with more than 70% being in mitosis (see FACS analysis in Figure 5, below).

Protein fractionation and nuclear matrix preparation

Cultured HeLa or K562 cells were harvested, washed twice in PBS and snap-frozen at -80 °C. The cell membranes were lysed by thawing into low-salt extraction buffer (10 mM Hepes, pH 7.9/1.5 mM MgCl₂/0.1 mM EGTA/0.5 mM dithiothreitol/5% glycerol). Nuclei were pelleted by centrifugation (600 g, 10 min). After one more wash, the nuclei were resuspended in the same buffer containing 0.35 M NaCl and nuclear proteins extracted on ice for 30 min. After centrifugation (14000 g, 4 °C, 25 min), the pellet was digested (30 min, 30 °C) with 300 units/ml RNase-free DNase I (Boehringer Mannheim) in a digestion buffer consisting of 20 mM Tris/HCl, pH 7.5/5 mM MgCl₂/

0.25 M sucrose. Further extraction of the digested pellet was performed twice in a high-salt buffer consisting of 2 M NaCl/0.2 mM MgCl₂/10 mM Tris/HCl, pH 7.5, leaving nuclear matrix as the insoluble pellet. All buffers contained a cocktail of protease inhibitors (Complete[™], Roche).

Western-blot analysis

The protein content of the different fractions was determined using the protein-assay reagent (Pierce) with BSA used as standard. Appropriate protein amounts were precipitated with trichloroacetic acid prior to PAGE performed on 7.5% or 5.5% polyacrylamide slab gels containing 0.1% SDS. The gels were transferred on to polyscreen PVDF membranes (Millipore), incubated with the affinity-purified 1455 reagent recognizing a C-terminal peptide of HIRA, described previously [24], or with the affinity-purified 484 antiserum (Agrobio) prepared as described below. Commercial monoclonal antibodies were used as controls at dilutions indicated by the manufacturers. Detection of immune complexes was accomplished using appropriate dilutions of peroxidase-coupled goat anti-mouse or anti-rabbit secondary reagents (Pierce) followed by detection using the ECL system (Amersham Pharmacia Biotech).

Preparation of protein extracts from asynchronous and mitotic cells

For exponentially growing HeLa cells, nuclear extracts were prepared by resuspending a pellet of isolated nuclei in a buffer consisting of 10 mM Hepes potassium salt, pH 7.9, 1.5 mM MgCl₂, 0.1 mM EGTA and 0.5 mM dithiothreitol with the addition of 150, 300 or 600 mM NaCl. Extraction was performed (30 min, 4 °C) with constant agitation. Residual nuclear material was pelleted by centrifugation (30 min, 10600 g). Exactly the same procedure was followed for protein preparations from cell cultures enriched in mitotic cells after incubation in the presence of drugs preventing the normal microtubule polymerization process. In this situation, a large proportion of the cells are blocked at a stage in mitosis when the nuclear membrane has broken down. This results in protein preparations that technically should not be referred to as nuclear. However, if anything, the procedure results in the loss of a proportion of nuclear proteins during the purification process, along with the soluble 'cytosolic' proteins. In this article, as everywhere else in the literature, we will thus refer to 'nuclei' or 'nuclear preparations' for want of a better designation.

Antibodies

The rabbit antiserum 484 (Agrobio) was generated by immunization with a recombinant form of glutathione S-transferase (GST) fused to amino acids 10–1017 of HIRA, from the previously described pGEX-HIRA construct [24], and produced in *Escherichia coli* BL21. Purification of the fusion protein was performed by overnight incubation (4 °C) on a rotating wheel in the presence of glutathione-agarose beads, followed by elution in the presence of 500 mM Tris, pH 8.0/100 mM glutathione (30 min, 4 °C on a rotating wheel). After trichloroacetic acid precipitation, the GST-HIRA protein was purified by electrophoresis in a preparative slab gel that was stained with 0.3 M CuCl₂. The negatively stained fusion protein was sliced and recovered from the gel by electrotransfer. A series of control Western-blot stainings demonstrated that this serum detects the same band of 112 kDa as detected with 1455 antiserum [24]. Affinity purification was performed as described in [24] on the same GST-HIRA fusion protein that was used as the immunogen.

