Cohesin Defects Lead to Premature Sister Chromatid Separation, Kinetochore Dysfunction, and Spindle-assembly Checkpoint Activation*

Received for publication, July 9, 2002, and in revised form, August 21, 2002
Published, JBC Papers in Press, August 27, 2002, DOI 10.1074/jbc.M206836200

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Scc1/Med1 is a component of the cohesin complex that plays an essential role in sister chromatid cohesion in eukaryote cells. Knockout experiments of this gene have been described in budding yeast, fission yeast, and chicken cells, but no study has been reported on human Scc1 thus far. In this study, we found that an N-terminally truncated human Scc1 shows a dominant-negative effect, and we examined the phenotypes of human cells defective in Scc1 function. Scc1 defects led to failure of sister chromatid cohesion in both interphase and mitotic cells. Interestingly, four chromatids derived from two homologues occupied four distinct territories in the nucleus in chromosome painting experiments. In mitotic Scc1-defective cells, chromatids were disjoined with normal condensation, and the spindle-assembly checkpoint was activated. We also found that, although the disjoined kinetochore (half-kinetochore) in Scc1-defective cells contains CENP-A, -B, -C, and -E normally, it apparently does not establish the kinetochore-microtubule association. These results indicate that Scc1 is essential for the association of kinetochores with microtubules.

In eukaryotic cells, the replicated DNA (sister chromatid) remains connected to each other from the end of the S phase until the onset of anaphase. This sister chromatid cohesion ensures that the two daughter cells inherit an identical set of genetic information. It is well established that the sister chromatid cohesion is accomplished by a phylogenetically conserved protein complex called cohesin (reviewed in Ref. 1). Cohesin was identified genetically for the first time in budding yeast, C. elegans, and chicken, and human (5–8). However, the exact behaviors of cohesin differ among species. Specifically, Scc1 dissociates from chromosomes in a single step of the metaphase-to-anaphase transition in budding yeast, mediated by its proteolysis by a protease called Esp1 or separase (9). In contrast, the dissociation occurs in two steps in vertebrates, from the arm regions in prophase and from the centromeric region in the metaphase-to-anaphase transition (5, 10, 11). The bulk Scc1 dissociation in prophase occurs independently of separase, and is regulated by Polo-like kinase in Xenopus (12), whereas the Scc1 dissociation from kinetochores at the metaphase-to-anaphase transition in human cells is achieved by the proteolysis of Scc1 by Esp1 (13).

Scc1 also plays an important role in kinetochore function. In budding yeast, it was reported that kinetochore-microtubule association can be established, but sister kinetochores frequently associate with monopoles (14). It was therefore concluded that Scc1 is important for the bipolar attachment of the sister kinetochores. A similar observation was reported in Scc1-depleted chicken DT40 cells, albeit the number of sister chromatids showing monopolar attachment was small (8). Because the centromeres in higher eukaryotes have a much larger size and are much more complex than those in budding yeast, it is possible that Scc1 plays different roles in kinetochore function in higher eukaryotes. In this study, we found that the N-terminally truncated hScc1 (human Scc1) shows a dominant-negative effect in human cells. By exploiting this allele, we examined the phenotypes of Scc1-defective human cells.

MATERIALS AND METHODS

Expression Vectors—The full-length hScc1 cDNA and the deletion mutants, hScc1ΔN (287–631 aa) and hScc1ΔC (1–415 aa), were subcloned into pEGFP expression vectors (Clontech) and pMX-puro retroviral expression vector (15). The retrovirus vectors were transfected into dN4 amphotropic packaging cells (a gift from Dr. G. P. Nolan), and allowed to produce infectious retroviruses.

