CHMP1 is a novel nuclear matrix protein affecting chromatin structure and cell-cycle progression

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Accepted 6 April 2001
Journal of Cell Science 114, 2383-2393 © The Company of Biologists Ltd

SUMMARY

The Polycomb-group (PcG) is a diverse set of proteins required for maintenance of gene silencing during development. In a screen for conserved partners of the PcG protein Polycomblike (Pcl), we have identified a new protein, human CHMP1 (CHromatin Modifying Protein; CHarged Multivesicular body Protein), which is encoded by an alternative open reading frame in the PRSM1 gene and is conserved in both complex and simple eukaryotes. CHMP1 contains a predicted bipartite nuclear localization signal and distributes as distinct forms to the cytoplasm and the nuclear matrix in all cell lines tested. We have constructed a stable HEK293 cell line that inductively overexpresses CHMP1 under ecdysone control. Overexpressed CHMP1 localizes to a punctate subnuclear pattern, encapsulating regions of nuclease-resistant, condensed chromatin. These novel structures are also frequently surrounded by increased histone H3 phosphorylation and acetylation. CHMP1 can recruit a PcG protein, BMI1, to these regions of condensed chromatin and can cooperate with co-expressed vertebrate Pcl in a Xenopus embryo PcG assay; this is consistent with a role in PcG function. In combination, these observations suggest that CHMP1 plays a role in stable gene silencing within the nucleus.

Key words: MeSH, Gene silencing, Nuclear matrix, Chromatin, Histones, DNA replication, S phase

INTRODUCTION

Epigenetic modifications are used to record and stabilize changes in the transcriptional activity of genes. One memory system, first discovered in Drosophila, uses the antagonistic action of repressors (Polycomb-group; PcG) and activators to maintain earlier developmental decisions about the activity of specific targets (Gould, 1997; Jacobs and van Lohuizen, 1999; Pirrotta, 1999). The PcG comprises a large, structurally diverse set of nuclear proteins, which functions both to select target genes and regulate their transcription.

Both biochemical and genetic studies suggest that PcG silencing is mediated through modification of chromatin structure. For example, a purified PcG complex has been demonstrated to inhibit nucleosome remodeling by SWI/SNF proteins in vitro (Shao et al., 1999). Furthermore, one component of this complex has been shown to directly interact with the nucleosome core (Breiling et al., 1999). Changes in histone covalent modification may also play a direct role in silencing. Studies in Drosophila demonstrate a genetic interaction between the PcG and dMi-2, which encodes a component of a nucleosome remodeling/histone deacetylase complex (Kehle et al., 1998; Wade et al., 1998; Zhang et al., 1998). Finally, one member of the PcG, human EED, has been demonstrated to interact physically with a histone deacetylase (van der Vlag and Otte, 1999).

Genetic analysis and theoretical considerations suggest that establishment or maintenance of PcG silencing requires the activity of proteins during DNA replication and mitosis. The Drosophila PcG gene cramped is expressed only during S-phase and interacts with the DNA replication gene mus209 (Yamamoto et al., 1997). At mitosis, the Drosophila PcG protein CCF is associated with mitotic chromosomes and is required for their proper condensation (Kodjabachian et al., 1998). By contrast, at mitosis the majority of transcription factors and most PcG proteins are no longer chromatin bound (Buchenau et al., 1998). The temporal relationship between the action of cramped, ccf and other PcG genes has not been defined.

Most of the 15 identified Drosophila PcG proteins have been demonstrated to have structural and functional counterparts in vertebrates (Gould, 1997) and some PcG target genes may also be conserved (Bel et al., 1998; Hanson et al., 1999; Sattijn and Otte, 1999). By contrast, only a few PcG proteins have structural relatives in Caenorhabditis elegans (Holdeman et al., 1998; Kelly and Fire, 1998; Korf et al., 1998), Arabadopsis (Preuss, 1999) or budding yeast (Nislow et al., 1997; Stankunas et al., 1998). The absence of conserved PcG components in these organisms suggests that specific aspects of this silencing mechanism arose later in evolution. In addition, it may indicate that highly conserved, multifunctional PcG components common to all eukaryotes might be under-represented in Drosophila genetic screens for new PcG members.

The PcG has been estimated to include 30-40 gene products (Jurgens, 1985; Landecker et al., 1994), with about half of these molecularly identified. These chromatin-associated proteins have been shown to function together through cis-acting regulatory elements (Chan et al., 1994; Chiang et al., 1995; Christen and Bienz, 1994), but are found in several biochemically distinct multiprotein complexes. The best
characterized is a complex of eight polypeptides called PRC-1 (Shao et al., 1999) and a second, distinct complex that includes Enhancer-of-Zeste and Extra Sex Combs (Denisenko et al., 1998; Jones et al., 1998; Sewalt et al., 1998; van Lohuizen et al., 1998). Some PcG components have not been included in either complex and may have distinct roles. For example, Pcl shows genetic interactions with many PcG genes and encodes a protein (Polycomblike) that colocalizes with other PcG proteins on polytene chromosomes, but does not co-purify with other known PcG proteins. Identification of physical partners for Pcl may help to unify our understanding of the action of PRC-1 and Enhancer-of-Zeste complexes and might reveal distinct gene regulatory mechanisms.