The anti-poly(ADP-ribose)polymerase (PARP) and the anti-lamin B monoclonal antibodies were purchased from Clontech and Oncogene Research Products, respectively. For immunofluorescence detection of histones, a monoclonal reagent recognizing all core histones was used (Roche).

Immunofluorescence analysis

For immunofluorescence staining of *in situ*-prepared nuclear matrix, HeLa cells were extracted directly on coverslips following the procedure described by Spector et al. [28]. In brief, in the first fractionation step, soluble proteins were removed by extraction in cytoskeletal buffer (CSK, 10 mM Pipes, pH 6.8/300 mM sucrose/100 mM NaCl/3 mM MgCl₂/1 mM EGTA) containing 0.5% Triton X-100. Chromatin proteins were released by DNase I digestion and extraction with 0.25 M ammonium sulphate. Nuclear matrices were then fixed with 2% paraformaldehyde (20 min, room temperature) in PBS and processed further for immunofluorescence staining, which was performed essentially as described in [24]. Developing reagents consisted of species-specific Alexa Fluor 488-conjugated (Molecular Probes) and Texas Red-conjugated (Jackson Laboratories) goat anti-mouse Ig or anti-rabbit Ig antisera, which were used at a dilution of between 1/400 and 1/800. Stained cells were observed, signals acquired and optical sections generated as described in [24]. To allow a visual comparative analysis of the resistance of the different nuclear molecules to the nuclear-matrix-preparation procedure, image acquisitions were performed with fixed acquisition settings.

Phosphatase treatment

Nuclear 0.35 M NaCl extracts (35 µg) were incubated (1 h, 37 °C) with various amounts of calf intestinal phosphatase (CIP; New England Biolabs) in a buffer consisting of 100 mM NaCl, 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂ and 1 mM dithiothreitol.

Half-life measurement of the HIRA protein

For metabolic labelling, K562 cells (30 × 10⁶) were harvested and washed twice with a long-term labelling medium consisting of 90% cysteine/methionine-free RPMI (ICN) and 10% complete RPMI (Life Technologies) supplemented with 10% FCS. Cells were incubated for 16–18 h in 30 ml of the same medium in the presence of 100 µCi/ml of a mixture of [³⁵S]methionine and [³⁵S]cysteine (Trans ³⁵S-Label, ICN) in a humidified 5% CO₂ incubator (at 37 °C). Cells were then collected, washed once with warm RPMI/10% FCS and 5 × 10⁶ cells were seeded in separate flasks. The entire cell contents of each flask were harvested at different time points followed by washing in PBS and snap-freezing at –80 °C.

For immunoprecipitation experiments, cells were lysed while vortexing (30 min, 4 °C) in 50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 10 mM MgCl₂, 0.5% Igepal Ca-630 (Sigma), 1 mM EDTA and the Complete antiprotease cocktail (Roche). After centrifugation (10600 g, 10 min, 4 °C), 500 µg of supernatant were precleared by incubation (4 h at 4 °C) once with Protein A/G-agarose (Santa Cruz Biotechnology) and once with Protein A/G-agarose preincubated with preimmune serum as recommended by the manufacturer. The precleared lysate was adjusted to 150 mM NaCl and 0.25% Igepal Ca-630 and subjected to overnight immunoprecipitation on a rotating wheel at 4 °C using an irrelevant rabbit antiserum or the affinity-purified 484 anti-HIRA reagent. The mixtures were incubated further (4 h at 4 °C) with 50 µl of Protein A/G-agarose which had been preincubated

overnight in the presence of 500 µg of total non-radioactive K562 lysate. After 10 washes in 50 mM Tris/HCl, pH 8.0, 0.25% Igepal Ca-630, 1 mM EDTA and 150 mM NaCl, the immunoprecipitates were submitted to SDS/PAGE and analysed by autoradiography or Western blotting.