Induction of hScc1ΔN Polypeptide in HT1080 Cells—HT1080 Tet-On cells, which constitutively express the tet-responsive trans-activator, were originally established and kindly provided by Dr. Y. Ishizaka (International Medical Center, Tokyo, Japan) (16). An effector plasmid, pTO/hScc1ΔN, was constructed by inserting hScc1ΔN (287–631 aa) to pTO that possesses the tet-responsive DNA element and the hygromycin selection marker gene (16). HT1080 Tet-On cells were transfected with pTO/hScc1ΔN and pTO-mock. Cells were cultured in the presence of G418 (400 µg/ml) and hygromycin (50 µg/ml). Twenty clones showing resistance to both drugs were selected and analyzed for inducible expression of hScc1ΔN after incubation with 1 µg/ml doxycycline for 48 h. One clone that showed the lowest background expression without induction and the highest expression with induction was used in this study.

Western Blotting—Western blotting was performed according to Ref. 11.

Indirect Immunofluorescence (IF) Experiments—In all IF analyses, cells were grown in chamber slide glasses (Falcon). Cells were fixed with 100% methanol for 20 min at –20 °C and permeabilized with 0.1% Triton X-100 for 10 min. Fixed cells were pre-incubated in blocking solution (0.1% bovine serum albumin and 0.1% skim milk in PBS).
followed by incubation with primary antibodies for 1 h at 37 °C in the blocking solution. Cells were then washed three times with shaking in PBS, and incubated with secondary antibodies for 1 h at 37 °C. The cells that were washed with PBS three times were mounted in the mounting solution containing 0.25 μg/ml propidium iodide (PI) or TOTO3, and examined by laser confocal microscopy (Zeiss). IF analysis for 3F322 antigen was performed according to Ref. 17.

DNA Probes and Fluorescence in Situ Hybridization (FISH)—Two cosmid clones, c112–156 and pBR12, specific for chromosome 12 short arm telomere and the chromosome 12 centromere, respectively, were kindly provided by Dr. K. Okumura (Mie University, Mie, Japan) (18). FISH experiments were performed using samples fixed with methanol:acetic acid essentially as described in Ref. 19 with slight modifications. Slides were taken from −20 °C storage and dried at room temperature for 30 min. Nuclei were treated with 1 mg/ml pepsin (Sigma) in 10 mM HCl solution. Cells were washed with PBS and dehydrated gradually by rinsing them in succession with 70, 85, and 100% ethanol. Cellular DNA was denatured at 80 °C in 50% formamide in 2× SSC solution and dehydrated in succession with 70, 85, and 100% ice-cold ethanol, c112–156 and pBR12 probes were labeled with biotin-16-dUTP by the nick-translation method according to the supplier’s protocol (BMY). Biotin-labeled probes were precipitated in presence of carrier salmon sperm DNA (Sigma) and competitor human Cot-1 DNA (BMY). Probes were then dissolved in hybridization solution (50% formamide (v/v), 10% dextran sulfate in 2× SSC), and denatured at 75 °C for 10 min. Denatured probes were pre-annealed at 37 °C for 30 min. Then, they were added to denatured nuclei and hybridized at 37 °C for 16 h in a humidified chamber. After hybridization, the slides were washed with 50% formamide in 2× SSC solution at 42 °C (three times, 5 min each) and with 2× SSC solution at 62 °C (three times, 5 min each). The biotinylated probe was detected using fluorescein-labeled avidin Fab fragment (Vector Laboratories), and finally nuclei were washed three times with 0.1% Tween 20 in 4× SSC solution at 42 °C. The nuclei were counterstained with PI and maintained in anti-fade medium (Vector Laboratories). The images were examined by laser confocal microscopy (Zeiss).

