Human Chromatid Cohesin Component hRad21 Is Phosphorylated in M Phase and Associated with Metaphase Centromeres*

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Sister chromatids duplicated in S phase are connected with each other during G2 and M phase until the onset of anaphase. This chromatid cohesion is essential for correct segregation of genetic material to daughter cells. Recently, understanding of the molecular mechanisms governing chromatid cohesion in yeast has been greatly advanced, whereas these processes in mammalian cells remain unclear. We report here biochemical and cytological analyses of human Rad21, a homologue of the yeast cohesin subunit, Scc1p/Mcd1p. hRad21 is a nuclear phosphorylated protein. Its abundance does not change during the cell cycle, and it becomes hypophosphorylated in M phase. Most hRad21 is not associated with chromatin when the nuclear envelope breakdown takes place in prophase. However, a detailed analysis of the spread chromosomes indicated that hRad21 remains associated with prometaphase-like chromosomes along their entire lengths. The mitotic chromatin-bound hRad21 becomes dissociated in a highly regulated manner because hRad21 remains specifically at the centromeres but disappears from the arm regions on metaphase-like chromosomes. Interestingly, hRad21 at the metaphase centromeres appears to be present at the inner pairing domain where the two sister chromatids are supposed to be in intimate contact. These results suggest that hRad21 has a critical role in chromatid cohesion in human mitotic cells.

Accurate chromosome segregation during mitosis into two daughter cells is one of the requirements for stable genome maintenance. Until recently, the molecular mechanisms regulating chromatid cohesion had not been well understood. However, recent studies, mostly conducted in budding yeast, have outlined the process generally (reviewed in Ref. 1). Concurrent with DNA replication, the sister chromatids become connected along their entire length. Several distinct groups of proteins are involved in establishing and maintaining chromatid cohesion. The most intensively investigated protein complex, budding yeast cohesin, consists of four subunits proteins, Scc1p/Mcd1p, Scc3p, Smc1p, and Smc3p (2–4), and serves as a physical glue between sister chromatids. Cohesin is phylogenetically conserved. Rad21p in fission yeast and human Rad21 (hRad21) are homologues of Scc1p/Mcd1p (5, 6). Smc1p and Smc3p are found in budding yeast, Xenopus, and mammals (7–9). They are members of the SMC (structural maintenance of chromosome) protein family that is characterized by the presence of coiled-coil domains and ATPase domains (reviewed in Ref. 10). Very recently, two Scc3p homologues, SA1 and SA2, have been found in Xenopus and human cells (11). Interestingly, in Xenopus, two distinct classes of cohesin, termed x-cohesinSA1 and x-cohesinSA2, are present. These two complexes share Xenopus (X)SCC1, XSCC2, and XRAD21 and differ via containing either XSA1 or XSA2 (11). Immunodepletion of Xenopus cohesin from egg extracts led to a failure of chromatid cohesion (8). Therefore, the cohesin complex is likely to be conserved in all eukaryotes, including humans.

However, the precise roles of cohesin may be different between budding yeast and other eukaryotes. In budding yeast, Scc1p/Med1p abruptly dissociates from chromatin at the onset of anaphase (2, 4). In contrast, ~95% of Xenopus cohesion molecules (XSMC1, XSMC3, and XRAD21) dissociate from chromatin at the entry of mitosis, much earlier than the metaphase-anaphase transition (8). Similarly, in indirect immunofluorescence (IF)1 experiments, it has been found that human Smc1p and mouse Rad21 (called PW29) are mostly excluded from mitotic chromosomes (9, 12). Therefore, most cohesins are apparently absent on metaphase chromatids in higher eukaryotes. It is not known what molecules or conditions are responsible for the chromatid cohesion immediately before anaphase. Two models have been proposed to explain the apparent inconsistency between the timings of the cohesin-chromatin dissociation and the mitotic chromatid separation (8). The first model proposes that cohesin molecules are responsible for interphase-specific chromatid cohesion and that some yet unidentified mitosis-specific cohesion machinery is responsible for the chromatid cohesion from prophase until the onset of anaphase. The second model hypothesizes that the same cohesin complex is required for both interphase- and mitosis-specific chromatid cohesions. However, in this model, the complex dissociates from chromatin in two steps, whereby most cohesin is released from chromatin at the entry into mitosis. The remaining cohesin connects chromatids in metaphase and dissociates from chromatin at the onset of anaphase. The recent discovery of Xenopus (XSA) proteins revealed that a small population of XSA1 is associated with the metaphase chromosomes formed in Xenopus cell-free extracts, thus supporting the second model (11).