RESULTS

Significant amounts of HIRA associate with the nuclear matrix

A number of nuclear proteins contain a so-called AT hook, a small sequence motif centred around a conserved glycine-arginine-proline (GRP) tripeptide that is often preceded and followed by basic amino acids; this motif appears sufficient to confer the corresponding proteins with DNA-binding property [29]. A nonapeptide (KKKKGRPRK) that fitted this description perfectly was found at positions 640–648 of the human HIRA amino acid sequence and at the equivalent position in each HIRA homologue identified in mouse, *Xenopus*, chick and *Fugu* (Table 1). Since most AT-hook-containing proteins are found associated with the nuclear matrix, the insoluble material that remains after extraction of all soluble proteins from the nucleus, we decided to investigate the subnuclear distribution of HIRA molecules. Exponentially growing K562 cells were treated as described in the Experimental section to obtain fractions of cytosolic, nuclear and matrix-associated proteins. Lamin B is a component of the lamina and as such is almost entirely retained with nuclear matrix material [30]. In contrast, PARP has been reported to partition essentially among soluble components of the cell nucleus, with usually less than 4% of the molecules remaining in the nuclear matrix [31]. Our Western-blot analysis performed with commercial antibodies to lamin B and PARP reproduced these results, most of the lamin B signal being detected in our matrix preparation (Figure 1B, lane 7), whereas PARP was mainly recovered in the soluble 0.35 M NaCl extract (Figure 1C, lane 2). A longer exposure (Figure 1D) revealed that a small fraction of nuclear PARP was also present among proteins more tightly associated with chromatin (Figure 1D, lane 6) and in the nuclear matrix (Figure 1D, lane 7). Thus nuclear proteins had been correctly partitioned during the procedure.

Staining of the same blot with the affinity-purified 484 anti-serum raised to human HIRA revealed that HIRA molecules were distributed between the soluble 0.35 M salt extract (Figure 1A, lane 2) and the nuclear matrix (Figure 1A, lane 7). Identical results were obtained with different fractionation procedures, including use of 0.25 M ammonium sulphate instead of 2 M NaCl for protein extraction as described by Penman and collaborators [32], or protein extraction preceded by, instead of following, DNase treatment [33] (results not shown). Although difficult to precisely quantify, the proportion of HIRA molecules

Table 1 Conservation of the AT-hook motif in HIRA proteins from various species

The AT hooks identified in the amino acid sequences of the HIRA homologues in the indicated species have been aligned. Numbers refer to positions in the amino acid sequences.

Species	Amino acid position and sequence	Reference
<i>Homo sapiens</i>	⁶⁴⁰ KKKKGRPRK ⁶⁴⁸	[1]
<i>Mus musculus</i>	⁶³⁸ KKKKGRPRK ⁶⁴⁶	[2]
<i>Xenopus laevis</i>	⁶³⁵ KRKKGRPRK ⁶⁴³	C. Scamps and M. Lipinski, unpublished work
<i>Gallus gallus</i>	⁶⁴⁴ KKKKGRPRK ⁶⁵²	[3]
<i>Fugu rubripes</i>	⁶⁴⁰ KRKKGRPRK ⁶⁴⁸	[5]

Figure 1 A fraction of HIRA protein is associated with the nuclear matrix

Nuclei prepared from K562 cells (lane 1) were extracted with 0.35 M NaCl (lane 2). The pellet (lane 3) was digested with DNase I (lane 4). After centrifugation, the supernatant was harvested (lane 5) and the pellet extracted in 2 M NaCl (lane 6), leaving the nuclear matrix fraction (lane 7). Equal amounts of proteins (40 µg) were resolved by electrophoresis on a 7.5% polyacrylamide gel and immunoblotted using the affinity-purified 484 anti-HIRA reagent (A) and commercial antibodies to lamin B (B) and PARP (C, D).

Figure 2 Immunofluorescence staining of *in situ*-prepared nuclear matrix

Staining of DNA, lamin B, HIRA and histones was performed on HeLa cells extracted with CSK buffer (upper panels) and on nuclear matrices prepared *in situ* from whole HeLa cells (lower panels).

that partitioned with nuclear matrix was clearly intermediate (approx. 10–20 %) between those observed for the mostly soluble PARP enzyme (about 2 % matrix-associated) and the integral component of the matrix, lamin B (close to 100 % matrix-associated).

