Characterization of fission yeast cohesin: essential anaphase proteolysis of Rad21 phosphorylated in the S phase

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Cohesin complex acts in the formation and maintenance of sister chromatid cohesion during and after S phase. Budding yeast Scc1p/Mcd1p, an essential subunit, is cleaved and dissociates from chromosomes in anaphase, leading to sister chromatid separation. Most cohesin in higher eukaryotes, in contrast, is dissociated from chromosomes well before anaphase. The universal role of cohesin during anaphase thus remains to be determined. We report here initial characterization of four putative cohesin subunits, Psm1, Psm3, Rad21, and Psc3, in fission yeast. They are essential for sister chromatid cohesion. Immunoprecipitation demonstrates stable complex formation of Rad21 with Psm1 and Psm3 but not with Psc3. Chromatin immunoprecipitation shows that cohesin subunits are enriched in broad centromere regions and that the level of centromere-associated Rad21 did not change from metaphase to anaphase, very different from budding yeast. In contrast, Rad21 containing similar cleavage sites to those of Scc1p/Mcd1p is cleaved specifically in anaphase. This cleavage is essential, although the amount of cleaved product is very small (<5%). Mis4, another sister chromatid cohesion protein, plays an essential role for loading Rad21 on chromatin. A simple model is presented to explain the specific behavior of fission yeast cohesin and why only a tiny fraction of Rad21 is sufficient to be cleaved for normal anaphase.

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Sister chromatid cohesion is an important cellular process essential for accurate chromosome segregation [Mi

yazaki and Orr-Weaver 1994; Yanagida 1995; Nasmyth 1999]. If the sisters were not linked, they would diffuse apart, which makes it difficult for cells to handle chromosomes for accurate segregation. In fact, aberration in this linkage may lead to chromosomal instability and aneuploidy, which are seen in the majority of cancers [Lengauer et al. 1998]. This linkage between sister chromatids also allows them to align properly, which may help in repairing chromosomal DNA damage by homologous recombination.

Sister chromatid cohesion is established during DNA replication and is assumed to be maintained until anaphase, a mitotic stage when sister chromatid separation takes place. Recent studies have demonstrated the presence of a multiprotein complex required for sister chromatid cohesion, called cohesin [Hirano 1999; Nasmyth et al. 2000]. In Saccharomyces cerevisiae, the cohesin subunits are Smc1p, Smc3p, Scc1p/Mcd1p, and Scc3p, whose defects induce premature sister chromatid separation [Guacci et al. 1997; Michaelis et al. 1997; Toth et al. 1999]. In frog, homologs of Smc1p, Smc3p, Scc1p/ Mcd1p, and Scc3p form the complex with an additional subunit. Immunodepletion of the frog subunits causes defects in sister chromatid cohesion [Losada et al. 1998, 2000].

Two subunits of the cohesin complex belong to members of the structural maintenance of chromosomes (SMC) superfamily [e.g., Hirano 1999]. SMC has the globular domains at the N and C termini and a central hinge region consisting of long coiled coils. These globular domains interact with ATP and possibly also with DNA. The function of the hinge region is unknown. SMC proteins are also essential for mitotic chromosome condensation (Sutani and Yanagida 1997; Kimura et al. 1997; Sutani et al. 1999) and sex chromosome dosage compensation [Lieb et al. 1998], and DNA repair

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[Lehmann et al. 1995]. These proteins are mostly bound to other non-SMC subunits. The budding yeast cohesin contains two non-SMC subunits, Scc1p/Mcd1p and Scc3p. Scc1p/Mcd1p is similar to Schizosaccharomyces pombe Rad21, originally identified as a protein involved in DNA repair (Birkenbihl and Subramani 1992, 1995; Tatebayashi et al. 1998).

Besides the cohesin complex, several other proteins are known to be required for sister chromatid cohesion. S. pombe Mis4, homologous to Scc2p in S. cerevisiae and to Nipped-B in Drosophila (Rollins et al. 1999), collectively called adherin, is needed for cohesion and becomes essential during the S phase (Furuya et al. 1998). S. cerevisiae Eco1p/Ctf7p is necessary for the establishment of cohesion only in the S phase (Skibbens et al. 1999; Toth et al. 1999). A fission yeast homolog Esol is also needed for sister chromatid cohesion (Tanaka et al. 2000). Other proteins that may interact with cohesin subunits and play a possible role in cohesion are Trf4p in S. cerevisiae (Castano et al. 1996) and BimD in Aspergillus nidulans (Holt and May 1996). These sister chromatid cohesion proteins are implicated in the normal progression of the S phase.

Existence of sister chromatid cohesion proteins was first presumed by the observation that sister chromatid separation may depend on the destruction of proteins other than mitotic cyclins (Holloway et al. 1993; Surana et al. 1993; Imiger et al. 1995). Proteins such as a glue or cohesion protein were thus considered (Miyazaki and Orr-Weaver 1994). Very recent findings in budding yeast suggest that dissociation of the cohesin complex from the chromosomes requires the cleavage of Scc1p by a protease under the control of Esp1p (Ciosk et al. 1998; Uhlmann et al. 1999). Esp1p, which is similar to fission yeast Cut1, is bound to Pds1p, a presumed inhibitor of anaphase similar to fission yeast Cut2. On anaphase proteolysis of Pds1p, which is ubiquitinated by anaphase promoting complex [APC]/cyclosome, Esp1p is liberated to degrade Scc1p, thus resulting in sister chromatid separation (Uhlmann et al. 1999). We are interested in whether the same mechanisms can be applied to fission yeast, as the same set of separin/Cut1 and securin/Cut2 complexes exists (Funabiki et al. 1996; Kumada et al. 1998). On the contrary, most of the cohesin complex was found to be dissociated from chromosomes at prophase, well before anaphase, in Xenopus (Losada et al. 1998). Also, in human and mouse, most of the cohesin exists outside the chromosomes before metaphase (Schmiesing et al. 1998; Darwiche et al. 1999). In higher eukaryotes, the protein complex other than cohesion may act for sister chromatid cohesion until anaphase. Alternatively, a residual fraction of cohesin remaining on the chromosomes may be sufficient for the association of sister chromatids until anaphase.