In this paper, we describe the biochemical and cytological behaviors of hRad21. We aimed to better understand the roles of hRad21 particularly in metaphase. We observed a small but significant population of hRad21 associated with colcemid-in-
duced mitotic chromosomes. These results suggest that the mitotic cohesion is mediated by cohesin, further underscoring the conserved mechanisms regulating chromatin cohesion and separation in eukaryotes.

**EXPERIMENTAL PROCEDURES**

Cloning of hRad21 cDNA and Preparation of Anti-hRad21 Antibodies—Full-length hRad21 cDNA was isolated from a HeLa cell cDNA library by reference to the published hRad21 cDNA sequence (6). The cloned cDNA was completely sequenced. The resulting sequence was identical to the published clone. The full-length hRad21 cDNA was then subcloned into pGEX5X-1 (Amersham Pharmacia Biotech), glutathione S-transferase-fused hRad21 was expressed in *Escherichia coli*. The recombinant protein contained in the inclusion body was denatured and purified using Prep Cell Model 491 (Bio-Rad). The purified protein was mixed with Freund’s complete adjuvant and injected into rabbits to obtain the anti-h-Rad21 antisera. To generate the anti-C-hRad21 antibodies, an oligopeptide possessing a C-terminal amino acid sequence of hRad21 (QQAILTQEEFP)YD, amino acids 606–619) was conjugated with keyhole limpet hemocyanine and was used to immunize the rabbits. The resulting antibodies were purified by affinity chromatography. Mouse anti-α-tubulin monoclonal antibody was purchased from Sigma. Mouse anti-PCNA, anti-β-tubulin, and anti-cyclin B1 monoclonal antibodies were purchased from Santa Cruz Biotechnology. Anti-CENP-B, anti-lamin B, and anti-cyclin B1 monoclonal antibodies were purchased from Santa Cruz Biotechnology. Anti-PCNA, anti-lamin B, and anti-cyclin B1 monoclonal antibodies were purchased from Santa Cruz Biotechnology.

Western Blotting—Western blotting was performed according to Ref. 13. Briefly, membranes were first pretreated for 1 h in Block-Ace solution (Dai Nippon Pharmaceuticals). Then all subsequent incubations and washes were carried out in 1× TNT buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20). Membranes were incubated for 1 h with primary antibodies at room temperature, followed by three washes. Then membranes were incubated for 30 min with horseradish peroxidase-conjugated anti-rabbit antibodies (Amersham Pharmacia Biotech), followed by three washes. Signals were detected using an ECL kit (Amersham Pharmacia Biotech).

**RESULTS**

**hRad21 Protein Does Not Change Its Abundance during Cell Cycle**—Budding yeast Scc1p/Mcd1p is a nuclear protein that associates with chromatin from late G1 through the metaphase-anaphase transition (2, 3). Scc1p/Mcd1p abundance is strictly regulated in a cell cycle-dependent manner. The protein is absent in early G1, accumulates in S, G2, and metaphase, and declines in anaphase (2, 3). In addition, SCC1/MCD1 mRNA is absent in early G1 and most abundant in late G2/S (2, 3). hRad21 mRNA is most abundant in late S through G2 (6). Therefore, we first investigated hRad21 protein levels throughout the cell cycle.

Full-length human Rad21 (hRad21) cDNA was isolated from a HeLa cell cDNA library. Nucleotide sequencing of the obtained cDNA yielded a sequence of complete identity to the published one (6), thus having an ORF that potentially encodes a protein of 631 amino acid residues with a calculated molecular mass of 72 kDa.