We next sought confirmation of the presence of HIRA in the nuclear matrix using an independent procedure and different

Figure 3 HIRA protein in mitotic cells

(A) K562 cells were stained immunofluorescently for HIRA (lower panel) with DNA counterstained using DAPI (upper panel). (B) Mitotic floating cells collected from HeLa cells treated with demecolcine (Dc) and an asynchronous population of exponentially growing HeLa cells (As) were used to prepare cellular extracts in the presence of the indicated concentrations of NaCl. The HIRA molecules present in 50 µg of proteins loaded in each lane were detected using the affinity-purified 484 anti-HIRA antibody.

cells. Nuclear matrices were prepared *in situ* from HeLa cells grown directly on coverslips. These adherent cells were submitted to various extraction steps as described in the Experimental section, prior to fixation and processing for immunofluorescence staining. In the first fractionation step, soluble cytosolic and nucleoplasmic proteins were removed by extraction in CSK buffer in the presence of the detergent Triton X-100. Treatment with DNase I and extraction in the presence of 0.25 M ammonium sulphate then released the majority of chromatin-associated proteins. The remaining material consisted of structural and additional molecules associated with the matrix. The efficiency of chromatin digestion was assessed by the disappearance of the nuclear staining produced by the DNA stain DAPI (Figure 2, compare upper- and lower-left images). Double immunofluorescence staining experiments were performed using simultaneously an anti-lamin B monoclonal antibody and the anti-HIRA affinity-purified 1455 antiserum. As expected, the lamin B signal resisted extraction (Figure 2, compare upper and lower images). When the same fields were observed for HIRA staining, it was apparent that part of the staining had been retained throughout the matrix preparation procedure. Since

Figure 4 Mitotic phosphorylation of HIRA

Nuclear extracts (35 μ g per lane) prepared from nocodazole-arrested HeLa cells were incubated (1 h, 37 °C) in the absence (lanes 2) or presence (lanes 3–6) of various concentrations of CIP as indicated. Lanes 6 were loaded with nuclear extracts treated by CIP in the presence of 50 mM of the phosphatase inhibitor sodium EDTA. **(A)** Immunoblot analysis using the affinity-purified 1455 antibody; **(B)** the same membrane stained with Coomassie Brilliant Blue.

DAPI staining left the same preparation totally unlabelled (Figure 2, lower-left image), it appeared unlikely that the HIRA immunofluorescence signal could be due to any proportion of HIRA molecules still associated with chromatin. Indeed, under the same conditions, the extremely intense histone signal was virtually abrogated (Figure 2, lower-right panel), consistent with the notion that only a very small fraction, less than 5%, of total nuclear histones resists extraction during nuclear matrix preparation [28]. Together, the biochemical and immunofluorescence data demonstrated that a significant proportion of mammalian HIRA molecules are tightly associated with the nuclear matrix.

The HIRA protein is excluded from mitotic chromosomes and more readily extracted from synchronized mitotic cells

We have reported previously [24] (see also Figure 2) that in interphase cells, HIRA molecules are distributed in numerous foci scattered throughout the nucleus, with the exception of nucleoli. Figure 3(A) displays interphase and mitotic cells stained for nuclear DNA and HIRA. It can be seen that the HIRA distribution pattern differed between the two cell subsets, the HIRA signal being clearly excluded from condensed chromosomes in mitotic cells. Since phosphorylation events have been implicated in the exclusion from condensed mitotic chromosomes for other nuclear proteins, including transcription factors [34] and members of chromatin-remodelling complexes [35], we next asked whether this could also be the case for HIRA. The classical procedure to prepare nuclei was applied to a HeLa cell population enriched in mitotic cells following treatment with demecolcine, followed by protein extraction in the presence of different salt concentrations. Western-blot analysis of the various protein fractions extracted from demecolcine- and mock-treated cells was performed to reveal HIRA. HIRA molecules isolated from asynchronous control cells behaved homogeneously with regard

to their electrophoretic migration pattern (Figure 3B, As). In these cells, 300 mM salt was necessary for efficient HIRA extraction, as expected for a chromatin-associated protein. A higher salt concentration (600 mM) did not extract more HIRA protein. In the corresponding extracts from demecolcine-treated cells, a novel band was detected in addition to the normally migrating species (Figure 3B, Dc). This slower form of HIRA was more readily extractable, as seen in the lane loaded with the 150 mM salt extract. In 300 and 600 mM salt, HIRA molecules were distributed equally between the normally migrating and the slower forms. An increase in the salt concentration again did not yield any additional HIRA material.