We therefore addressed the question of whether fission yeast cohesin is dissociated in anaphase, as in budding yeast, or is largely dissociated from chromosomes before mitosis, as in vertebrate cells. To answer this, the cohesin complex had to be identified in fission yeast first. However, only Rad21, a homolog of Scc1p, had been previously investigated (Birkenbihl and Subramani 1995; Tatebayashi et al. 1998) when we began this investigation. By looking for homologs of Smc1p, Smc3p, and Scc3p, all of their counterparts in S. pombe designated Psm1, Psm3, and Psc3, respectively, were identified, and the presence of the cohesin complex was established by analysis of the immunoprecipitates. Chromatin immunoprecipitation [CHIP] and immunofluorescent and green fluorescent protein [GFP]-tagging microscopy showed that the S. pombe cohesin complex differed in one important aspect from vertebrates and budding yeast; namely, the majority of cohesin was located in the nucleus and seemed to be bound to chromatin throughout the cell cycle. To our surprise, the level of associated Rad21 detected by CHIP did not change during the highly synchronous progression from metaphase to anaphase. As in budding yeast, however, a small population of Rad21 was cleaved at the onset of anaphase, and this cleavage was essential for proper progression of anaphase. These results are interpreted in the Discussion section, in which we present a simple model to explain the specific behavior of S. pombe cohesin.

Results

Identification of fission yeast cohesin genes

to characterize the cohesin complex in S. pombe, three genes homologous to S. cerevisiae [SMC1, SMC3, and SCC3] were identified. Two homologous sequences were found in the database ([SPAC10F6.09C and SPAC17H9.20P]. The psm3 gene encodes a 1194-amino acid polypeptide [molecular mass = 137 kD] interrupted with one intron [indicated in Fig. 1A by the arrows]. The N-terminal, central, and C-terminal regions were 40.7%–62.8% identical to budding yeast Smc3p. The Psm3 sequence was more similar to human hSMC3 in the N and C termini than to S. cerevisiae Smc3p. The sequence in the database for psc3 similar to Scc3p was partial, so we obtained the whole genomic clone from a cosmid that contained the 11.5-kb genomic DNA fragment obtained was used as the probe to isolate a cosmId with a presumed inhibitor of anaphase. By looking for homologs of Smc1p, Smc3p, and Scc3p, all of their counterparts in S. pombe designated Psm1, Psm3, and Psc3, respectively, were identified, and the presence of the cohesin complex was established by analysis of the immunoprecipitates. Chromatin immunoprecipitation [CHIP] and immunofluorescent and green fluorescent protein [GFP]-tagging microscopy showed that the S. pombe cohesin complex differed in one important aspect from vertebrates and budding yeast; namely, the majority of cohesin was located in the nucleus and seemed to be bound to chromatin throughout the cell cycle. To our surprise, the level of associated Rad21 detected by CHIP did not change during the highly synchronous progression from metaphase to anaphase. As in budding yeast, however, a small population of Rad21 was cleaved at the onset of anaphase, and this cleavage was essential for proper progression of anaphase. These results are interpreted in the Discussion section, in which we present a simple model to explain the specific behavior of S. pombe cohesin.

Fission yeast cohesin subunits are essential for sister chromatid cohesion, and their defects lead to blocking mitosis

To examine defective phenotypes of the cohesin subunit genes, gene disruption was performed by one-step re-

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A

Smc1
Rad21/Scc1/Mcd1

\[\text{Δpsm1}\]

\[\text{Δpsm3}\]

\[\text{Δrad21}\]

\[\text{Δpsc3}\]

\[\text{rad21-K1}\]

\[\text{wt}\]

B

Figure 1. S. pombe cohesin genes and their disruption phenotypes. (A) Schematic representations of cohesin subunits. S. pombe subunits designated Psm1, Psm3, Rad21, and Psc3 are homologs of budding yeast Smc1p, Smc3p, Scc1p, and Scc3p, respectively. The conserved regions are hatched. The arrows indicate the positions of introns in the S. pombe genes. Except for Rad21, cohesin subunits were first identified in this study. (X) Xenopus; (h) human, (Dm) Drosophila; (Mm) mouse. (B) Phenotypes of gene-disrupted Δpsm1, Δpsm3, Δrad21, and Δpsc3. Gene-disrupted cells were observed after germination (33°C for 10 h) using DAPI for DNA and anti-tubulin (TUB) antibody. Aberrant segregation phenotypes of disrupted cells were very similar. Temperature-sensitive rad21-K1 (36°C for 4 h) and wild-type mitotic cells (26°C) are shown as control. The bar, 10 µm. (C) Localization of Mis12–GFP, a GFP-tagged kinetochore protein, in cohesin gene disruption mutant cells. Gene-disrupted cells (Δpsm1, Δrad21) expressing Mis12–GFP were observed after DAPI staining. The GFP dots represent kinetochores, the number of which exceeded more than three, indicating that sister kinetochores were prematurely separated. (D) The LacO repeat was integrated in rad21-K1 mutant about 30 kb apart from the inner centromere region of chromosome I and the repeat was visualized by the LacI–NLS protein tagged with GFP (Straight et al. 1996; Nabeshima et al. 1997). Cells grown at 26°C were shifted to 36°C for 4 h, and interphase cells were measured for the frequencies revealing two GFP signals (two examples shown in the left panel).

placement using the S. pombe ura4+ gene as the marker. Disruption of psm1+, psm3+, rad21+, and psc3+ was verified by genomic Southern hybridization of heterozygous diploid cells [data not shown]. Tetrad analysis of the sporulated diploids indicated that only two spores were viable, and both were Ura−. All these genes were, hence, essential for cell viability.