Two different rabbit antisera were raised, one (anti-hRad21 antibody) against the glutathione S-transferase-fused recombinant full-length hRad21 protein expressed in *E. coli* and the other (anti-C-hRad21 antibody) against a C-terminal synthetic oligopeptide (see “Experimental Procedures”). These antibodies were purified by affinity chromatography using the respective cognate antigens. Following hRad21 cDNA transfection both antibodies recognized the recombinant hRad21 protein that was expressed in the 293T cells (human kidney cells transfected with SV40 T antigen) (Fig. 1A). Both antibodies also recognized an endogenous protein in untransfected 293T cells that showed the same mobility in SDS-PAGE with that of the overexpressed hRad21 (Fig. 1B). These reactions were specific because preincubating the antibodies with the antigens prior to Western blotting prevented detection of hRad21 (Fig. 1A and B).

The apparent molecular mass of the hRad21 protein (about 120 kDa), as estimated using SDS-PAGE electrophoresis, was greater than that predicted from its supposed amino acid sequence. Similar observations were made with extracts of backgrounds yeast Scc1p (2), fission yeast rad21 protein (16), and *Xenopus* Rad21 homologue, XRAD21 (8). To confirm that the 120-kDa band indeed represents hRad21 protein, we transcribed and translated hRad21 cDNA in *vitro* using a rabbit reticulocyte lysate system. Recombinant proteins were synthesized in the presence of either [35S]methionine or cold methionine and then
analyzed by SDS-PAGE. Labeled proteins were detected by autoradiography, whereas unlabeled proteins were subjected to Western blotting with anti-hRad21 and anti-C-hRad21 antibodies. As shown in Fig. 1C, the [35S]methionine-labeled hRad21 protein synthesized in vitro was detected as a 120-kDa protein band after SDS-PAGE electrophoretic migration. Because this 120-kDa protein was specifically recognized by both anti-hRad21 and anti-C-hRad21 antibodies (Fig. 1C), we concluded that it is indeed hRad21 protein.

HeLa cells were synchronized using a thymidine and aphidicolin double-block protocol (Fig. 2). The cells were harvested at intervals after release from the block and first extracted by 1% Triton X (Fractions T), followed by 0.5 M NaCl (Fractions N). The insoluble pellet fractions (Fractions P) were also examined. These fractions were analyzed using Western blotting with (+) or without (−) preabsorption of the antibodies with the cognate antigen. A, antih-Rad21 and anti-C-hRad21 antibodies recognize the endogenous hRad21 protein derived from untransfected 293T cells. 293T whole cell extracts were analyzed by Western blotting with (+) or without (−) preabsorption of the antibodies with the cognate antigens. B, anti-hRad21 and anti-C-hRad21 antibodies specifically recognize the recombinant hRad21 protein synthesized in rabbit reticulocyte lysates. Protein synthesis in rabbit reticulocyte lysates was done in the absence of exogenous DNA (−) or in the presence of pcDNA3 (Mock) or the full-length hRad21 cDNA expression vector (hRad21). Proteins synthesized in the presence of cold methionine were analyzed by Western blotting with anti-hRad21 and anti-C-hRad21 antibodies (left and middle panels). Proteins synthesized in the presence of [35S]methionine were separated by SDS-PAGE and visualized by autoradiography (right panel).

We found that hRad21 was accurately extracted in 0.5 M NaCl but not in 1% Triton X-100 at all examined stages of the cell cycle (Fig. 2B). We also found that hRad21 was not present in Fractions P (data not shown). Furthermore, hRad21 was not extracted by DNase I (data not shown). These results indicate that hRad21 is not a soluble protein or a protein loosely binding to chromatin. hRad21 may be associated with nuclear structures at all cell cycle stages including M phase.