Chromosome Painting—Cells were fixed with methanol:acetic acid and denatured in 50% deionized formamide in 2× SSC solution for 2 min. The slides were transferred quickly to a coplin jar containing 70% ice-cold ethanol and incubated for 5 min. Then, the slides were incubated in succession with 85 and 100% ice-cold ethanol, each for 5 min, and finally air-dried. An aliquot of the digoxigenin-labeled whole chromosome 12 painting probe (BMY) was denatured for 10 min at 75 °C and pre-annealed at 37 °C for 30 min. The probe was added to the denatured nuclei, covered with a cover glass, and sealed with rubber cement. The slides were then transferred to a humidified chamber and incubated therein at 37 °C for 16 h. Then, the slides were washed twice with 50% formamide in 2× SSC solution for 15 min in a shaking water bath at 42 °C, followed by washing two times with 0.1% Tween 20 in 2× SSC solution at room temperature. Anti-digoxigenin-rhodamine Fab fragment (BMY) was diluted with PBS/bovine serum albumin solution and incubated with nuclei for 60 min in a humidified chamber at 37 °C. Slides were washed twice with 0.1% Tween 20 in 2× SSC solution, and DNA was counterstained with 0.1 μg/ml PI in PBS solution. The images were examined by laser confocal microscopy (Zeiss).

RESULTS

N-terminally Truncated Form of hScc1 Shows a Dominant-negative Effect—Because Scc1 functions by forming a complex with Scc3, Smc1, and Smc3, it was expected that some deletion mutants of Scc1 might show a dominant-negative effect. Accordingly, we transfected the hScc1 cDNA encoding full-length, N-terminally or C-terminally truncated Scc1 (deleted for aa 1–286 or 416–631, respectively; Fig. 1A) fused with the N-terminal GFP into 293T cells (EGFP-hScc1, EGFP-hScc1ΔN, or EGFP-hScc1ΔC, respectively). Scc1 contains a proline-rich region and a glutamate-rich region at the C-terminal half, which are absent in hScc1ΔC and retained in hScc1ΔN. The two truncated Scc1 proteins contain the putative nuclear localization sequence at their centers. Consistently, all EGFP-fused hScc1 proteins localized in the nuclei, where endogenous Scc1 is present (11), whereas EGFP-mock protein found in the cytoplasm (Fig. 1B). We next analyzed the cell cycle profiles of these transfectedants. Cells at 48 h after the transfection were fixed, stained with PI, and analyzed by FACScan. To examine specifically the cells expressing the recombiant proteins, we first sorted the EGFP-positive cells and then analyzed the cell cycle profile of this population. Although overexpressing EGFP-hScc1 and EGFP-hScc1ΔC as well as EGFP-mock did not change the cell cycle profile, a significant decrease of the G1 fraction and an increase of the G2+M fraction were observed in EGFP-hScc1ΔN-overexpressing 293T cells (Fig. 1C). The fraction of G2+M cells in EGFP-hScc1ΔN-overexpressing 293T cells was ~40%, which is more than twice of those observed in the other transfectants. These results suggest that overexpressing hScc1ΔN interferes with the normal cell cycle progression, leading to cell cycle arrest at G2/M.

hScc1ΔN Leads to Premature Sister Chromatid Separation in Normal Human Fibroblasts—The above results suggested that hScc1ΔN has a dominant effect on endogenous hScc1 protein. To investigate this possibility, we examined the sister chromatid cohesion in hScc1ΔN-expressing normal human fibroblast MRC-5 cells. Cells were infected with retroviral expression vector encoding FLAG-tagged wild-type hScc1 (wt-hScc1), hScc1ΔN, or hScc1ΔC mutant protein. The infected cells were analyzed at the indicated time after the completion of infection without drug selection (0 h). In a parallel control experiment, the transfection efficiency was estimated to be ~40%. First, we monitored the proliferation rates. The wt-hScc1- and hScc1ΔC-expressing MRC-5 cells grew as well as the mock-infected cells (Fig. 2A). In contrast, the hScc1ΔN-expressing MRC-5 cells grew less efficiently, which was evident at 72 h. We speculated that the impaired cell growth was a result of the cohesion defect. To test this possibility, asynchronously growing infected MRC-5 cells at 48 h were examined by FISH experiments using pBR12, a probe specific for the chromosome 12 centromere (referred to as 12CEN probe) (18). We found that the 12CEN signals in the wt-hScc1- and hScc1ΔC-expressing populations showed either one of the following three patterns: two separate spots, one spot and one set of twin spots, and two sets of twin spots. These three patterns were interpreted as two 12CENs unreplicated, one 12CEN replicated and the other unreplicated, and both 12CENs replicated, respectively. Fig. 2B(a) shows one representative wt-hScc1-expressing cell that remained to replicate two homologous 12CENs. In contrast, we found that a significant fraction of the hScc1ΔN-expressing populations showed three or four separately located FISH spots, suggesting failure of cohesion at the chromosome 12 centromere most possibly in G2 cells (Fig. 2B, b).