The mitosis-specific form of HIRA is hyperphosphorylated

We next investigated whether the retarded migration of mitotic HIRA molecules was due to phosphorylation. Proteins extracted from mitotically enriched HeLa cells were submitted to Western-blot analysis (Figure 4A). The amount of retarded HIRA molecules somewhat varied from one experiment to the other, probably due to varying proportions (from 70 % to 90 %) of cells blocked in mitosis in different experiments; in the present case, virtually all HIRA molecules in the extract migrated with the retarded pattern (Figure 4A, lane 1). Treatment with CIP caused HIRA molecules to migrate more quickly (Figure 4A, lanes 3–5) compared with untreated (Figure 4A, lane 1) or mock-treated (Figure 4A, lane 2) extracts, and this effect was abrogated in the presence of the phosphatase inhibitor sodium EDTA (Figure 4A, lane 6). Similar results were obtained when protein extracts were submitted to the action of λ -phosphatase (results not shown). Since the migration pattern of other proteins in the various extracts was not affected, as revealed by Coomassie Blue staining (Figure 4B), the HIRA migration differences were not due to an electrophoretic artefact. Together, these results indicated that

Figure 5 The mitotic mobility shift affects HIRA molecules not associated with nuclear matrix

Fractions were prepared and loaded in the gel as in Figure 1. The left- and right-hand panels correspond to control and floating HeLa cells harvested from nocodazole-treated cultures, respectively. A FACS analysis of the two populations stained for DNA content using propidium iodide is shown above the corresponding blots. Equal amounts of proteins (35 μ g) were loaded in each lane for immunoblot analysis using the affinity-purified 1455 anti-HIRA antibody. The short and long arrows point to the quickly and slowly migrating forms of HIRA, respectively.

mitotically enriched cells contain hyperphosphorylated forms of HIRA whose biochemical features include increased extractability in lower-salt buffer (150 mM NaCl) and decreased electrophoretic mobility.

These experiments were performed with cells incubated for 16 h in the presence of nocodazole. Although a long-established procedure to obtain large numbers of cells blocked in mitosis, this incubation period in the presence of a microtubule-depolymerizing drug could result in damaged cells and a physiologically irrelevant hyperphosphorylation of HIRA. To address this possibility, we performed several experiments. First, we verified that nocodazole-treated cells were not irreversibly damaged. DNA staining and FACS analysis performed after replacement of the nocodazole-containing medium by drug-free medium indicated that 35% and 56% (compared with 3% in the drug-arrested population) of the cells were in G_1 140 and 180 min after nocodazole removal, respectively. Secondly, we repeated the nocodazole treatment using shorter incubation periods or lower drug concentrations. The same gel retardation was obtained for HIRA molecules after 8 h of nocodazole treatment or with as little as 10% of the usual drug concentration (results not shown). A final indication that HIRA hyperphosphorylation is indeed related to mitosis rather than a side effect of nocodazole treatment was provided using a classical 'shake-off' procedure that allows the recuperation of mitotically enriched floating cells from an asynchronous population of exponentially growing HeLa cells. In our hands, this procedure reproducibly yielded 25–28% of mitotic cells, compared with approx. 3% in the control asynchronous population. Again, similarly retarded HIRA products were detectable in the corresponding lane of the gel electrophoresis (results not shown). We therefore conclude that the hyperphosphorylation of HIRA molecules that occurs in mitotically arrested cells following drug treatment reproduces modi-

fications that affect HIRA at the time of mitosis in a normally cycling population.

The hyperphosphorylated form of HIRA is not associated with the nuclear matrix

Having observed differences in the subnuclear distribution of HIRA molecules between interphase and mitosis, and in light of the existence of hyperphosphorylated forms of HIRA in mitotic cells, we next asked whether these features were indicative of different properties of HIRA with regard to the nuclear matrix. Nuclear matrices were prepared from asynchronous and floating (mitotically enriched) HeLa cells obtained after nocodazole treatment. As seen in the FACS analysis displayed in Figure 5, greater than 80% of cells harvested from the nocodazole-treated culture had a DNA content corresponding to the G_2/M phases of the cell cycle, as compared with 11% in the control asynchronous population. Upon microscopic examination, the proportion of mitotic cells was 3.6% in the latter population, compared with 72% in the former. In control cell extracts (Figure 5, Western blot, left-hand panel), no retarded HIRA band was observed and there were no detectable migration differences between the different fractions (Figure 5, left-hand panel, lanes 2–7). HIRA molecules present in mitotically enriched HeLa cells (Figure 5, Western blot, right-hand panel) were also found distributed between the soluble and matrix-associated fractions. However, contrasting with the asynchronous population, the migration of HIRA molecules varied between fractions. Retarded HIRA molecules (Figure 5, right-hand panel, long arrow) were abundant in the 'nuclear' fraction (Figure 5, right-hand panel, lane 1), where they accounted for more than half of the total HIRA signal. It is readily apparent that the vast majority of the retarded HIRA molecules partitioned in the