hRad21 Is Hyperphosphorylated in M Phase—It has been shown that fission yeast Rad21p and budding yeast Scclp are phosphorylated from S phase to anaphase (16). We next examined whether hRad21 is phosphorylated in a cell cycle-dependent manner. For this purpose, HeLa cells were synchronized following a thymidine and aphidicolin double-block protocol. The cells were harvested at intervals after being released from the block. Prior to each harvest, the cells were cultured for 1 h in the presence of [32P]orthophosphate. A parallel culture was used for determining the cell cycle by FACscan analysis. hRad21 was immunoprecipitated using anti-hRad21 antibodies from cell extracts containing approximately the same number of cells. The immunoprecipitates were separated in an SDS-PAGE gel, and the proteins were blotted onto a membrane. First, the membrane was subjected to autoradiography (Fig. 3B). Then the same membrane was analyzed for total hRad21 levels using a Western analysis with anti-C-hRad21 antibodies (Fig. 3C). From these experiments, it was found that hRad21 is phosphorylated most intensely at 10, 12, and 14 h after release from the block. Because the FACscan analysis revealed that these samples were derived mostly from M phase cells, these results imply that hRad21 becomes hyperphosphorylated in M phase.

To test the hypothesis that hRad21 is hyperphosphorylated in M phase more vigorously, we repeated the experiment in a quantitative manner. HeLa cells were arrested at G2/S following a thymidine and aphidicolin double-block protocol. A portion of the cells were released from the block and cultured for 8 h and then arrested at the following metaphase by incubating...
the cells with colcemid for 4 h. These G1/S-arrested and metaphase-arrested cells, along with the control exponentially growing cells, were labeled with [32P]orthophosphate for 2 h prior to each harvest. FACscan analysis of parallel cultures indicated that the two populations of cells were indeed arrested prior to each harvest. FACscan analysis of parallel cultures from 0,4,8,10, and 12 h after release from the block, A, Western blotting analyses for hRad21, cyclin B1, and α-tubulin. The cells harvested from the synchronized culture (indicated by the harvest time after block release), as well as an asynchronous exponentially growing culture (Expo), were first extracted with 1% Triton X-100 (T) and then with 0.5 M NaCl (N). The proteins were run in a SDS-PAGE gel and analyzed in Western blotting experiments using anti-C-hRad21, anti-cyclin B1, and anti-α tubulin antibodies. Cell cycle stages deduced from A and C, and the cyclin B1 abundance are shown above. C, the insoluble fractions (I) were analyzed for PCNA by Western blotting with anti-PCNA antibodies.

Nevertheless, when we calculated relative phosphorylation levels by dividing the autoradiography intensities by the Western blot intensities, we saw consistent cell cycle-specific changes in hRad21 phosphorylation levels both among the 3× samples and among the 9× samples (Fig. 4D). In both comparisons, the colcemid-arrested cells showed 3-fold increases in the specific phosphorylation levels of hRad21 compared with the G1/S-arrested cells. Therefore, we concluded that hRad21 is hyperphosphorylated in M phase.

In the autoradiographs of interphase immunoprecipitates, we observed three major and several minor 32P-labeled bands additional to hRad21 (Figs. 3B and 4B). The apparent molecular masses of the major bands as determined from SDS-PAGE were about 150, 140, and 80 kDa (Fig. 3B). Because these three bands were not reactive with anti-hRad21 antibodies in the Western blotting analysis, we concluded that these three bands are not hRad21. We interpret these as being hRad21-associated phosphorylated proteins and have designated them p150, p140, and p80. Interestingly, the phosphorylation levels of hRad21 and its putative associated proteins (p150, p140, and p80) in the metaphase-arrested cells were significantly higher than those found with the interphase cells (Figs. 3B and 4B). Furthermore, additional phosphorylated proteins, with apparent molecular masses of 180, 95, and 85 kDa, were specifically found in the hRad21 immunoprecipitate derived from the metaphase-arrested cells. These three proteins were interpreted as putative hRad21-associated proteins and designated p180, p95, and p85. Although we do not know the identity of these bands, the results suggest that cohesin components may be coordinately modified and/or regulated by phosphorylation during M phase.