We used the yeast two-hybrid system, with mammalian Pcl as bait, to identify a novel potential component of the PcG, called CHMP1 (pronounced chimp-1; CHromatin Modifying Protein; CHarged Multivesicular body Protein). CHMP1 is widely conserved across eukaryotes and plays a cytoplasmic role in protein sorting to the multivesicular body (Howard et al., 2001). Endogenous mammalian CHMP1 is also present in the interphase nuclear matrix and mitotic chromosome scaffold. When overexpressed, CHMP1 has potent effects on nuclear structure and DNA replication. In addition, exogenous CHMP1 targets a PcG protein to condensed chromatin and functions like a PcG protein in a Xenopus misexpression assay. Thus, CHMP1 has a potential dual function, with distinct roles in membrane trafficking and nuclear gene regulation.

MATERIALS AND METHODS

Plasmid constructions
pLexA-mPcl1 and pLexA-dPcl were constructed for two-hybrid analysis by introducing the full-length open reading frames from mPcl1 (Yoshitake et al., 1999) and Drosophila Pcl (Lonie et al., 1994) into the BamHI site of pLex-A. The Drosophila Pcl cDNA was kindly provided by R. Saint. For subcloning, the human CHMP1 open reading frame was isolated by reverse-transcription from human placental RNA followed by PCR with primers 5'-GCGCAATTCTACCACTGGCAGATCCGGTCCATGTGA-3' and 5'-GGCA-GGATCCATACGGGGGCAGACGGTGCAA-3'. The resulting EcoRI/BamHI fragment was subcloned into a modified version of pC52+ (a gift of David Turner) to generate pCS-hCHMP1. pIND-CHMP1 was constructed by excising CHMP1 from pCS-hCHMP1 with HindIII/Xhol and introducing it into the corresponding sites of pIND (Invitrogen).

Two hybrid screen
Saccharomyces cerevisiae strain L40, transformed with pLex-mPcl1, was used to screen an E9.5/10.5 mouse embryo cDNA fusion library as described previously (Hollenberg et al., 1995). The bait plasmid (containing ADE2) was eliminated from each primary positive by isolating a red colony (Chen et al., 1996). Each library positive was then tested for β-galactosidase activity after mating with derivatives of yeast strain AMR70 that express LexA-mPcl1 or LexA-dPcl.

Fractionation of cells and tissues
Nuclear matrix was isolated as described previously (Belgrader et al., 1991), with minor modifications. Cells were washed and collected in cold PBS and pelleted by centrifugation at 1000 rpm for 5 minutes. From this point on, all buffers contained a protease inhibitor cocktail consisting of 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 1 mM E-64 and 1 mg/ml each of bestatin, leupeptin and aprotinin (Sigma Immunochemicals). The cell pellet was resuspended in 1.5 ml lysis buffer (10 mM Tris-HCl, pH 7.4, 2.5 mM MgCl2) and transferred to ice for 15 minutes. Cells were lysed with 25-60 strokes of a dounce homogenizer and the lysate was gently layered over a 1.5 ml sucrose cushion (0.5 M sucrose, 10 mM Tris-HCl, pH 7.4, 2.5 mM MgCl2). Nuclei were pelleted by centrifugation at 5000 g for 10 minutes at 4°C in a Sorvall HB4 rotor. The upper layer consisting of cytoplasmic extract was collected and concentrated to 100 µl in a centricron 10 column. The nuclear pellet was stripped of membranes by resuspension in 200 µl of Triton extraction buffer (0.5% Triton X-100, 10 mM Tris-HCl pH 7.4, 2.5 mM MgCl2), followed by incubation on ice for 15 minutes. Membrane depleted nuclei were collected by centrifugation at 5000 g for 10 minutes in a microfuge at 4°C. Chromatin and soluble proteins were removed by resuspension in 150 µl of DNase I digestion buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl2) containing 50 U of DNase I and incubated at 4°C for 1 hour. This was followed by addition of 150 µl of 2X High Salt buffer (10 mM Tris-HCl pH 7.4, 1.45 M NaCl), and further incubation for 15 minutes at 4°C. The sample was centrifuged at 10,000 g for 10 minutes at 4°C and the supernatant saved as the DNase I High Salt fraction. The nuclear matrix pellet was resuspended in 150 µl of RIPA buffer (50 mM Tris-HCl pH 7.4, 5 mM DTT, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS).