We next examined the spread chromosomes in mitotic cells. In mitotic cells in wt-hScc1-expressing populations, two 12CEN-positive chromosomes were observed in all cases examined (Fig. 2B, c), suggesting that the ectopic expression of wt-hScc1 does not interfere with the normal chromatid cohesion in mitosis. However, in hScc1ΔN-expressing populations, the number of condensed chromosomes was frequently larger than those found in the parental MRC-5 cells and wt-hScc1-expressing populations (Fig. 2B, d-f). Most importantly, four 12CEN-positive chromosomes were frequently observed separately in hScc1ΔN-expressing populations. It was often observed that one of the 12CEN-positive chromosomes was completely separated from others (for example, d and f). However, it was unlikely that the four FISH signals represented four pairs of chromatids in tetraploid cells, because we did not find any significant increase in the number of hScc1ΔN-expressing cells with hyperploidy (data not shown). These observations indicate that two pairs of sister chromatids are disjoined in hScc1ΔN-expressing cells. We also noticed that the chromosomes in hScc1ΔN-expressing populations were condensed normally as in parent MRC-5 cells.

To determine the fate of these cells, we monitored the later time points after retrovirus infection. When DNA and actin
were stained with PI and anti-actin antibodies, respectively, we found that a significant number of cells in hScc1N-expressing populations contained multiple nuclei in a single cell after 72 h of infection (Fig. 2C). In contrast, neither wt-hScc1- nor hScc1ΔC-expressing populations displayed such phenotypes (data not shown). The amounts of DNA in these nuclei varied. These results suggest that cohesin-defective cells show defects in chromosome segregation and form multiple nuclei in a single cell.

Sister Chromatids Appear to Be Separated Completely in hScc1N-expressing HT1080 Cells—To investigate the effect of hScc1N in greater detail, we used an inducible expression system in human fibrosarcoma HT1080 cells that we confirmed to contain near-diploid chromosomes (data not shown). Using this approach, we were able to induce hScc1N expression by the addition of doxycycline to the culture medium (hScc1N-HT1080 cells; see “Materials and Methods”). hScc1N was endowed with a C-terminal FLAG epitope that allowed its detection by FLAG-monoclonal antibodies. No detectable hScc1N was expressed in the absence of doxycycline; in contrast, hScc1N was induced upon the addition of doxycycline, which reached a plateau 2–3 days after the addition (Fig. 3A).

By immunostaining with anti-FLAG antibody, we found that the hScc1N protein was localized in the hScc1N-HT1080 cell nuclei (data not shown). The uninduced hScc1N-HT1080 cells, as well as mock-treated HT1080 cells with or without doxycycline addition, proliferated at a similar rate (data not shown). However, as expected, hScc1N-HT1080 cells that had been induced to express hScc1N protein showed a significantly reduced growth rate (data not shown). In the following experiments, hScc1N-HT1080 cells grown in the presence or absence of doxycycline for 48 h were analyzed.