hRad21 Is a Nuclear Protein Mostly Excluded from Chromosomes in Mitosis—We next examined the subcellular localization of hRad21 in the asynchronous HeLa cells by IF experiments using anti-hRad21 antibodies. HeLa cells were fixed with methanol, with or without being permeabilized in 0.1% Triton X-100. The samples were stained with TOTO3 to stain the DNA and examined by IF using anti-hRad21 and anti-α tubulin antibodies. hRad21 was detected in interphase nuclei and was not present in nucleoli (Fig. 5). When the cells were pretreated with Triton X-100 prior to fixation, most of the α-tubulin was extracted to disappearance, whereas hRad21 remained. These results confirm the biochemical results described above and indicate that hRad21 is a nuclear protein associated with nuclear structures.

If hRad21 is involved in sister chromatid cohesion, as has been proposed, one would expect the subcellular localization of hRad21 to change in M phase. However, it has been reported that most Xenopus Rad21 is dissociated from prophase-like chromosomes (8). We therefore were interested in the intracellular distribution of hRad21 in metaphase cells. Two fixation methods, one employing cold methanol and the other involving paraformaldehyde (see “Experimental Procedures”), were used. We found these two protocols essentially gave rise to the same results, and the results obtained from methanol-fixed cells are shown in Fig. 6 and described below. Asynchronous HeLa cells stained with DNA dye TOTO3 were examined by IF using anti-α tubulin and anti-hRad21 antibodies (Fig. 6). Although some hRad21 seemed to be associated with chromatin during early prophase when the two centrosomes are not yet separated, a significant amount was already dissociated from chromatin (Fig. 6, row a). hRad21 was quite heterogeneously distributed in late prophase cells, with some fraction of it still apparently bound to chromatin (Fig. 6, row b). However, in metaphase and anaphase, it seemed that most hRad21 had completely dissociated from the chromatin and was associated with the spindles (Fig. 6, rows c–e). The microtubule-hRad21

FIG. 2. hRad21 protein levels do not change during cell cycle. HeLa cells were synchronized at early S phase following a thymidine and aphidicolin double-block protocol. The cells were harvested at intervals after release from the block. A, FACscan analysis. The cells were stained with PI, and the DNA contents were analyzed by FACscan. B, Western blotting analyses for hRad21, cyclin B1, and α-tubulin. The cells harvested from the synchronized culture (indicated by the harvest time after block release), as well as an asynchronously growing culture (Expo), were first extracted with 1% Triton X-100 (T) and then with 0.5 M NaCl (N). The proteins were run in a SDS-PAGE gel and analyzed in Western blotting experiments using anti-C-hRad21, anti-cyclin B1, and anti-α tubulin antibodies. Cell cycle stages deduced from A and C, and the cyclin B1 abundance are shown above. C, the insoluble fractions (I) were analyzed for PCNA by Western blotting with anti-PCNA antibodies.

A 3-fold intensity difference relative to the 3× samples indicates that the intensities reliably reflect protein abundance in this range of protein amounts. In contrast, autoradiography intensities of the 9× samples displayed more than a 3-fold intensity difference relative to the 3× samples and therefore seemed to lack a linear dose-response relationship. Nevertheless, when we calculated relative phosphorylation levels by dividing the autoradiography intensities by the Western blot intensities, we saw consistent cell cycle-specific changes in hRad21 phosphorylation levels both among the 3× samples and among the 9× samples (Fig. 4D). In both comparisons, the colcemid-arrested cells showed 3-fold increases in the specific phosphorylation levels of hRad21 compared with the G1/S-arrested cells. Therefore, we concluded that hRad21 is hyperphosphorylated in M phase.

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association was particularly obvious in telophase and cytokinesis, because part of the hRad21 was colocalized at midbody. hRad21-chromatin association resumed during cytokinesis and completed in nascent two daughter cells.