Stable line construction and cell culture
The IND-CHMP1 stable line was obtained by Lipofectamine (Gibco BRL) transfection of EcR-293 cells (human embryonic kidney, stably expressing the hybrid edcsyone receptor, Invitrogen). HEK293 cells were cultured on plates or eight-well Nunc SuperCell culture slides (Intermountain Scientific) in Dulbecco's modified eagle medium (DMEM) with 10% fetal calf serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin (Gibco BRL), 300 µg/ml Zeocin (Invitrogen) and 800 µg/ml G418 (Calbiochem). Cells were allowed to recover for 18 hours after plating before muristerone (10 µM) induction.

Immunocytochemistry and antibodies
Cultured cells were fixed with 4% paraformaldehyde in PBS, washed with PBS and allowed to dry on the slide. Slides were incubated 30 minutes in blocking solution (PBS with 10% normal sheep serum, 0.1 mg/ml bovine serum albumin, and 0.05% Triton X-100). Primary antibodies in blocking solution and secondary antibodies in PBS with 0.05% Triton X-100 were added consecutively for 1 hour each at room temperature.

Rabbit polyclonal anti-CHMP1 (1:300) was made by injection into rabbits of GST-CHMP1 fusion protein mixed with Freund's incomplete adjuvant (Pocono Rabbit Farm & Laboratory Inc.). The antibody was subsequently purified over a MBP-CHMP1 Affigel-10 column and eluted with low pH. Other antibodies include polyclonal phospho-histone H3 (Cat# 06-570 1:200) and polyclonal anti-acetylated histone H3 (Cat# 06-599, 1:100) from Upstate Biotechnology, and monoclonal anti-BMI1 (a kind gift from Maarten van Lohuizen, 1:300). Secondary antibodies are anti-rabbit Alexa 488 (Molecular probes, 1:300) and Cy3 (Jackson ImmunoResearch Laboratories Inc.; 1:300) or anti-mouse Cy3 (Jackson ImmunoResearch Laboratories Inc.; 1:300). Hoechst 33258 and propidium iodide (Molecular Probes) were included at 1.0 µg/ml. Samples were visualized with a Zeiss Axiosoplan microscope or by confocal laser scanning microscopy on a BioRad MRC 1000. Bromodeoxyuridine (BrdU) labeling was performed according to instructions provided with the product (Becton Dickenson). TUNEL was performed according to instructions included in the kit (Boehringer Mannheim). Mitotic shake-off and spreads were performed as described (Chandler and Yunis, 1978). The stabilized nuclear matrix was prepared as described previously with modifications (Nickerson et al., 1997). IND-CHMP1 cells were grown on chamber slides and incubated for 24 hours with 10 µM ponasterone. Cells were washed with cold PBS and soluble proteins were removed
by extraction in CSK buffer (10 mM Pipes, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, protease inhibitors) for 5 minutes at room temperature. Cells were then crosslinked by treatment with 4% paraformaldehyde in CSK for 5 minutes at room temperature and washed three times with CSK buffer for 5 minutes at room temperature. After washing in CSK, cells were digested with 0.25, 125, or 500 U/ml of DNase I for 50 minutes at room temperature in digestion buffer (10 mM Pipes, pH 6.8, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂ protease inhibitors). Cells were then washed with 0.25 M ammonium sulfate extraction buffer (10 mM Pipes, pH 6.8, 250 mM ammonium sulfate, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, protease inhibitors). The ammonium sulfate extraction buffer was aspirated and cells were washed three times with cold PBS followed by standard immunocytochemistry.

**Western analysis**

Antibodies used were affinity-purified polyclonal anti-CHMP1 (1:1000), monoclonal anti-α-tubulin (Research Biochemicals International, 1:10,000), and chicken polyclonal anti-matrin-4 (kind gift of Ronald Berezney, 1:2000). Bound antibodies were detected by HRP-conjugated secondary antibodies and enhanced chemiluminescence.

**Flow cytometry**

A single-cell suspension was prepared by trypsin digestion and titration. The cells were fixed in cold 50% ethanol with vortexing and stored at -4°C. After washing, the cells were stained with a modified, standard propidium iodide staining protocol (Thornthwaigte et al., 1980). Samples were then analyzed on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). A total of 20,000 cells were analyzed for each cell line and growth condition. Raw single parameter histogram data was fit with the Multicycle program (Phoenix Flow Cytometry, Inc., San Diego, CA) to estimate the G1, S and G2/M phases of the cell cycle.

**Xenopus injection assay**

Embryos were injected with capped, in vitro transcribed RNA and were analyzed morphologically and through RNA in situ hybridization, as described previously (Yoshitake et al., 1999).

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**RESULTS**

**Identification of a conserved Pcl partner**

The yeast two-hybrid system was used to screen for partners of the mammalian PclG protein mPcl1 (Yoshitake et al., 1999). The full-length mPcl1 open reading frame was fused to LexA and used to screen a mouse embryo cDNA fusion library (Hollenberg et al., 1995). Each positive was also tested for two-hybrid interaction with Drosophila Pcl (Lonie et al., 1994) to define the subset of proteins which interact with the conserved structural features of mPcl1 (Yoshitake et al., 1999). This procedure isolated a single library clone with equivalent Pcl and mPcl1 two-hybrid signals, which corresponded to PRSM1 (Scott et al., 1996), a gene frequently deleted in sporadic breast carcinoma (Whitmore et al., 1998). Surprisingly, a reading frame alternative to that reported to encode the PRSM1 metalloprotease was fused in-frame to the library activation domain coding sequence. This distinct reading frame is contained entirely within PRSM1 (Fig. 1A) and encodes a novel protein of 196 amino acids, called CHMP1.