Sister chromatid cohesion in these cells was examined by FISH experiment using cosmid clones cCI12–156 (Fig. 3B) and pBR12 (Fig. 3C), which recognize the telomere at the short arm of chromosome 12 and the centromere of the same chromosome, respectively. The cell cycle stage was determined by using a laser scanning cytometer (reviewed in Ref. 20). This apparatus scans and records the two-dimensional position and the PI intensity, thereby determining the cell cycle stage for individual cells in a population on a slide glass. After the recording, cells of interest can be recalled and analyzed for a second chromo-probe. In both experiments using cCI12–156 or pBR12, two single spots and two sets of twin spots were observed in the uninduced hScc1N-HT1080 cells at G1 and G2 phases, respectively (Fig. 3B, a and b; data not shown). In contrast, four completely separated FISH spots were frequently observed in induced G2 cells (Fig. 3B, c). These four spots were not derived from four homologous chromosomes in tetraploid cells, because no significant fraction of tetraploid cells was detected. As for the pBR12 probe specific for the chromosome 12 centromere, four FISH spots were observed in 25% of the total population of the induced hScc1N-HT1080 cells and in 3% of the uninduced cells (Fig. 3C). We therefore concluded that the expression of hScc1N also caused premature sister chromatid separation in this system. The four spots did not show any tendency of clustering into two groups; rather, they appeared to distribute randomly. The average distances between one FISH spot to the nearest one in the induced cells with four spots were 10.87 and

![Fig. 1. Overexpression of EGFP-hScc1N leads to G2/M arrest. A, schematic structures of full-length and truncated forms of hScc1 used in this study. B, nuclear localization of Scc1 mutant proteins. Expression vectors encoding N-terminally EGFP-fused Scc1 proteins were transfected to 293T cells, and localization of the overexpressed proteins was examined by immunofluorescence microscopy. C, a, cell cycle profile of EGFP-Scc1-expressing 293T cells. 293T cells transfected with the cDNA encoding the indicated proteins were first sorted for EGFP-positive cells using FACSscan. These cells expressing the recombinant proteins were then analyzed for DNA contents by PI staining. C, b, fractions of G1, S, and G2+M cells deduced from a.](image-url)
Dominant Negative Allele of hScc1

It was interesting to know if the entire chromatids of chromosome 12 were disjoined in the hScc1ΔN-expressing cells. To address this question, a similar analysis was conducted using a chromosome 12 painting probe. The uninduced G2 cells showed two separate chromosome 12 territories (Fig. 3E, a). In contrast, four distinct territories were observed in a significant fraction of the induced G2 cells (Fig. 3E, b). These results demonstrate that the four sister chromatids of two chromosomes 12 are separated in the G2 phase. The four chromatids appear to be randomly positioned in nuclei. There was no recognizable DNA connection between these remote chromatin masses, suggesting that the chromatids were completely separated without major DNA tangling.

**Spindle Microtubules Are Not Associated with Kinetochores in hScc1ΔN-expressing HT1080 Cells**—Next, we analyzed the M phase of the induced hScc1ΔN-HT1080 cells. Because it was not possible to synchronize HT1080 cells, we examined asynchronous cells by PI staining and immunostaining with anti-α-tubulin and anti-γ-tubulin antibodies (Fig. 4A). In uninduced cells, we observed normal metaphase and anaphase cells (Fig. 4A, a, d, and e). In contrast, we frequently observed abnormal anaphase-like configurations (hereafter referred to as pseudo-anaphase) in induced cells (Fig. 4A, b, c, and f). Despite the fact that the bipolar spindle was evident in IF experiments using anti-α- and anti-γ-tubulin antibodies, the chromosome DNA was excluded from the area occupied by the spindle. Indeed, it appeared that there was no kinetochore-microtubule association, and that the chromosome mass was mechanically divided into two or three groups by the spindle. Moreover, the orientation of the polar spindle was perpendicular instead of parallel to the splitting orientation of chromosome masses.