Figure 6 Metabolic labelling and pulse-chase analysis reveals HIRA as a stable protein

(A) Autoradiogram (6 h exposure) of extracts prepared from K562 cells that have been metabolically labelled for 18 h and submitted to immunoprecipitation using the immunoaffinity-purified 484 antiserum at 0 (lane 2), 6 (lane 3) and 24 (lane 4) h into the chase. Lane 1 was loaded with material precipitated using an irrelevant rabbit antiserum. (B) Materials precipitated with an irrelevant antiserum (lane 1) or the immunoaffinity-purified HIRA-specific 484 antiserum (lanes 2–4) were transferred to PVDF membranes and revealed by staining with 484. Lanes 2–4 were loaded with precipitates obtained at 0, 24 and 36 h after beginning of the chase, respectively. In both panels the arrows indicate the positions of the HIRA protein.

soluble protein subset (Figure 5, right-hand panel, lane 2), being totally undetectable in the matrix preparation (Figure 5, right-hand panel, lane 7).

The observation that the hyperphosphorylated forms of HIRA detected during mitosis are excluded from the nuclear matrix suggest that this modification enhances the biochemical extractability of HIRA, which probably reflects its decreased association with chromatin material *in vivo*.

The endogenous HIRA is a long-lived protein

Nuclear proteins phosphorylated during the mitotic phase of the cell cycle are often targeted for degradation immediately following mitosis, presumably to prevent their presence in the cell at an untimely stage in the next cell cycle. The finding that the HIRA protein was subject to mitotic phosphorylation prompted us to explore its turnover. Various synchronization protocols revealed a lack of major quantitative variations in the amounts of HIRA protein throughout the cell cycle (Y. Lécluse and M. Lipinski, unpublished work), either because HIRA is rapidly synthesized and degraded or more likely because it is a stable protein. To answer this question, we undertook a determination of the half-life of HIRA using a classical pulse-chase approach. K562 cells were metabolically labelled using ^{35}S followed by immunoprecipitation of endogenous HIRA. A representative autoradiogram is shown in Figure 6(A). Despite the presence of numerous cysteine and methionine residues in its amino acid sequence, a long incubation period (16–18 h) in the presence of radioactive cysteine and methionine was required for a specific band of approx. 112 kDa to appear in the HIRA immunoprecipitates, consistent with the known migration of HIRA [24]. Thus the HIRA protein appears not to be actively synthesized in these exponentially growing cells. Probably because of the length of the labelling period, and despite extensive preclearing, numerous bands were reproducibly visible in addition to HIRA (Figure 6A, arrow), all of them non-specific as seen in the immunoprecipitation carried out with an irrelevant antiserum (Figure 6A, lane 1). A Western-blot analysis performed on similar samples further verified the position of HIRA after migration under the same experimental conditions (Figure 6B). The latter experiment also demonstrated that equivalent amounts of HIRA protein were reproducibly precipitated up to 36 h after the beginning of the chase (Figure 6B). Three pulse-chase ex-

periments were performed. In each case, the radioactive HIRA band decreased only slightly at 6 h (Figure 6A, lane 3) after the beginning of the chase (Figure 6A, lane 2), confirming that HIRA is a stable protein. Only at later time points did the intensity of the band clearly diminish (see the 24 h time point, Figure 6A, lane 4). Quantification analyses indicated that 25 % of the initial amount of radioactive HIRA was consistently present in material immunoprecipitated at 24 h leading to a half-life determination of approx. 12 h for this protein.