CHMP1 is closely related to another human protein, CHMP1.5, and has structural counterparts in many eukaryotes including Drosophila, C. elegans, Dictyostelium, Arabidopsis and S. cerevisiae (Fig. 1B; C; D.R.S. and S.M.H., unpublished). A search for structural motifs identifies only a predicted bipartite nuclear localization signal (Fig. 1C; aa 20-35), which is also present in human CHMP1.5 and Drosophila, Dictyostelium and Arabidopsis CHMP1, but absent in CHMP1 from C. elegans and budding yeast. A BLAST similarity search (Altschul et al., 1997) for other human proteins related to CHMP1 shows that it is most closely related to BC-2, a protein upregulated in the nuclear matrix of breast adenocarcinoma (Keesee et al., 1999), followed by more distant proteins involved in vesicle trafficking in S. cerevisiae (Bast et al., 1998; Howard et al., 2001).

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**Fig. 1.** CHMP1 is a conserved eukaryotic protein. (A) Conceptual translation products CHMP1 and PRSM1 from alternative reading frames of the human PRSM1 mRNA. (B) Phylogenetic tree for CHMP1 and CHMP1.5 from human (H.s.), and CHMP1 from Drosophila melanogaster (D.m.), C. elegans (C.e.) and S. cerevisiae (S.c.). ClustalX was used to align proteins and calculate the distance between proteins for tree construction (Thompson et al., 1997). (C) All CHMP1 proteins from the tree are aligned. Amino acids that are identical in at least three proteins are highlighted.
A modified form of CHMP1 is nuclear matrix-associated

The two-hybrid interaction between CHMP1 and a PcG protein suggested a nuclear localization and function for CHMP1. To test this possibility, we analyzed the endogenous subcellular distribution of CHMP1 in a variety of established cell lines using an affinity-purified polyclonal antiserum to CHMP1. Western blot analysis of a whole-cell extract from human HEK293 cells (Fig. 2A, ‘w’) shows a doublet consisting of 32 kDa and 35 kDa species. These two forms are likely to represent differential CHMP1 post-translational modification. Each can be competed by pre-incubating the CHMP1 antibody with an excess of CHMP1 fusion protein (data not shown) and both are increased by overexpression from a human CHMP1 cDNA in HEK293 cells (Fig. 3B). Subcellular fractionation of HEK293 cells into cytoplasmic (c), nuclear DNaseI/high salt (ds) and matrix (m) fractions reveals that the smaller and larger CHMP1 forms are restricted to the cytoplasm and nuclear matrix, respectively (Fig. 2A). A cytoplasmic marker, α-tubulin, is undetectable in noncytoplasmic fractions, whereas nuclear matrix marker matrin-4 (Nakayasu and Berezney, 1991) is absent from the cytoplasmic fraction, indicating the fidelity of our fractionation procedure. This partitioning of the two CHMP1 forms into distinct subcellular compartments also occurs in each of six other human cell lines tested (Fig. 2B).

Although the matrix form of CHMP1 never localizes to the cytoplasm, some lines (HeLa, MCF7, HT-29 and JAR) show a detectable level of the 32 kDa form of CHMP1 in the nuclear matrix. This suggests that both CHMP1 forms are sometimes matrix-associated.

Subcellular compartmentalization of the two CHMP1 species also occurs in a murine system. An established mouse cell line (C2C12 myoblasts) and primary mouse embryo fibroblasts (MEF) show accurate fractionation of the two CHMP1 subcellular forms (Fig. 2C). Whole-tissue extracts from mouse indicate that both CHMP1 forms are widely expressed, with increased levels of the 35 kDa form in adult heart, kidney and liver (Fig. 2D). Overall, these analyses demonstrate that CHMP1 is expressed in diverse mammalian cell types as distinct cytoplasmic and nuclear forms.