To interpret these results quantitatively, we measured the relative frequencies of cells showing the metaphase, anaphase, or pseudo-anaphase configurations in induced and uninduced populations. The pseudo-anaphase was defined by the appearance that the orientation of interpolar microtubules (when stained with anti-α-tubulin) or the interpolar axis (when stained with anti-γ-tubulin) was perpendicular to the splitting orientation of chromosomes. When more than 500 cells that appeared in metaphase, anaphase, or pseudo-anaphase were scored, 18% of induced cells, in contrast to 3–4% of uninduced cells, showed the pseudo-anaphase configuration (Fig. 4B). We therefore concluded that pseudo-anaphase cells were specifi-
Fig. 3. Separation of sister chromatids in hScc1ΔN-expressing HT1080 cells. A, hScc1ΔN protein was fully expressed at 48 h after induction. Whole-cell extracts were prepared from hScc1ΔN-HT1080 cells (hScc1ΔN) and from a control cell line transfected with the empty vector (Mock). Extracts (25 μg of protein) prepared from the indicated cells cultured in the presence (+) or absence (−) of doxycycline (Dox) at the indicated time were examined for the expression of recombinant protein using anti-FLAG antibodies. B, FISH analyses of the telomere at the short arm of chromosome 12. hScc1ΔN-HT1080 cells that had been cultured in the absence (−) or presence (+) of doxycycline were analyzed using the cCI12–156 probe. α, two single spots in uninduced G1 cells; β, two twin spots in uninduced G2 cells; γ, four separate spots in induced G2 cells. Scale bar represents 10 μm. C, summary of the percentage of cells containing different numbers of pBR12-positive spots among the total population of indicated cells. Over 1000 nuclei were examined for each case. D, nuclear area is increased in induced G2 cells containing four separate pBR12-positive spots. Images of 30 nuclei each from induced (with four FISH spots) and uninduced (with two twin FISH spots) were recorded by a laser confocal microscope. Nuclei areas were measured using a computer software, and the average values are shown. E, apparently complete separation of sister chromatids in induced hScc1ΔN-HT1080 cells. Induced (Dox +) and uninduced (Dox −) cells were examined by painting of chromosome 12. DNA was stained with TOTO3. Scale bar represents 10 μm.
Kinetochore dysfunction in hScc1ΔN-expressing HT1080 cells. A, abnormal spindle-chromosome organization in hScc1ΔN-expressing HT1080 cells. Induced (Dox +) and uninduced (Dox −) hScc1ΔN-HT1080 cells were analyzed by IF for α-tubulin and γ-tubulin (green). DNA was stained by PI (red). α and e, anaphase in an induced cell. Scale bars represent 10 μm. B, relative frequencies of metaphase, anaphase, and pseudo-anaphase cells in induced and uninduced hScc1ΔN-HT1080 populations. The operation definition of pseudo-anaphase cells is given in the text. More than 500 cells stained with anti-α-tubulin (α-tubulin) or anti-γ-tubulin (γ-tubulin), together with PI for DNA, were scored for their chromosome configurations. C, loss of kinetochore-microtubule association in hScc1ΔN-expressing HT1080. CENP-C and α-tubulin were detected by IF using specific antibodies (green and red, respectively), and DNA was stained with TOTO3 (blue) in induced (Dox, +) and uninduced (Dox, −) cells. Scale bar represents 10 μm.
proteins were positive in most, if not all, pseudo-anaphase cells (85, 86, and 86% for hMad2, 3F3/2, and cyclin B1, respectively). This analysis confirmed that the spindle checkpoint is activated in pseudo-anaphase cells. Moreover, it suggests that tension is not applied to kinetochores in pseudo-anaphase cells, because it is known that the 3F3/2 epitope disappears from kinetochores by sensing the tension applied to kinetochores (29). Taken together, we conclude that the cells expressing hScc1\N are arrested at the metaphase-anaphase transition as a result of the activation of the spindle-assembly checkpoint.

DISCUSSION

In this report, we describe the phenotype of Scc1-defective human cells in interphase and the M phase. The results presented here are consistent with the established role of Scc1 in sister chromatid cohesion. However, as discussed below, some phenotypes are unexpected from those known with Scc1-defective lower eukaryotic cells.