To understand the relationship of the nuclear envelope breakdown and hRad21 dissociation from chromatin, we examined asynchronous cells by IF using anti-hRad21 and anti-lamin B antibodies (Fig. 7). At entry to mitosis, a major population of hRad21 was already excluded from chromatin of cells that had been already undergone nuclear envelope breakdown (Fig. 7, row a). It was not possible to determine whether this fraction of hRad21 had once associated with the chromatin and then dissociated from it in prophase or whether the fraction had never associated with the chromatin. However, some additional fraction of hRad21 apparently dissociated as the chromatin further condensed (Fig. 7, row b). At the exit of M phase, a fraction of hRad21 remained in the cytoplasm even when the nuclear envelope had apparently reformed (Fig. 7, row c). Therefore, it is suggested that hRad21 might be imported to nucleus actively. Eventually, nearly all hRad21 was localized inside the nuclei (Fig. 7, row d).

Significant Fractions of hRad21 Are Associated with Prometaphase Chromosomes and Metaphase Centromeres—Because of the background signals produced by the chromatin-unbound hRad21 in mitotic cells, it was difficult to determine whether hRad21 associates with condensed chromosomes in M phase or not. We therefore examined spread prometaphase-like and metaphase-like chromosomes to clarify whether hRad21 is present on condensed chromosomes. To this end, synchronized HeLa cells were treated with colcemid, and the spread chromosomes were analyzed by IF. We found that significant amounts of hRad21 associated along the axes of the less condensed prometaphase-like chromosomes (Fig. 8A, row a). These signals were not observed when the antibody was preabsorbed by the antigen and when chromosomes were stained by normal rabbit IgG, indicating that the signals were specific (data not shown). The hRad21 signals appeared heterogeneous along the length of chromosomes. To better understand the relative distribution of hRad21 on the chromosomes, we also localized centromeres using antibodies recognizing CENP-B, a protein that binds to centromeric alphoid DNAs. We found that hRad21 was localized at both the centromeres and the arm regions on prometaphase-like chromosomes (Fig. 8A, row a).

In contrast, when we analyzed further condensed metaphase-like chromosomes, we found that hRad21 became localized at centromeres (Fig. 8A, row b). Images under a high magnification revealed that hRad21 mostly disappeared from the arm regions and specifically localized at centromeres of metaphase-like chromosomes (Fig. 8B). Interestingly, when CENP-B signals were recognized as two spots, hRad21 was detected not only at CENP-B-positive regions but also at inner regions between the two sister CENP-B signals (Fig. 8B, rows a and c). Taken together, these observations strongly suggest that hRad21 in M phase is dissociated from chromatin in two steps. The arm region-associated hRad21 is dissociated during the prophase to metaphase transition, whereas the centromere-bound hRad21 remains associated with metaphase-like chromosomes.

**DISCUSSION**

It is estimated that about 70% of total Scc1p/Mcd1p is associated with chromatin in budding yeast during metaphase (18), whereas vertebrate cohesin molecules are mostly excluded from chromatin in prophase (8, 9). Therefore, it has not been determined whether cohesin plays a role in the mitotic chromatin cohesion in higher eukaryotes. In this study, we have found that a small but significant fraction of hRad21 is localized at centromeres of colcemid-induced metaphase-like chromosomes. Moreover, hRad21 and its potential associated proteins are hyperphosphorylated in M phase. These results highly suggest that hRad21 has a critical role in the chromatin cohesion and separation in M phase, as has been demonstrated in budding yeast.