The occurrence of two CHMP1 forms suggested that CHMP1 might be modified in a cell-cycle-dependent fashion. To test this possibility, HEK293 cells were synchronized and fractionated at distinct phases of the cell cycle. Western analysis of CHMP1 demonstrated that the ratio of cytoplasmic to nuclear CHMP1 is largely invariant throughout interphase (D.R.S., T.N. and S.M.H., unpublished). Within mitotic cells the larger, 35 kDa form of CHMP1 is found in the chromosome scaffold fraction. In contrast to this western blot analysis, M-phase is the only point during the cell cycle when nuclear CHMP1 becomes visible by immunocytochemistry. During telophase, CHMP1 is distributed in a punctate arrangement on the condensed chromosomes, but cannot be seen in the nucleus immunocytochemically at earlier stages of mitosis or in interphase nuclei (D.R.S., T.L.H., T.N. and S.M.H., unpublished). Cytoplasmic CHMP1, however, is readily visible by immunocytochemistry and exhibits a perinuclear localization (Fig. 3C, 0 hours) (Howard et al., 2001). In combination, western analysis and immunocytochemistry demonstrate that nuclear CHMP1 is present within the nuclear matrix or chromosome scaffold at all stages of the cell cycle, but has an epitope that is usually masked from antibody recognition in situ.

Exogenous CHMP1 is modified and nuclear matrix-associated

Overexpression in mammalian cells was used to study the functional potential of CHMP1. All attempts to generate a stable line that constitutively overproduces CHMP1 were unsuccessful, regardless of the species origin of CHMP1 (D.R.S. and S.M.H., unpublished). Therefore, we relied upon an ecdysone-inducible expression system (No et al., 1996) to
CHMP1 functions in the nucleus

Fig. 3. Exogenous CHMP1 is tightly regulated and enters the nucleus. All experiments use IND-CHMP1 cells and exogenous CHMP1 induction with 10 μM muristerone. (A) Total RNA was harvested at the indicated times of induction and analyzed by northern blot for CHMP1, with CHO-B as an internal control. Exogenous CHMP1 RNA does not comigrate with exogenous and is not detected with this shorter exposure. (B) Cells were harvested at 0 hours and 24 hours of induction, fractionated and analyzed as in Fig. 2. CHMP1 species that comigrate with endogenous (closed arrows) or are novel (open arrow) are indicated. (C) Cells were induced for the indicated times, fixed and double labeled with anti-CHMP1 (red) and Hoechst 33258 (blue). Note the cytoplasmic fibers at 3 hours and 9 hours. (D) Cells were induced for 24 hours, including treatment with colcemid (200 ng/ml) for the final 10 hours to enrich for mitotic cells.

Metaphase spreads were prepared (Chandler and Yunis, 1978) and stained for CHMP1 and DNA. The inset shows an enlarged region from the spread. c, cytoplasm; m, nuclear matrix solubilized by detergent; w, whole cell with complete detergent solubilization.

generate a derivative of HEK293 cells, IND-CHMP1, which conditionally overexpresses CHMP1. Northern blot analysis demonstrates a tightly regulated, rapidly inducible CHMP1 (Fig. 3A). Exogenous CHMP1 RNA is virtually undetectable prior to induction, whereas a strong signal appears at 1.5 hours and is close to steady state by 3 hours (Fig. 3A, compare 0, 1.5, 3 and 6 hours).

Biochemical fractionation of IND-CHMP1 cells demonstrates that the ratio of cytoplasmic to nuclear CHMP1 is not dramatically altered during induction (Fig. 3B). The intensity of the cytoplasmic and nuclear matrix forms of CHMP1 are both comparably increased (compare whole cell, ‘w’, for 0 and 24 hours). Overexpression does alter the ratio of CHMP1 forms within the nuclear matrix fraction (m). In addition to upregulation of the 35 kDa CHMP1 matrix form (top arrow), both the 32 kDa form (bottom arrow) and a novel intermediate form (open arrow) become prominent in the nuclear matrix after a 24 hour induction, which suggests saturation or disruption of normal modification mechanisms.

IND-CHMP1 was analyzed by immunocytochemistry at various times of induction to define further the subcellular distribution of exogenous CHMP1. At early times of induction (Fig. 3C, 3-9 hours), CHMP1 produces a transient cytoplasmic fiber pattern (3 hours) and mixed cytoplasmic fiber nuclear punctate pattern (9 hours). At 24 hours post induction, nearly all cells exhibit a very intense nuclear punctate CHMP1 pattern and less intense cytoplasmic diffuse signal. The extreme intensity of the punctate nuclear CHMP1 signal at 24 hours post induction made simultaneous visualization of both the nuclear and less intense cytoplasmic diffuse patterns impossible without greatly over exposing the images (Fig. 3C, 24 hours). In contrast to the transient production of CHMP1 cytoplasmic fibers, the nuclear pattern of CHMP1 persists for at least three days. This nuclear pattern is not reversible by muristerone removal, even though exogenous CHMP1 mRNA becomes undetectable within 6 hours, indicating that nuclear CHMP1 is extremely stable in these cells (T.N. and S.M.H., unpublished). The suggestion that CHMP1 is closely associated with chromatin in the nucleus is supported by analysis of mitotic IND-CHMP1 cells. After induction, metaphase spreads show that CHMP1 is localized to the axial component of highly condensed chromosomes, the chromosome scaffold (Fig. 3D) (Earnshaw and Laemmli, 1983). Therefore, immunocytochemical analysis of exogenous CHMP1 in both interphase and mitotic cells strongly supports the nuclear localization of endogenous CHMP1, detectable only by western with our antibody during interphase.