Cohesion Defects in Interphase of hScc1\N-expressing Cells—In interphase, the expression of hScc1\N apparently did not affect the progression of the S phase, because the cells containing 4C DNA contents appeared as expected. Previously, it was reported that DNA replication occurs normally in fission yeast Scc1\ts mutant at the restrictive temperature, as well as in cohesin-depleted Xenopus oocyte extracts and in Scc1-depleted chicken cells (5, 8, 30), supporting this notion. Interestingly, we found that the four sister chromatids derived from two homologous chromosomes 12 occupied four separate territories that appeared to distribute randomly in hScc1\N-expressing HT1080 cells in the G2 phase. This result further supports the notion that DNA replication occurs normally in hScc1\N-expressing cells, and indicates that Scc1 is essential for the establishment or maintenance of cohesion in interphase cells. However, the apparently complete disjoining of the four sister chromatids was not straightforwardly expected because it is known that two daughter DNAs produced from one supercoiled DNA by the semiconservative replication mechanism are mutually concatenated (reviewed in Ref. 31). Topoisomerase II is believed to be responsible for resolving the catenation (32), although it is not known at which stage of the cell cycle topoisomerase II decatenates the intertwined two chromatids in vivo. It has been proposed that the decatenation reaction mediated by topoisomerase II is mechanistically coupled with
DNA replication (33). Because we found four physically separated sister chromatids in cohesin-defective cells in the G2 phase, the decatenation reaction should occur independently of the sister chromatid cohesion in the late S or early G2 phase. However, we cannot exclude the possibility that some microscopic connection between sister chromatids escaped detection by FISH experiments in this study.

Even when the decatenation reaction occurs while the cohesion is not formed, there is no reason to expect that the sister chromatids be separated from each other, because the sister chromatids are presumably positioned closely immediately after DNA replication. It is evident that chromosomes are too large to move simply by heat diffusion. Therefore, there should be some kind of mechanical force, either passive or active, which leads to the separation of the sister chromatids in cohesion-defective interphase cells. Further study is necessary to characterize this intranuclear movement of cohesion-defective chromatids.

We also noticed that the nuclear volume, which was inferred by the area occupied by the nucleus on a slide glass, was consistently larger in cohesion-defective cells than in control cells. No significant size difference was observed between the induced and uninduced G2 phase cells, suggesting that the large nuclear volume was related to the failure of cohesion. Because the DNA contents of the two types of cells are the same (4C), this observation may be explained by either the abnormal compaction of DNA or the abnormal nuclear organization in the cohesion-defective interphase cells.

**Mitosis in Cohesion-defective Cells**—Mitotic sister chromatids also showed abnormal cohesion in hScc1ΔN-expressing cells. The four 12CEN signals were frequently separated in these cells. Importantly, these cells showed no evidence of progression to anaphase; the high level of cyclin B1 and the persistent localization of 3F3/2 and Mad2 indicated that the spindle-assembly checkpoint was activated. Therefore, it is concluded that the expression of hScc1ΔN leads to disjoining of the mitotic sister chromatids before entry to anaphase, which presumably reflects the cohesion defects in interphase. We found that at least some of the 12CEN signals were localized remotely from others. However, this result should be interpreted cautiously because the chromosome spreading technique does not necessarily preserve the in vivo structure. Accordingly, we do not know whether the sister chromatids are completely disjoined or not in hScc1ΔN-expressing cells.

Chromosome condensation of the disjoined sister chromatids in hScc1ΔN-expressing cells appeared normal. This result suggests that condensation occurs independently of sister chromatid cohesion, as reported previously in Xenopus and chicken (5, 8).

**Failure in Sister Chromatid Cohesion Leads to Defective Kinetochores**—The observation of multiple 12CEN signals indicates that sister centromeres are frequently disjoined in hScc1ΔN-expressing cells. These “half-kinetochores” maintained some structural features of kinetochores, including CENP-A, -B, -C, and -E. However, it was recently reported that INCENP (inner centromere protein) is absent at the half-kinetochores in Scc1-knockout DT40 chicken cells (8), indicating that all structural features of kinetochores are not maintained in cohesion-defective cells. Consistently, we found that the half-kinetochores in hScc1ΔN-expressing cells frequently did not associate with microtubules. This notion was supported by our observations that there were few, if any, kinetochores associating with monopoles or congregating at the equator in these cells. Moreover, the kinetochores in pseudo-anaphase were very frequently positive not only for hMad2, which senses the relative amount of attaching microtubules (26), but also for the 3F3/2 epitope, which senses the tension applied to kinetochores (29). This result is also consistent with the idea that the microtubule-kinetochore association is defective in hScc1ΔN-expressing cells. Similar conclusions were obtained in a recent study using fission yeast (36).