DISCUSSION

A major reorganization of chromatin takes place during mitosis when duplicated chromosomes must be condensed prior to their separation and incorporation into the reconstituting nucleus of each daughter cell. Chromatin-associated proteins and protein complexes are obviously affected by this process. Contrasting with the situation in interphase when HIRA molecules are scattered throughout the nuclear space [24], we have shown here that this histone-binding protein is excluded from condensed mitotic chromosomes. In cells blocked in mitosis following drug treatment, the electrophoretic migration of HIRA was retarded and this was due to phosphorylation events. This post-translational modification of HIRA was not a side effect of drug treatment as it was also observed with low doses of nocodazole, with shorter incubation times and in non-treated mitotically enriched cells obtained by a shake-off procedure. Hyperphosphorylated forms of HIRA displayed an increased extractability from chromatin, 150 mM NaCl being sufficient to extract significant amounts of HIRA protein in drug-arrested mitotic cells, whereas 300 mM was necessary in non-synchronized cells.

Other nuclear proteins that are similarly phosphorylated in mitosis are subsequently degraded and totally absent in early- G_1 -phase cells [36]. This is clearly not the case for HIRA, which remains readily detectable and gets progressively dephosphorylated as the cells progress into G_1 of the next cell cycle (results not shown). Indeed, our pulse-chase experiments indicated that HIRA undergoes a slow turnover. The protein is not actively synthesized and it takes 16–18 h of labelling *in vivo* for sufficient amounts of radioactive HIRA to become detectable by immunoprecipitation and autoradiography. The stability of the protein is reflected in the approx. 12 h that were necessary for precipitated HIRA to lose half of its radioactive content in the chase experiments. In conclusion, HIRA is neither synthesized nor degraded actively during the cell cycle, suggesting that its mitotic phosphorylation affects its molecular interactions rather than its stability. When the composition of the HIRA-containing high-molecular-mass complex is solved (this is work in progress in our laboratory), it will be interesting to examine whether HIRA molecular partners vary between mitotic and interphase cells.

One possible reason why HIRA gets dissociated from chromatin could be that otherwise it would be detrimental to chromosome condensation or to any other mitosis-associated chromatin-affecting event. Alternatively, this mitotic redistribution of HIRA could precede and allow retargeting to different regions of chromatin in daughter compared with parental cells. The single HIRA protein in higher eukaryotes and its two structural homologues in *S. cerevisiae*, Hir1p and Hir2p, define a family of nuclear proteins that are likely to function in regulation of gene transcription, presumably via an impact at the chromatin level, as suggested by their evolutionarily conserved histone-binding properties. Studies in avian [15] and murine [8] models have demonstrated that HIRA is essential for normal processes during early embryogenesis. We propose that one way

in which HIRA is important in development is as part of protein complexes that specify the chromatin status of genomic regions targeted by sequence-specific DNA-binding transcription factors such as the homeodomain protein Pax-3 [25]. A nonapeptide perfectly fitting the consensus for AT-hook sequences [29] is present and evolutionarily conserved in HIRA proteins from higher eukaryotes. AT-hook-containing proteins bind the minor groove of DNA with a preference for AT-rich sequences but without further nucleotide specificity. Matrix- or scaffold-associated DNA regions (MARs or SARs) that function as boundary elements for transcriptional domains have been shown to be bound by AT-hook-containing proteins with global roles in regulation of cellular functions in defined lineages [37]. Components in yeast [38] and mammalian [39] chromatin-remodelling complexes contain AT hooks that are essential to their functions. We suggest that HIRA utilizes its AT hook to further tether HIRA-containing complex(es) to chromatin.

Using both biochemical and immunofluorescence techniques we have found that a significant albeit minor number of the nuclear HIRA molecules were tightly associated with the nuclear matrix in both non-synchronized and mitotic cells. From their electrophoretic behaviour, these particular HIRA molecules were not subject to the same phosphorylation event that modified the migration and biochemical properties of extractable HIRA. Physically, the nuclear matrix provides interfaces for the attachment of multi-protein and multi-enzyme complexes involved in DNA and RNA metabolism [40]; functionally, it is involved in the regulation of DNA replication [30,41], RNA transcription and splicing [42,43] or cellular differentiation [44]. In this regard, HIRA belongs to a growing group of molecules that includes DNA-binding transcription factors [45], ATPases such as those found in mammalian Swi/Snf complexes [46], histone acetyltransferases and deacetylases [47,48], protein kinases [49,50] and the DNA replication factor PCNA (proliferating-cell nuclear antigen) [51]. Those HIRA molecules that remain in the nuclear matrix in a non-modified form during mitosis are likely to engage in molecular associations different from those of extractable HIRA. They could also be important in the reassembly of HIRA-containing multi-protein complexes following mitosis. These questions clearly call for additional studies.

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