Gene-disrupted cells were germinated in the absence of uracil. They were observed by fluorescence microscopy using DAPI for DNA and antitubulin antibody for microtubules [Fig. 1B]. The wild-type cells are shown at the bottom. Interestingly, all disrupted strains produced phenotypes similar to those of temperatureresensitive (ts) rad21-K21 mutant at the restrictive temperature (36°C). The phenotypes were defective in mitosis, revealing the short spindle along which condensed chromosomes were scattered. Similar phenotypes were also found in adherin mutant mis4–242 cells [Furuya et al. 1998; Goshima et al. 1999]. These arrest phenotypes appeared to be caused by the Mad2-dependent spindle checkpoint, as the double mutant lacking mad2 exited mitosis without the delay [K. Furuya, Y. Toyoda, and M. Yanagida, in prep.].

To assess whether precocious separation took place, behavior of the kinetochores was examined in Δpsm1 and Δrad21 gene-disrupted cells. For this purpose, plasmid-carrying Mis12–GFP (an essential kinetochore protein tagged with GFP, Goshima et al. 1999) was introduced into the heterozygous diploids, and the GFP signals were observed in haploid segregants germinated in the absence of uracil [Fig. 1C]. Mis12–GFP signals were dispersed into five or six signals in Δrad21 and Δpsm1 haploid cells (10% frequency), whereas they were not observed in the wild-type control. Sister cohesion was thus at least partly impaired in the gene-disrupted cells.

The defect of the sister chromatid cohesion was also verified in ts rad21-K21 mutant cells (Tatebayashi et al. 1998) by examining the centromere DNA. For this, the Lac repressor tagged with GFP and nuclear localization signal [NLS] was expressed and associated with the LacO DNA repeats, which were integrated into the lys1 locus near the cen1 [Straight et al. 1996; Nabeshima et al. 1997]. Interphase rad21 mutant cells frequently revealed the two GFP signals, which presumably represented the separated sister pericentromere DNAs. The frequency of cells with the two GFP cen1 dots increased to 25% after 4 h at 36°C, higher than the wild-type control (Fig. 1D). These results showed that sister cohesion was impaired in ts-mutant as well as in gene-disrupted cells.

A stable complex formation by Psm1, Psm3, and Rad21 but not by Psc3

To examine whether Psm1, Psm3, Rad21, and Psc3 formed the complex in vivo, strains were constructed in which the genes for Rad21, Psc3, and Psm3 were tagged...
with 3xHA or 8xMyc, integrated into the chromosome under the native promoter. These tagged proteins were identified by immunoblotting (Fig. 2A). Polyclonal antibodies raised against the recombinant protein (aa 1–631) were used to detect Psm1. Rad21–3HA produced multiple phosphorylated bands over 100 kD (Birkenbihl and Subramani 1995), whereas Psc3–3HA, Psm1, and Psm3–8Myc showed the expected 116, 140, and 148 kD bands, respectively. No band was detected in the control wild-type cells [wt] when antibodies against HA or Myc were used. Upper bands of Rad21 were produced by phosphorylation because they were lost after phosphatase treatment [Fig. 2B], as reported previously (Birkenbihl and Subramani 1995).

Rad21 was immunoprecipitated by anti-HA antibody using extracts of the doubly integrated strain with Rad21–3HA and Psm3–8Myc under their native promoters. Immunoprecipitated proteins were silver stained (Fig. 2C). Two intense silver bands around 140 kD, which were not present in the wild-type extracts, were identified as Psm1 and Psm3–8Myc by immunoblotting (Fig. 2D). Although precipitated Rad21–3HA was clearly detected by immunoblotting, it was not seen as a single silver-stained band in immunoprecipitates, probably because it was multiply hyperphosphorylated (Fig. 2C). It was also highly susceptible to proteolysis during extract preparation.

No silver-stain band corresponding to Psc3 was detected in the immunoprecipitates. We examined by immunoprecipitation whether Psc3–3HA was bound to Rad21 and/or Psm1. Anti-HA antibody was used to immunoprecipitate Psc3–3HA produced by the integrated gene under its native promoter. Neither Psm1 nor Rad21 was coprecipitated with Psc3–3HA (Fig. 2E). This result was unexpected because Scc3p, the homolog in budding yeast, is a subunit of cohesin. In fission yeast, Psc3 might loosely bind to the cohesin complex. Another possibility is that only the insoluble chromatin-bound fraction of Psc3 would be able to form the complex with other subunits. Alternatively, Psc3 might interact with cohesin only during a particular cell cycle stage such as the S phase. Immunoprecipitation was done for cells arrested in the presence of hydroxyurea, but Psc3 was not coprecipitated with Rad21 or Psm1 (data not shown). Psc3, thus, might form the complex with an undetectable fraction of cohesin or not be a stable member of the cohesin subunits in fission yeast.

Viability loss of rad21-K1 and phosphorylation of Rad21 protein occurs during the S phase

To examine whether Rad21 becomes essential when cells traverse the G1/S phase, rad21-K1 mutant cells were first arrested in the G1 phase by nutrient starvation at 26°C and then released to the rich medium at the restrictive temperature (36°C). Under the culture conditions, the traverse of cells from the G1 to the M phase could be monitored. Viability of rad21-K1 decreased 2 h after the release, at the timing of the S phase (Fig. 3A), as verified by the FACS pattern. The amounts of cellular DNAs in wild-type and rad21-K1 cells shown at right indicated that replication occurred between 2 and 4 h in rad21-K1. The defective phenotype observed in the sub-