The spindles appeared normal, because two spindle poles probed by the anti-γ-tubulin antibodies and the interpolar microtubules probed by the anti-α-tubulin antibodies were evident. Therefore, the lack of kinetochore-microtubules association appears to be caused by defective kinetochores, not by any defect in the spindle. These findings strongly suggest that prematurely disjoined centromeres cannot possess functional kinetochores. It is possible that these observations are a direct result of the inactivation of an uncharacterized function of hScc1, and are independent of the premature chromatid separation.

These observations are different from those obtained in scc1-budding yeast cells (14). In budding yeast cells depleted for Scc1p, the half-kinetochores still associated with the microtubules, but the sister kinetochores (a pair of half-kinetochores) frequently aggregated at the same SPB (spindle pole body). This observation was interpreted as follows: sister chromatid cohesion at the centromeric region facilitates the establishment of bipolar attachment of microtubules with the sister chromatids. The reason for the difference in cohesion defect impact on microtubule-kinetochore association between yeast and human remains to be determined. One possibility is that the yeast chromosome is sufficiently small to be pulled by a single microtubule associated with the kinetochore (34). The half-kinetochores in higher eukaryotes may have a defect in organizing an integrated kinetochore structure that associates with multiple microtubules. However, because the genetic approaches in the yeast study and this study are different (deletion of the gene versus the dominant-negative allele), future studies are necessary to confirm the different phenotypes shown by yeast and human cells.

We also observed an anaphase-like configuration in hScc1ΔN-expressing cells. However, this is by no means anaphase, because 1) there is no kinetochore-microtubule attachment (discussed above); 2) the direction of chromosome separation is perpendicular, instead of parallel, to the interpolar axis; 3) the chromosomes group into two or more masses for which the DNA contents differ; and 4) the spindle-assembly checkpoint is activated in these cells (discussed above).

In the absence of kinetochore-microtubule association, how were the chromosomes divided into groups in these cells? We observed that the chromosomes were frequently excluded from the center of the equator. Instead, they clustered into two groups around the two ends of the equator, with the interpolar microtubules penetrating between the two groups of chromosomes. It is known that chromosome arms are pushed by microtubules in the direction away from the spindle pole (“polar ejection force” or “polar wind,” reviewed in Ref. 35). If chromatids do not achieve the kinetochore-microtubule association, one would expect that the chromatids be excluded from the vicinity of the spindle pole by the polar ejection force. In this case, the chromosomes will be positioned at regions farthest from the spindle poles. We hypothesize that the characteristic chromosome positioning and grouping in hScc1ΔN-expressing cells is caused by the polar ejection force.

We frequently observed multinuclear cells after overexpressing of the dominant-negative hScc1 by retrovirus vectors in normal human fibroblast MRC-5 cells. These cells appeared to be 2N and had not undergone end replication. Therefore, the groups of chromosomes most probably underwent chromosome decondensation and nuclear envelope formation after adapta-
tion of the activated spindle-assembly checkpoint. However, the lack of cytokinesis may be a result of mechanical inability, rather than checkpoint mechanisms, because the direction of the spindle (and the to-be cytokinesis) is perpendicular to the dividing orientation of chromosomes.

Acknowledgments—We are grateful to Dr. T. Kitamura for pMX-puro and pMX-neo, Dr. G. P. Nolan for dNX cells, Dr. Y. Okumura for FISH probes, Dr. F. Ishizaka for HT1080 Tet-On cells, and Dr. G. J. Gorbsky for 3F3/2 antibodies. We also acknowledge the excellent secretarial work of F. Nishizaki-Nakayama, A. Orii, and A. Katayama, and technical assistance by M. Tamura.

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