In human and mouse cells, an electron microscopy study revealed that sister chromatid arm regions are morphologically distinguishable with each other prior to metaphase, whereas...
FIG. 4. hRad21 and its putative associated proteins are hyperphosphorylated in M phase. A, exponentially growing (Expo), G1/S-arrested (G1/S), and colcemid-arrested (Meta) HeLa cells were analyzed for their DNA contents by FACs. B, extracts containing the same 32P counts of cells were subjected to immunoprecipitation with either anti-hRad21 antibody (1×, 3×, and 9×), or with preimmune normal rabbit immunoglobulin (P). Three different amounts of protein (1×, 3×, and 9×) were analyzed for each type of immunoprecipitates. Immunoprecipitates were separated by SDS-PAGE and blotted onto a membrane. The membrane was then exposed to an x-ray film to visualize the labeled proteins. The position of hRad21, as identified by Western blotting in C, as well as the positions of six additional proteins (p180, p150, p140, p95, p85, and p80) are indicated. Relative signal intensities of labeled hRad21 are shown at the bottom of the figure. Values were normalized such that the value of the 3× exponentially growing cells equaled one unit. C, Western blotting of hRad21 with the same membrane that was analyzed in B. Relative signal intensities of the total hRad21 are shown at the bottom of the figure. Values were normalized to the 3× exponentially growing cells. The slight discrepancy in the positioning of hRad21 from left to right in the gel was caused by a horizontal difference in protein migration rates in the gel. D, specific labeling efficiencies of hRad21. Intensities of the labeled hRad21 protein observed in 3× and 9× samples of B were divided by the total hRad21 intensities observed in the 3× and 9× samples of C. Calculated values were normalized to the exponentially growing cells in the 9× sample. Expo, exponentially growing cells; G1/S, G1/S-arrested cells; M, metaphase-arrested cells.

the centromeric regions are connected until the onset of chromatid segregation in anaphase (19). This implies that chromosomes may be separated in a two-step process in mammalian cells. In the first step, the arm regions are disjoined during prophase to metaphase. At this stage, the remaining cohesion at centromeres serves as a hinge connecting the two sister chromosomes. In the second step that happens at the metaphase-anaphase transition, the centromere cohesion is dissolved, and the two liberated chromatids separate and move toward centrosomes by pulling forces exerted by spindles. Such a two-step cohesion-separation event has been suggested cytologically in a variety of animals such as Drosophila, chicken, muntjac, mouse, and human (reviewed in Ref. 20).

The changes of hRad21 distribution that we have found with mitotic chromosomes are remarkably well correlated with these cytological observations. We have found that hRad21 is present along the entire length of chromosomes during prophase to prometaphase. However, the arm-associated hRad21 disappeared and only the centromere-associated hRad21 remains on the colcemid-induced metaphase-like chromosomes. Therefore, the dynamics of hRad21 distribution on mitotic chromosomes perfectly corresponds to the morphological changes of cohesion between mitotic chromatids. These results suggest that cohesion containing hRad21 is responsible for the chromatid cohesion in M phase. After this paper was submitted for publication, it was reported that a small population of SA1 protein is associated with metaphase chromosomes both in Xenopus egg cell-free extracts and Chinese hamster ovary cells (21). Even more recently, mouse Scc1/Rad21 protein expressed ectopically in HeLa cells was shown to be specifically associated with centromeric regions in metaphase chromosomes (22). Our study indicates that the endogenous hRad21 protein behaves as the ectopically expressed protein and further substantiates the likelihood of a highly regulated distribution of cohesin complex on vertebrate mitotic chromosomes as revealed in the above-mentioned studies.

Scc1p/Mcd1p has been shown to associate with specific regions along the chromosome arms, especially at centromere-proximal regions (23–25). Especially, yeast cohesion becomes further preferentially localized at centromere-proximal regions on metaphase-like chromosomes induced by a microtubule inhibitor methyl 2-benzimidazolecarbamate (23). Therefore, in both yeast and humans, cohesin is enriched at centromere-proximal regions just prior to the onset of anaphase. It is tempting to speculate that these particular cohesin fractions associated with centromeres are the target of the APC (anaphase promoting complex)-dependent pathway that triggers anaphase. The recent study by Waizenegger et al. (22) supports this hypothesis by analyzing the biochemical and cytological behaviors of HeLa cells cohesin and mitotic processes in Xenopus cell-free extract.

Interestingly, we found that the centromere-associated hRad21 on the metaphase-like chromosomes appeared to be present between two sister CENP-B signals in some cases. Molecular cloning of the alphoid repeats on human chromosome 21 identified two different classes of alphoid repeats, α21-I and α21-II (26). α21-I contains the CENP-B box to which CENP-B binds, but α21-II does not. Correspondingly, it has
been reported that α21-I is precisely colocalized with CENP-B signals on spread metaphase-like chromosomes, but α21-II distributes over a broader area. Our observation that hRad21 distributed in broader regions that included the sister CENP-B signals and the inner paring domain located between the two CENP-B signals, suggests that a subset of alphoid DNAs similar to α21-II may be involved in sister chromatid cohesion at centromeres.