Figure 2. Stable complex formation of cohesin subunits. (A) Detection of Rad21, Psm1, Psm3, and Psc3 by immunoblot using anti-HA [12CA5], anti-Myc [9E10] monoclonal antibodies or polyclonal antibody against the recombinant Psm1 protein. Extracts of the strains chromosomally integrated with Rad21–3HA, Psc3–3HA, or Psm3–8Myc under the native promoter were used. Wild-type control produced no band by anti-HA or anti-Myc antibodies. (B) Extracts of cells expressing tagged Rad21–3HA were treated with (+) or without (−) a phosphatase and a phosphatase inhibitor (10 mM sodium orthovanadate) at 30°C for 30 min. Upper modified bands were diminished and the lowest bands were intensified after treatment. (C) To identify stable complex formation of cohesin subunits, a strain chromosomally integrated with Rad21–3HA and Psm3–8Myc under the native promoter was constructed and employed for immunoprecipitation using antibodies against HA. Silver staining of the precipitated proteins is shown. Proteins present in the precipitates from the integrant strain but not from the wild-type corresponding to Psm3–8Myc and Psm1. (D) Extracts of cells prepared in (C) were examined by immunoblot using antibodies against anti-HA, anti-Myc, and anti-Psm1 antibodies. Psm1 and Psm3–8Myc were coprecipitated with Rad21–3HA. (E) Extracts of cells expressing tagged Psc3–3HA were prepared, and immunoprecipitation was performed using anti-HA antibodies. Precipitates obtained were immunoblotted using anti-HA, anti-Rad21, and anti-Psm1 antibodies. Neither Psm1 nor Rad21 was coprecipitated with Psc3–3HA.
examined the degree of phosphorylation of Rad21 under phophorylated form of Rad21 is abundant during S/G2. We Subramani 1995), which indicates that the hyperphosphorylation bands of Rad21 increased during the S phase. Wild-type cells which occurred 2° replication was somewhat delayed in Judging from the FACS pattern, the completion of DNA suggested that Rad21 became essential during replication. sequent mitosis was similar to that found in gene-disrupted Δrad21 cells [data not shown]. These results suggested that Rad21 became essential during replication. Judging from the FACS pattern, the completion of DNA replication was somewhat delayed in rad21-K1.

Rad21 was phosphorylated, producing multiple bands, and this phosphorylation was proposed to be functionally relevant as the phosphorylated form was diminished in a radiosensitive mutant rad21-45 [Birkenbihl and Subramani 1995]. It was also shown that phosphorylation of Rad21 was extensive in the S/G2 phase [Birkenbihl and Subramani 1995], which indicates that the hyperphosphorylated form of Rad21 is abundant during S/G2. We examined the degree of phosphorylation of Rad21 under a culture condition of wild-type cells, first nitrogen starved in the G1 phase and then released to the rich medium. As seen in the immunoblot pattern (Fig. 3B), hyperphosphorylated forms of Rad21 were already abundant 2 h after the release, corresponding to the onset of the S phase, and reached the maximum after 4 h [the FACS analysis shown below indicated the S phase from 2–4 h]. A faint upper band seen in nitrogen-starved cells was due to an unknown cause and probably not caused by phosphorylation, as the same band was obtained after protein phosphatase treatment [data not shown]. Rad21 was reported to be hypophosphorylated in the G1 arrested cdc10-129 mutant [Birkenbihl and Subramani 1995] and we obtained the same result [data not shown]. It was then found that the level of hyperphosphorylated Rad21 decreased in rad21-K1 cultured at both 26° and at 36°C for 4 h [Fig. 3C]. The higher degree of Rad21 phosphorylation was, thus, in parallel with more established sister chromatid cohesion.

CHIP reveals abundance of cohesin subunits in the centromere regions

To examine whether the cohesin complex interacts with specific chromosome regions, CHIP was performed using various DNA probes. Rad21-3HA, Psm1–3HA, or Psc3–3HA integrated cells under their native promoters were grown exponentially and fixed with 1% formaldehyde and then extracts were immunoprecipitated with anti-HA antibody after genomic DNAs were sheared by sonication to an average length of 800 bp. DNAs coprecipitated with Rad21–3HA, Psm1–3HA, or Psc3–3HA were amplified by the PCR method using primers whose chromosomal locations are illustrated in Figure 4A. They abundantly reside either at the centromeric and pericentromeric regions in chromosome I, but the level was much less at the arm region near the cdc2° gene of chromosome II (Fig. 4B). The cnt1 and dg are the probes for the inner and outer centromere regions, respectively, whereas lys1 is in the pericentric arm probe of chromosome I [Takahashi et al. 1992]. Another 10 probes [a1–a10] were derived from a single cosmid c1750 [Machida et al. 2000].

The cohesin subunits were clearly distinguished from Mis6, an authentic centromere protein, which was restricted only in the inner centromere region essential for chromosome segregation [Saitoh et al. 1997; Takahashi et al. 2000]. In these experiments, the amount of template DNA was optimized to generate the PCR products within a linear range by serial dilutions of total or coprecipitated DNAs [Tanaka et al. 1999]. The amplified coprecipitated DNAs with Rad21–3HA, Psm1–3HA, or Psc3–3HA were then examined by calculating the percentage of coprecipitated DNA compared with total DNA [Fig. 4C]. The maximum distribution of the cohesin subunits was found in the repetitive outer centromere region, containing dg and dh repeats, which is not vital to the centromere function in chromosome segregation [Takahashi et al. 1992]. The low levels of coprecipitated DNA derived from the arm region were vari-

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**Figure 3.** Essential role of Rad21 and its phosphorylation during the S phase. (A) The nitrogen-starved culture of rad21-K1 mutant at 26°C was transferred to the complete medium at 36°C for 8 h. Cell viability and the DNA content were measured by plating and FACScan analysis, respectively. The wild-type control is also shown. Cell viability in the mutant culture initially arrested at the G1 phase decreased during the S phase, which occurred 2–4 h after the shift. (B) Upper phosphorylated bands of Rad21 increased during the S phase. Wild-type cells integrated with the Rad21–8Myc gene were nitrogen starved at 26°C and shifted to the complete medium at 36°C for 8 h as in (A). Cell extracts made were immunoblotted using anti-Myc antibody. Hyperphosphorylated forms of Rad21 were abundant in 2–4 h and subsequently hypophosphorylated form increased (upper panel). Note that the single upper band faintly seen in the arrested cells was not hyperphosphorylated (see text). (C) Upper phosphorylation bands of Rad21 were examined in rad21-K1 mutant. Wild-type cells (lane 1), rad21-K1 at 26°C (lane 2) and at 36°C for 4 h (lane 3). Upper phosphorylated forms were greatly diminished in rad21 mutant cells.
able depending on the primers used. More extensive analysis is needed to determine how these proteins are distributed along the entire arms.