CHMP1 arrests cells in S-phase

Exogenous CHMP1 produces strong effects on cell-cycle progression. After treating IND-CHMP1 cells with muristerone for 24 hours, the mitotic index drops tenfold (Fig. 4A). In addition, the fraction of cells replicating their DNA and thus able to incorporate BrdU, decreases fourfold (Fig. 4A). These effects are not a result of increased apoptosis, as measured by TUNEL staining, and therefore must reflect alterations in the cell cycle. To define the cell-cycle effect, the percentage of cells in each phase was determined using flow cytometry of propidium-iodide-stained nuclei. Fig. 4B shows the distribution of cells in G1, S and G2/M. DNA content is unaffected by muristerone in the parental activator line and is very similar to that observed in IND-CHMP1 prior to induction. By contrast, CHMP1 induction produces a significant increase in S-phase cells by 24 hours (data not shown), with about 80% of the cells showing an S-phase DNA content at 48 hours (Fig. 4B). Not only are many more cells in S-phase, but a greater fraction are in late S-phase relative to early S-phase, as indicated by the steep upward slope of the S-phase peak. The poor correspondence between the fraction of cells with S-phase DNA content (80%) and those actively replicating DNA (10% BrdU positive) (compare Fig. 4A,B), suggests that cells have arrested or are strongly inhibited in their transit through S-phase, with a stronger effect during late S-phase.

CHMP1 alters chromatin structure

The punctate pattern produced by CHMP1 in the nucleus is accurately mirrored by both Hoechst 33258 and propidium-iodide fluorescence (Fig. 5A). As these two dyes bind to DNA through distinct mechanisms (Harshman and Dervan, 1985; Pjura et al., 1987; Wilson et al., 1985), the similar effect on both strongly suggests that CHMP1 is locally increasing
nuclear DNA concentration through chromatin condensation. Surprisingly, laser scanning confocal microscopic analysis reveals that CHMP1 and the DNA dyes are not perfectly colocalized. Instead, CHMP1 predominantly localizes to the exterior regions of these new subnuclear structures, with condensed chromatin inside (Fig. 5A). This lack of internal antibody staining is not a consequence of general antibody inaccessibility: a PcG antibody against BMI1 penetrates the structure readily (Fig. 7).

Because modified chromatin structure is expected to coincide with changes in histone modification (Kuo and Allis, 1998), the subnuclear distribution of phosphorylated (Ser10; P-H3) and acetylated (Lys9,14; Ac-H3) histone H3 levels were measured with specific antibodies (Hendzel et al., 1997). Prior to induction, P-H3 occurs at high levels only in mitotic cells and Ac-H3 shows a speckle pattern uniformly distributed throughout the nucleus (Fig. 4A; Fig. 5B, 0 hours). CHMP1 induction produces localized increases in P-H3 and Ac-H3 levels (Fig. 5B, 24 hours), with these modified histones accumulating in a relatively stable or very transient fashion, respectively, in a subset of nuclei. Phospho-H3-positive regions have no correspondence to centromeric heterochromatin as demonstrated by double staining with a CENP-B antiserum (D.R.S., T.L.H. and S.M.H., unpublished). Therefore, this does not represent classical mitotic chromosome condensation that initiates near centromeres (Hendzel et al., 1997; Van Hooser et al., 1998). Neither CHMP1, P-H3, nor Ac-H3 localizes predominantly within regions of de novo condensed chromatin. Instead, each surrounds the Hoechst-dense DNA in a shell-like structure in what are likely to be coincident patterns (Fig. 5A,B; Fig. 7). Therefore, overexpressed CHMP1 defines the exterior of nuclear bodies, which show modified histones at the periphery and condensed chromatin within the interior.

**CHMP1 bodies are nuclease resistant**

The nuclear CHMP1 structures have been further defined by measuring the nuclease resistance of the components relative to bulk chromatin. We have relied on a previous study which demonstrated that chromatin in fixed nuclei is readily accessible to nuclease digestion (Nickerson et al., 1997). CHMP1-expressing cells were freed of membrane, fixed, digested with increasing concentrations of DNase I for 1 hour and analyzed by fluorescent microscopy for localized DNA content relative to CHMP1 or P-H3. As shown in Fig. 6, DNase I selectively degrades chromatin that is external to CHMP1 structures (compare Hoechst at 50 U vs. 0 U), supporting the conclusion that increased dye binding corresponds to condensed, nuclease-resistant chromatin. A higher concentration of DNase I can efficiently remove Hoechst-stained, condensed chromatin (Fig. 6, 200 U) (Nickerson et al., 1997), but has no impact on CHMP1 and P-H3 staining intensity. This DNA-independent nuclear matrix localization of CHMP1 is consistent with the cell fractionation analysis (Fig. 2A, ds). By contrast, the nuclease resistance of the P-H3 signal is completely unexpected and significantly greater than on mitotic chromosomes (D.R.S. and S.M.H., unpublished).