Several aspects of the behavior of hRad21 are different from that of yeast Scc1p/Mcd1p. We found that hRad21 levels do not change during the cell cycle. This finding is different from that of budding yeast, wherein Scc1p/Mcd1p levels have been found to fluctuate throughout the cell cycle. We found that only a small fraction of hRad21 associated with prophase chromatin. The remaining and major population of hRad21 did not locate to chromatin in the earliest prometaphase cells, wherein the nuclear envelope breakdown had already occurred. We could not determine whether the major fraction of hRad21 we observed had once bound with chromatin and dissociated in early prophase or whether it had been unbound to chromatin throughout the cell cycle. Nevertheless, we conclude that only a minor fraction of hRad21 contributes to chromatid cohesion in M phase, a situation different from that of yeast Scc1p/Mcd1p (18). This observation suggests that hRad21 may play a role additional to chromatid cohesion. In support of this notion, the mitotic hRad21 protein, the majority of which is dissociated from chromatin (Figs. 6 and 7), was resistant to detergent extraction (Fig. 2B). Therefore, it is suggested that this “free” form of hRad21 is associated with other nuclear structures, such as nuclear matrix and spindles. We noted that a fraction of hRad21 appeared to closely associate with spindle midzones and midbody in anaphase and telophase, respectively. This behavior is reminiscent of that of the chromosome passenger proteins INCENP (inner cen/tromere proteins) (27). It has been recently proposed that a primary function of INCENP involves chromosome segregation processes (28). It may be interesting to investigate the possible roles of hRad21 in later stages of mitosis.

It is not known how the dissociation of hRad21 from mitotic chromatin is regulated. In budding yeast, Sec1p/Mcd1p is cleaved proteolytically in a manner dependent on Esp1p at the onset of anaphase. Similarly, it has been reported very recently that a small fraction of mammalian Sec1/Rad21 is proteolytically cleaved at the metaphase-to-anaphase transition (22). In this study, we could not demonstrate a similar proteolysis that...
specifically happened to anaphase hRad21. However, we have found that hRad21 is phosphorylated in a cell cycle-dependent manner. Significantly, hRad21 and its potentially associated proteins, p180, p150, p140, p95, p85, and p80, were correspondingly phosphorylated most heavily in M phase. Recently, it has been reported that Xenopus Scc3 homologues (XSA1 and 2) are phosphorylated by Cdc2-cyclin B in vitro. Phosphorylated SA proteins show low affinities for chromatin, and thus it has been proposed that this protein modification might prevent unbound SA proteins in M phase from reassociating with chromatin (11). Interestingly, p140 and p150, which were coimmunoprecipitated with anti-hRad21 in this study, showed apparent molecular masses in SDS-PAGE similar to those of human SA proteins (140 kDa) (11), suggesting that p140 and p150 may be human SA proteins. Xenopus cohesin contains a fifth unidentified component having an apparent molecular mass of 95 kDa in SDS-PAGE (8). p95 that was also coimmunoprecipitated with anti-hRad21 in this study may be the human counterpart of this Xenopus cohesin component. Identities of the other phosphorylated proteins (p180, p85, and p80) revealed in this study are not known. p180, p95, and p85 are particularly interesting because these phosphorylated proteins were not detected in the interphase hRad21 immunoprecipitates (Fig. 4B). These proteins may be phosphorylated strictly in mitosis or associated with the cohesin complex specifically in mitosis. Taken together, these results suggest that numerous compo-
nents of human cohesin are coordinately modified by phosphorylation in M phase, which may regulate the function of cohesin. Future investigations of hRad21 should undoubtedly be aimed at understanding how arm-associated and centromere-associated fractions of hRad21 are differentially regulated.

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