**Intracellular location of cohesion proteins**

Intracellular location of Rad21 was examined in the wild-type strain integrated with Rad21–Myc expressed from the native promoter. Fixed cells were observed by immunofluorescence microscopy using anti-Myc and SPB antibodies (Fig. 5A). Rad21–Myc was located mainly in the nuclear chromatin region from interphase through mitosis. Centromere-specific staining was not observed. GFP-tagged Rad21 integrated on a chromosome with the native promoter also did not show specific centromere localization in living and fixed cells (see below). No significant change was recognized in cells at different cell cycle stages, consistent with the previous study (Birkenbihl and Subramani 1995). Basically identical nuclear localization was obtained for Psm1 and Psm3 (data not shown).

Immunoprecipitation experiments suggested that Psc3 might not be a subunit of the stable complex. We therefore determined whether Rad21 and Psc3 were distinctly distributed in the nucleus. Psc3–HA integrant cells were doubly stained as shown in Figure 5B. The individual and merged images taken in the same cells indicated that the location of Psc3–HA was very similar but did not appear to be identical to that of Rad21. Basically identical results were obtained under different staining conditions. Although Rad21 was found in the nucleus throughout the cell cycle, its interaction with chromatin might be altered during the cell cycle. The procedure developed for visualizing cell cycle-dependent location of MCM proteins in *S. pombe* (Kearsey et al. 2000) was applied to Rad21 tagged with GFP. Cdc21, an MCM protein essential for replication, remained specifically in the nucleus only during the G1/S phase when the detergent washing step was introduced (Fig. 5C, top). If the detergent step was not used, Cdc21 remained in the nucleus throughout the cell cycle (data not shown). After detergent washing, however, no cell cycle-dependent nuclear localization was observed for Rad21–GFP. Rad21–GFP was found in the nucleus throughout the cell cycle regardless of the absence or presence of the detergent washing procedures.

**Cleavage and phosphorylation change of Rad21 during metaphase–anaphase transition**

To examine whether the chromatin interaction of Rad21–HA altered during the progression from mitotic...
metaphase to anaphase, the CHIP experiment was performed using extracts of cold-sensitive β-tubulin mutant, *nda3-KM311* (Fig. 6A). Mutant cells were first arrested at mitotic prophase by culturing them at 20°C for 8 h and then released to the rapid progression into metaphase followed by anaphase using the temperature shift to 36°C, the permissive temperature. A highly synchronous cell cycle advance took place from the mitotic prophase to anaphase within 10 min (Hiraoka et al. 1984). The transition from metaphase to anaphase occurs very rapidly and highly synchronously at ∼6 min as seen by the destruction of mitotic cyclin Cdc13 (Fig. 6A, *H9251*-Cdc13) and the peak of cells showing a dividing nucleus in the early anaphase (filled circles in the bottom time course of Fig. 6A). Under these culture conditions, the levels of three probes, *cnt1*, *dg*, and *lys1*, coprecipitated with antibodies against Rad21–HA, did not significantly alter during the transition from mitotic prophase to anaphase (0–9 min). The results were strikingly different from the behavior of Scc1p in budding yeast (Tanaka et al. 1999), suggesting that the bulk of Rad21 bound to these probe DNAs did not dissociate from chromatin during the mitotic transition in *nda3-KM311* cells. Basically the same result was obtained for other synchronous cultures by the release of G2-arrested *cdc25* mutant cells [data not shown].

We then addressed the question of whether Rad21 was cleaved during the mitotic transition as found for budding yeast Scc1p. Rad21 contains two potential cleavage R residues at the 179 and 231 amino acid positions (Fig. 6B; Uhlmann et al. 1999). It was examined whether the cleavage could be detected in the synchronous culture of *nda3-KM311*, and the cleavage pattern would be altered if the noncleavable mutation of Rad21 was introduced. Wild-type and two-substitution Rad21 mutants (the change to E from R) were tagged with Myc (wt-Myc, *R179E-Myc*, *R231E-Myc*) integrated into a chromosome of the *nda3-KM311* strain with the native promoter. Immunoblot patterns of those integrated *nda3-KM311* cells during the synchronous mitotic progression are shown in Figure 6C. The cleaved product was seen only after rather prolonged exposure of the immunoblot patterns (indicated by the arrow in the middle panel). In the short exposure (top panel), however, only multiply phosphorylated Rad21 bands were seen: No cleaved band was detected in the molecular mass range between 20 and 110 kD [data not shown]. The amount was <5% of the total Rad21, judging from the exposure time required for revealing the cleaved band. The intensity of the hyperphosphorylated uppermost band decreased ∼6 min after the shift in *nda3-KM311* integrated with the wild-type Rad21 tagged with
Myc (wt-Myc), whereas it remained significantly high in nda3-KM311 integrated with mutant R179E-Myc or R231E-Myc. Note that the hypophosphorylated lowest band increased the intensity.

The cleaved product appeared only transiently in anaphase (6 min) in wt-Myc. It was ∼25 kD smaller than the full-length hypophosphorylated Rad21–Myc in either wt-Myc or R179E-Myc (the position indicated by the arrow) but 20 kD smaller in R231E-Myc (the position indicated by the asterisk), suggesting that Rad21–Myc was transiently cleaved during the metaphase–anaphase transition.
cleaved at the presumed sites. This was confirmed by our observation that the cleaved bands of R179E-Myc and R231E-Myc migrated at the same positions as the N-terminally truncated 231 and 179 aa fragments, respectively (data not shown). Taken together, uppermost hyperphosphorylated Rad21 appeared to be cleaved during the metaphase-anaphase progression at the presumed sites, because the decrease of the band intensities was retarded in the noncleavable mutants of Rad21. Dephosphorylation or very fast de novo synthesis of Rad21 occurred in anaphase. Note that single cleavage mutants grew normally at any temperature but introduction of the double-mutant gene with the native promoter has not been successful, probably because of the toxic effect of the product.