**CHMP1 localizes PcG proteins to condensed chromatin**

CHMP1 was isolated as a Pcl partner and was therefore
hypothesized to participate in PcG function. In some cell types, such as the human osteosarcoma cell line U2OS, PcG proteins are colocalized at punctate subnuclear structures (Alkema et al., 1997). To investigate the relationship between these PcG structures and CHMP1 nuclear bodies, U2OS cells were transfected with CHMP1, then subsequently fixed and triple-labeled for CHMP1, the PcG protein BMI1 and DNA (Fig. 7). Approximately 90% of the transfected cells that overexpress CHMP1 no longer exhibit a punctate BMI1 pattern (D.R.S. and S.M.H., unpublished). Given that BMI1 has been demonstrated to lose its punctate distribution in late S-phase (Voncken et al., 1999), this result is consistent with the action of CHMP1 on cell-cycle progression in our inducible line (Fig. 3; Fig. 5; Fig. 6). In addition, they exhibit a punctate BMI1 distribution that colocalizes with condensed chromatin and is encapsulated by CHMP1 (Fig. 7). Transfected CHMP1 also recruits human Polycomb2 to condensed chromatin (D.R.S. and S.M.H., unpublished), suggesting that many components of this PcG multiprotein complex (Alkema et al., 1997) respond similarly to CHMP1 overexpression.

**CHMP1 misexpressed in Xenopus embryos produces a PcG phenotype**

A Xenopus embryo assay was used to compare the functional capabilities of CHMP1 and other PcG proteins. We showed previously that dorsal injection of RNA encoding distinct PcG proteins into four-cell Xenopus embryos produces similar effects: repression of En-2 and Rx2A, and disruption of normal anterior neural development (Yoshitake et al., 1999). This assay was used to compare the ability of CHMP1 and mPcl1 RNA to produce this characteristic PcG phenotype. As shown in Fig. 8A, unilaterally injected CHMP1 RNA can repress En-2 or shift its site of expression posteriorly, frequently producing more pronounced changes than mPcl1. The ability of mPcl1 and CHMP1 to cooperate in this assay was quantified using suboptimal concentrations of each RNA. Fig. 8B demonstrates a greater than additive effect on En-2 repression with co-injected CHMP1 and mPcl1, and an additive effect on shifting the En-2 site of expression. Also consistent with PcG function, CHMP1 strongly represses Rx2A and alters anterior neural development in a manner indistinguishable from other PcG
CHMP1 cooperates with mPcl1 and produces the entire spectrum of phenotypes observed with four other PcG RNAs (Yoshitake et al., 1999).

**DISCUSSION**

On the basis of the assumption that the structural conservation of Pcl from different phyla results from a conserved protein interaction, we have used the yeast two-hybrid system to identify CHMP1. This novel protein is found in a much broader spectrum of eukaryotes than Pcl, and is thus likely to carry out fundamental activities that are independent of Pcl. Our analyses in this and the accompanying study (Howard et al., 2001) are consistent with this notion and indicate that CHMP1 mediates distinct processes in both the cytoplasm and nucleus.

**CHMP1 subcellular localization**

Cell and tissue fractionation studies demonstrate that CHMP1 is not only ubiquitously expressed, but is always found in both cytoplasmic and nuclear matrix fractions. The accompanying study (Howard et al., 2001) demonstrates a cytoplasmic role for CHMP1 in membrane trafficking, indicating that cytoplasmic CHMP1 is not simply dormant protein awaiting its nuclear calling. Consistent with distinct functions for CHMP1 within the cell, we have observed a size difference between the cytoplasmic and nuclear forms, which is apparently the result of covalent modification. It is presently unclear how CHMP1 is partitioned into cytoplasmic and nuclear compartments. Although the putative modification may regulate nuclear entry, our observation of significant levels of the lower molecular weight, less-modified form of CHMP1 in the nucleus in some cell types argues against this simple distribution mechanism.

Within the nucleus, both CHMP1 species are tightly associated with the nuclear matrix. During mitosis, when the nuclear matrix disassembles, CHMP1 remains associated with the M-phase chromosome scaffold. Little is known about the extent to which components of the nuclear matrix are recruited for mitotic scaffold formation, although the biochemical similarity of these structures suggests some shared proteins. Further work will be required to determine if CHMP1 is complexed with a common set of proteins in both the interphase nucleus and mitotic scaffold. Given the proposed role of CHMP1 in stabilizing gene activity during cell division, it is tempting to speculate a function for CHMP1 in structuring the condensed chromosome to facilitate gene memory through M-phase. A similar role has been suggested for Drosophila CCF, a protein that cooperates with other PcG proteins and is essential for correct mitotic chromosome condensation (Kodjabachian et al., 1998).