Requirement of the cleavage for normal progression of anaphase

To determine if the cleavage of Rad21 is essential for sister chromatid separation, the single-cleavage mutants [R179E and R231E] or the double mutant [R179E R231E] were moderately overexpressed under the REP41 promoter in the wild-type cells (Fig. 6D). Neither the wild-type protein nor the single mutant proteins [R179E, R231E] affected cell proliferation when Rad21 under the inducible promoter was synthesized by depleting thiamine from the media. In contrast, overexpression of the double mutant Rad21 [R179ER231E] using the same inducible promoter became lethal (Fig. 6D). To investigate its cellular phenotype, liquid cell cultures were made in the absence of thiamine. Sister chromatid separation was indeed blocked in cells expressing the double mutant Rad21, even though the degradation of Cut2/securin normally took place, which was the indication of cell cycle progression into anaphase (two lower cells in Fig. 6E). Thus, expression of the double mutant Rad21 was highly inhibitory against sister chromatid separation in anaphase but not against Cut2 destruction. These results indicate that the cleavage of Rad21 occurring in anaphase may be essential for proper anaphase.

Recruitment of Rad21 requires fully functional Mis4

To examine whether location of Rad21 protein might be altered in mis4-242 mutant, rad21+ gene was tagged with GFP at the C terminus and integrated into the chromosome under the native promoter, and its fluorescence was observed in living cells [Fig. 7A]. The GFP signal was in the nuclear chromatin regions with punctate spots in the wild-type cells. However, in mis4-242 mutant cells, the punctate signals were present but significantly reduced even at the permissive temperature and virtually disappeared after 4 h at 36°C.

The CHIP experiment was done to determine whether the DNA probes cnt1, dg, and lys1 were coprecipitated in the wild-type and mis4-242 mutant equally well with anti-HA antibodies against Rad21–3HA. Both wild-type and mis4 strains were integrated with Rad21–3HA with the native promoter into the chromosome. Neither the centromere DNA fragments, cnt1 and dg, nor the pericentric arm DNA fragment, lys1, were amplified at all in mis4-242 after 4 h at 36°C (Fig. 7B, lane 7). Surprisingly, the amplified DNA fragments at cnt1 and dg were significantly reduced and the lys1 DNA fragment was hardly amplified even at the permissive temperature (Fig. 7B, lane 6). These results suggest not only that fully functional Mis4 is required for normal loading of Rad21 onto chromatin but also that a relatively low level of Rad21 association with chromatin could be sufficient for cells to survive. It should be stressed that mis4-242 could grow nearly normally at 26°C, whereas cohesin was only weakly bound to the chromatin region. Finally, hyperphosphorylated Rad21 in mis4-242 was decreased at 26°C, the permissive temperature, and greatly dimin-
lished at 36°C, the restrictive temperature; this result is similar to that in the rad21-K1 mutant [Figs. 3C, 7C].

Discussion
This article reports that all four genes of fission yeast cohesin subunits, Rad21, Psm1, Psm3, and Psc3, are essential for cell viability. Their gene disruption phenotypes are highly similar, which is consistent with the notion that they form the same essential complex reported in other organisms [Michaelis et al. 1997; Losada et al. 1998; Toth et al. 1999]. We were able to demonstrate that Rad21, Psm1, and Psm3 formed the complex. Precocious sister chromatid separation was observed in cohesin subunit mutants, strongly suggesting that fission yeast cohesin was also required to establish sister chromatid cohesion. As ts rad21-K1 mutant cells lost viability during the S phase, sister cohesion was likely to be established by the complex during the S phase. However, the cleavage of Rad21, although its level is rather fractional, must take place in anaphase, and overproduction of the noncleavable double Rad21 mutant protein blocks sister chromatid separation, strongly suggesting that Rad21 has a role in the anaphase as well as in the S phase.

Immunoprecipitation failed to show that Psc3, a homolog of Scc3p, was a part of the complex. Immunolocalization and CHIP showed that Psc3 behaved similarly [but not identically] to other cohesin subunits. Certain diversifications may thus exist in the subunit compositions of cohesin. In frog, a total of five cohesin subunits were reported [Losada et al. 1998, 2000]. Gene disruption results presented in this paper showed that the loss of any four subunits led to the same phenotypes, suggesting that the complex acts as a functional entity and that each of the subunits is essential for its function. Psc3 is probably functionally related to cohesin, and it might perform its essential function without complex formation. Alternatively, a small population of cohesin that carries essential function may consist of the four subunits. These are equally possible scenarios.

Rad21 was characterized by its hyperphosphorylation: Multiple bands produced became a single band after protein phosphatase treatment. Human and frog Scc1p/Rad21 homologs, in contrast, were detected as a single band by polyclonal antibodies [Losada et al. 1998; T. Matsusaka and M. Yanagida, unpubl.]. Whether vertebrate Rad21/Scc1p homologs are actually phosphorylated remains to be determined. Hyperphosphorylation of fission yeast Rad21 was a cell cycle–dependent phenomenon. It appeared to occur at the onset of S phase when the nitrogen-starved G1 cells were released in the rich medium.

Is this phosphorylation of Rad21 functionally relevant? It appears to be the target of proteolysis, but we have no definitive evidence that phosphorylation is functionally essential. The previous [Birkenbihl and Subramani 1995] and the present studies showed that hyperphosphorylated Rad21 was diminished both in radiosensitive rad21-45 and ts rad21-K1. In addition, phosphorylation was greatly reduced in mis4 mutant, in which recruitment of Rad21 to punctate nuclear signals and chromatin precipitates were greatly diminished. This hyperphosphorylation might be directly related to the activation of Rad21 for cohesion. Alternatively, phosphorylated Rad21 was possibly recruited to proper chromatin sites. Later in anaphase, phosphorylated Rad21 might be degraded. The previous work indicated that the phosphorylated region resided within the N-terminal 215 amino acids [Birkenbihl and Subramani 1995]; this region contains many S and T residues and has one Cdc2 site. A protein kinase (or kinases) responsible for hyperphosphorylation of Rad21 is likely to be active at the onset of the S phase. Its identification is of considerable interest.

CHIP experiment showed that Rad21, Psm1, and Psm3 were bound to centromere and pericentromere DNA probes much more abundantly than the probes present in the arm. It is very unlikely, however, that the cohesin complex functions as an essential kinetochore component, as do Mis6, Mis12, or Cnp1 in fission yeast [Saitoh et al. 1997; Goshima et al. 1999; Takahashi et al. 2000]. These kinetochore chromatin proteins are present exclusively in the central innermost centromere regions essential for maintaining chromosomes, whereas the cohesin proteins exist in a much broader region including the outer centromeric repeats dg and pericentric arm probes such as lys1 not required for correct chromosomal segregation. Indeed, centromere-specific chromatin [Takahashi et al. 2000] was maintained in rad21-K1 mutant cells [K. Furuya, K. Takahashi, and M. Yanagida, in prep.].

Important behavior of Rad21 was revealed during the metaphase–anaphase transition in nda3-KM311 mutant. First, chromatin-bound Rad21 did not seem to alter abruptly from metaphase [3 min] to anaphase [6 min] within the resolution of detection. Second, the same nuclear localization of Rad21 was observed during the cell cycle by regular and detergent-treated fluorescence microscopy that was successful in demonstrating the S phase–specific nuclear localization of MCM proteins in S. pombe. In contrast to the above two unchanged properties, the state of hyperphosphorylated Rad21 was grossly altered. Its level was dramatically decreased from metaphase to anaphase in nda3-KM311 mutant. This rapid decline was shown to be partly caused by proteolysis and also probably by protein dephosphorylation, as the intensity of the hypophosphorylated form increased from metaphase to anaphase. Among many phosphatases studied, only Dis2 PP1 phosphatase has been known to be activated from metaphase to anaphase because of the down-regulation of Cdc2 kinase [Ishii et al. 1996].

It is unknown whether only single protease is responsible for the decrease of Rad21. In budding yeast, Esp1 seems to be the sole protease for anaphase destruction of Scc1p [Uhlmann et al. 1999]. Because the cleavage sites of Rad21 were similar to those of Scc1p, the protease responsible for this cleavage is probably Cut1, a homolog to Esp1p. We, however, have not been able to examine whether proteolysis of Rad21 was delayed in cut1 mu-
tant cells, as inactivation of mutant Cut1 protein in the temperature-shift experiment requires extensively long time, whereas the cleavage took place nearly immediately after the temperature shift.

Is the cleavage of Rad21 in anaphase functionally essential? As only a very small fraction was cleaved, such cleavage might not affect overall function of Rad21. This cleavage, however, was most likely essential for a proper anaphase, as in budding yeast, because overexpression of noncleavable double mutant Rad21 protein (R179ER231E) in wild-type cells was highly toxic and prevented sister chromatid separation. In budding yeast, the cleavage of Scc1p appeared to be sufficient not only for sister chromatid separation but also for triggering the metaphase-to-anaphase transition (F. Uhlmann and K. Nasmyth, unpubl.). The cleavage of Rad21/Scc1p might be a general phenomenon because a small amount of human SCC1 is cleaved at the onset of anaphase (J.M. Peters, pers. comm.).

We propose a simple model to explain the specific behavior of Rad21 in fission yeast. First, Rad21 may be highly abundant in this organism, and the presence of its tiny fraction becomes functionally essential in anaphase. It is unknown whether this essential Rad21 fraction is entirely destroyed during the metaphase-anaphase progression. Second, the bulk of chromatin-associated Rad21 remains along chromosomes in anaphase without causing any harm to cells. It might be necessary to remain bound to chromatin for the establishment of cohesion at the next S phase because the duration of G1/S is very short in fission yeast. Third, the difference between the bulk and the fractional Rad21 that undergoes proteolysis may be caused by localization or protein modification or both. These hypotheses can explain many of the results that we obtained. The same localization and CHIP results during the mitotic transition are likely to represent the behavior of the bulk fractions of Rad21. Behavior of the functionally essential Rad21 may be obscured by its paucity. Note that the C-terminal fragment can associate with the other two cohesin subunits [A. Murakami and M. Yanagida, unpubl.], so that Rad21 could remain bound to chromatin even after it is cleaved on anaphase.

Another attractive hypothesis is that the essential population of Rad21 is located in restricted chromosome regions such as the centromeres. This would explain the paucity of functionally essential Rad21. Indeed, in human cells, a small amount of SCC1 remains bound to centromeric regions until the onset of anaphase (J.M. Peters, pers. comm.). Likewise, in fission yeast, cohesin subunits are abundantly present in the centromere regions, as they are in budding yeast. Furthermore, Rad21 hardly binds to the pericentric arm region, but little remains associated with centromere in mis4 mutant cells at the permissive temperature. Therefore, it may be possible that proteolysis of a very small fraction of Rad21 located at the centromere is essential for sister chromatid separation. No further results, however, support this hypothesis because no variation in the centromere regions was yet obtained from the CHIP experiments of the synchronous nda3-KM311 culture and localization of GFP-tagged Rad21.

There has been no definitive experimental evidence reported for the direct structural role of the cohesin complex for bridging the sister chromatids (Nasmyth 1999; Yanagida 2000). If cohesin is a catalyst rather than a structural link or glue between sister chromatids, the form free from chromatin may still be able to execute its function. In fact, factors interacting with SMC proteins have been reported to show various enzymatic activities. Bovine recombination complex-1 [RC-1] contains DNA polymerase ε and ligase III as well as SMC1 and SMC3 (Jesserberger et al. 1996). SbcC, an SMC family protein in Escherichia coli, interacts with SbcD, a nuclease [Connelly et al. 1998]. In addition, Trf4p in budding yeast, which is required for sister chromatid cohesion, was recently found as DNA polymerase κ (Wang et al. 2000). The presumed synthetic lethality of the rad21-K1 mutation with mis5-268 or cdc17-K42 [data not shown], which are defective, respectively, in an MCM protein [Takahashi et al. 1994] having DNA helicase [Ishimi 1997] and ligase activity, indicates the S phase role of Rad21 and also its implication in DNA metabolism.