**Chromatin modification through CHMP1 action**

The suggestion that CHMP1 might play a role in mitotic chromosome condensation is supported by its ability to produce regions of visibly condensed chromatin in the interphase nucleus. CHMP1 accumulates in specific subnuclear regions and promotes increased DNA dye fluorescence that is nuclease resistant. Surprisingly, CHMP1 does not perfectly colocalize with this structurally altered
chromatin, but forms a shell around it. As this shell frequently shows histone H3 phosphorylation and acetylation, it is likely to represent a region of gene transcriptional activity (Cheung et al., 2000; Clayton et al., 2000). In addition, it might be a region of chromatin restructuring (Sauve et al., 1999). Perhaps this region acts as a transition zone for genes that are either being incorporated into the condensed chromatin structure or are being transcriptionally activated. This shell would thus act as a boundary that includes the demarcation between active and inactive chromatin domains. The existence of CHMP1 within this shell suggests a possible role for CHMP1 in sequestering or sorting DNA elements. A functional interaction between PcG proteins and boundary elements has been observed in Drosophila and mammalian cells (Gerasimova and Corces, 1998).

One intriguing aspect of the CHMP1 structure is its strong resistance to nuclease digestion. The interior of the structure is more resistant to nuclease digestion, whereas the CHMP1-positive shell is extremely resistant. This property is shared with the phosphorylated histone H3 signal that surrounds condensed chromatin. This observation suggests that histones can become bound to the nuclear matrix at sites of chromatin restructuring, perhaps as substrates for matrix-bound remodeling complexes. Our analysis of histone H4 does not show acetylation on the CHMP1 shell; instead, histone H4 within the CHMP1 structure exhibits weak hypoacetylation (T.L.H. and S.M.H., unpublished). An understanding of this apparent difference between histone H3 and H4 acetylation awaits a more complete description of the changes in nucleosome structure in specific contexts.

These profound changes in chromatin structure are accompanied by strong effects on cell-cycle progression. CHMP1 induction results in a large increase in the percentage of cells with a late S-phase DNA content. This effect may stem directly from the highly condensed and inaccessible chromatin that CHMP1 overexpression produces. Many have noted a strong correlation between replication timing and gene activity (Sadoni et al., 1999), thus very late replication of chromatin within CHMP1 bodies would be consistent with gene silencing. Alternatively, CHMP1 overexpression may affect the expression of genes required for DNA replication, or trigger an S-phase checkpoint.

**CHMP1 is a candidate PcG protein**

Our observations suggest CHMP1 may function in PcG silencing. Overexpressed CHMP1 encapsulates proteins in the Polycomb complex and localizes them to visibly condensed chromatin. In addition, CHMP1 behaves like other PcG proteins in a Xenopus embryo injection assay. Finally, CHMP1 shows a two-hybrid interaction with Pcl proteins from Drosophila and mouse. We have been unable to co-immunoprecipitate CHMP1 and mouse Pcl when overexpressed, but this may be a consequence of inefficient complex formation at elevated protein levels. In addition, this interaction is likely to be transient, based on the close spatial relationship between BMI1 and CHMP1 (Fig. 7), but absence of colocalization. Because CHMP1 is more evolutionarily conserved than most PcG proteins, it may function as a component of an ancient, more fundamental gene regulatory mechanism. This would be analogous to the use of the NURD/Mi-2 complex in Drosophila PcG function (Kehle et al., 1998). A physical interaction between CHMP1 and the PcG may promote a structural modification mediated by multiprotein complex associated with CHMP1.

Our analysis suggests that CHMP1 is creating condensed, nuclease-inaccessible chromatin domains. Others have not seen evidence of nuclease resistance for PcG target genes, although the assays used were quite distinct from ours (Schlossherr et al., 1994). For example, our results with CHMP1 overexpression may amplify smaller differences in accessibility by increasing the volume of structurally modified sequences. The presence of PcG proteins on condensed chromatin is supported by immunocytochemical studies with BMI1 (Voncken et al., 1999). Future analyses of PcG silencing will promote our understanding of the relationship between gene activity, chromatin structure and subnuclear localization.

**Relationships between multivesicular body sorting and gene silencing**

A combination of our results (Howard et al., 2001) and the work of others (Babst et al., 2000; Watanabe et al., 1998; Xie et al., 1998) indicates that some proteins may play a dual role in sorting membrane and protein to an endosomal derivative, the multivesicular body, and in regulating nuclear gene expression. Defining the subset of sorting proteins involved in this dual activity, their mechanism of subcellular partitioning and any mechanistic similarities between multivesicular body formation and transcriptional regulation produced as a result of dual protein activity are areas of future interest.

We thank the following generous contributors for reagents: R. Berezney, R. Saint, E. M. Tan, and M. van Lohuizen. We greatly appreciate the help of Tony Bakke with the flow cytometric analysis, and thank Jan Christian for help with the Xenopus injections and their interpretation. We also thank Matt Thayer for critical comments on the manuscript and for his continued interest and encouragement. This work was supported by a grant from the National Institutes of Health (S. M. H. GM 52458-05).

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