Material and methods

Strains and media

The S. pombe haploid strains used were wild-type 972h− and its derivative strains: nda3-KM311 (Hiraoka et al. 1984), mis4-242 (Furuya et al. 1998), and rad21-K1 [Tatebayashi et al. 1998]. For gene disruption, a diploid strain was made by crossing haploid ST485 (h′ leu1 ura4 ade6-210) and ST486 (h− leu1 ura4 ade6-216). The culture media used were complete YPD [1% yeast extract, 2% Bactopeptone, and 2% glucose] and the minimal EMM2.

Epitope tagging, plasmid, and construction of Rad21 mutant strains

NotI restriction sites were introduced at the C termini of the coding regions of psm1+ , psm3+, rad21+, psc3+, and cut2+ genes by PCR. The NotI restriction sites were ligated with the 3xHA or 8xMyc or GFP tag sequence, and resulting genes were cloned into an integration vector, pYC6 carrying the S. pombe ura4− gene or pYC11 carrying the S. cerevisiae LEU2 gene. Resulting plasmids were introduced to either h− ura4 or h− leu strain, respectively. The arginine at aa 179 and 231 of Rad21 were replaced to glutamic acid by site-directed mutagenesis. The resulting mutated rad21 genes were cloned into plasmids pREP41 or pREP81 carrying the nmt1 promoter to induce overexpression of the mutant Rad21. The mutated rad21 genes were also introduced together with 654-nt upstream promoter, the S. pombe leu1− gene and 8xMyc tag into the leu1-32 locus of heterozygous rad21+ gene-disrupted diploid cells. Correct integration was confirmed by Southern blotting.

Antibodies

To obtain anti-Psm1 antibody, the N-terminal 1894-bp sequence of psml− gene was inserted in frame into pGEX-6p (Pharmacia). Glutathione S-transferase [GST]-Psm1 fusion protein was produced in E. coli by inducing expression with 1 mM...
of isopropyl-β-D-thiogalactoside [IPTG] for 4 h at 36°C. The fusion protein recovered as insoluble inclusion bodies was sonicated, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroeluted. The purified protein was used for immunization, and polyclonal antisera was obtained. Anti-Rad21 antibody was a gift of Dr. S. Subramani [Birkenbihl and Subramani 1995].

Isolation of psm1+, psm3+, and psc3+

To isolate the psm1+ gene, mixed oligonucleotides based on the conserved C-terminal amino acid sequences of S. cerevisiae, Xenopus, and human SMC1 homologs were made. They were 5'-GA(AG)AA(AG)ACIGTIGCIGCI(CT)TIGC-3' (encoding EKTVAAAL) and 5'-TTIA(GA)ICAGTGA[TG(TA)TGA]ATIAC(TG ATATGAANAC(TG(TG-3' (encoding QFIVISL on the complementary strand), and used for degenerated PCR. The resulting 176-bp PCR fragment, which coded for the amino acid sequence 78% identical to the C terminus of S. cerevisiae Smc1p, was employed as the hybridization probe to screen an S. pombe cosmId library, and cosmid 196 located on the long arm of chromosome II was obtained. An 11.5-kb Xhol–SacI fragment of cosmId 196 contained the full-length coding sequence of the psm1+ gene, and its nucleotide sequence was determined. A partial sequence of the psm3+ gene was determined in the database of the Sanger Center as an S. pombe hypothetical ORF and SPAC10F6.09C as a homolog of S. cerevisiae Smc3p. The partial sequence of the psc3+ gene was located on a cosmId SPAC17H9.20P. Using the N terminus 521-bp fragment in the cosmId as a probe, the rest of the genomic coding region of psc3+ was obtained from cosmId 456 located on the long arm of chromosome I and, its nucleotide sequence was determined.

Gene disruption

One-step gene replacement (Rothstein 1983) was employed. The 1.8-kb S. pombe wad2+ gene was replaced with the following coding fragments of the cohesin subunit genes: 0.75 kb EcoRV of psm1+, 2.25kb HpaI–BglII of psm3+, 0.7 kb KpnI–HindII of rad21, and 1.5 kb NcoI–EcoT22I of psc3+. The resulting disrupted genes were integrated into the chromosomes of diploid cells by homologous recombination. Gene disruptions were verified by Southern hybridization. Gene-disrupted cells were sporulated and dissected by tetrad analysis. The phenotypes of gene-disrupted cells were examined after germination. Heterozygous diploid cells were sporulated at 26°C, and Ura− spores were germinated in EMM2 liquid medium lacking uracil at 33°C. Germinated cells were fixed and stained with DAPI and anti-tubulin TAT1 antibody.

Immunoprecipitation

Exponentially growing cells [3 × 10^6] of wild-type, Rad21–3HA/ Psm3–3MYC, or Psc3–3HA integrated strains were disrupted with glass beads in modified TEG buffer (40 mM Tris-HCl at pH 7.5, 1 mM EDTA, 10% Glycerol ±0.1% NP-40, 150 mM NaCl, and 1 mM PMSF) containing protease inhibitors (1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin A). Extracts were then centrifuged [17,000g, 20 min] and treated with protein A-sepharose beads (50 μL) for 1 h, then incubated for 3 h with anti-HA monoclonal antibody 12CA5 [Boehringer] cross-linked to the protein A-sepharose beads (10 μL). Beads were washed five times with the modified TEG buffer. Proteins precipitated with the beads were separated by SDS-PAGE and detected by silver staining or immunoblotting.

Microscopy

The centromere DNA of chromosome I [cen1] in a living cell was visualized as described previously [Straight et al. 1996; Nabeshima et al. 1997]. Immunofluorescence microscopy was done using anti-Sad1, anti-tubulin TAT1, anti-HA 12CA5 (BAbCO), anti-Myc 9E10 (Calbiochem), and anti-Rad21 antibodies [Hagan and Hyams 1988; Birkenbihl and Subramani 1995; Hagan and Yanagida 1995]. Strains expressing GFP-fused proteins were observed either in living or fixed cells with methanol as described previously by Nabeshima et al. [1997]. In situ chromatin binding assay was performed as described [Kearsey et al. 2000].

CHIP

CHIP was performed as described previously [Saitoh et al. 1997]. The primers used are available upon request.

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