The RCC1 Protein, a Regulator for the Onset of Chromosome Condensation Locates in the Nucleus and Binds to DNA

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Abstract. The RCC1 gene, a regulator for the onset of chromosome condensation was found to encode a protein with a molecular mass of 45 kD, determined using the antibody against the synthetic peptides prepared according to the amino acid sequence of the putative RCC1 protein. The p45 located in the nuclei was released from the isolated nuclei, either by DNase I digestion or by treatment with 0.3 M NaCl. Consistently, p45 bound to the DNA-cellulose column was eluted with 0.3 M NaCl. After sequential treatment with DNase I and 2 M NaCl, almost all of the RCC1 protein were released from the nuclei. Thus, RCC1 protein locates on the chromatin and is not a component of the nuclear matrix. In mitotic cells, p45 is dispersed into the cytoplasm. Presumably, RCC1 protein plays a role in regulating the onset of chromosome condensation, at the level of transcription or of mRNA maturation.

Chromosome condensation occurs at the transition from G2 to M phase in the cell cycle, with the aid of maturation promoting factor (MPF)¹ (20, 36). MPF is present in a latent form in the Xenopus oocyte (7, 9) and is specifically activated at mitosis by the translation of an activating agent (for review see reference 10). It is therefore assumed that the interphase cells have a regulatory mechanism which represses expression of this activator protein. This view was supported by cell fusion experiments which revealed that mitosis was delayed in G2 phase cells fused with S phase cells (30), and by isolation of mutants with defects in negative regulatory factor(s) for MPF activation (23, 26). In eukaryotic cells, mitosis does not normally occur until DNA synthesis is completed, but, in these mutants, mitosis is prematurely induced at interphase under restrictive conditions.

In one such temperature-sensitive (ts) mutant, tsBN2, from the baby hamster kidney (BHK21) cell line (24, 26) premature chromosome condensation (PCC) occurs at 39.5°C. PCC is a phenomenon that was first identified in cell fusion experiments (30): after fusion with mitotic cells, interphase cells undergo rapid transformation resembling that of G2–prophase transition, leading to the formation of discrete chromosomes. In the tsBN2 cells, this phenomenon can be induced at the restrictive temperature without fusing mitotic cells. In addition to PCC, all mitotic-specific events (such as phosphorylation of histone H3, appearance of mitotic-specific antigens, and synthesis of p35 protein) occur in interphase tsBN2 cells at the nonpermissive temperature (2, 39, 40). These mitotic-specific events depend on the synthesis of a new protein (24).

Consistently, tsBN2 cells showing PCC possess the ability to condense the chromatin of interphase cells by cell fusion (14). Thus, chromosome-condensing factor is newly produced in tsBN2 cells at the nonpermissive temperature even in cases of arrest at interphase. As the tsBN2 mutation is recessive in hybrid cells (22), tsBN2 cells are considered to have a ts defect in the negative regulator for the onset of chromosome condensation, which inhibits the condensation until the G2 phase.

We cloned the human RCC1 gene, complementing tsBN2 mutation from HeLa cells, by DNA-mediated gene transfer (15). Using the Alu-free DNA fragment of the RCC1 gene, cDNA clones were isolated from Okayama–Berg’s cDNA library (25). While two of these complement the tsBN2 mutation, the 5' base sequence of these clones differs. Since both clones share the open reading frame of 1,263 bp, we considered that this open reading frame may encode the product of the RCC1 gene, RCC1 protein. According to the base sequence of the RCC1 cDNA, the putative RCC1 protein is estimated to have a molecular mass of 45 kD and seven homologous internal repeats of ~60 amino acids.

We prepared antibodies against the peptides of putative RCC1 protein, in attempts to identify the presence of human RCC1 protein in HeLa cells. Using these antibodies, human RCC1 protein was shown to be a new member of the nuclear DNA-binding protein family. It has a molecular mass of 45 kD, the same as estimated from the base sequence of the cloned cDNA.

Materials and Methods

Cell Lines and Culture Condition

tsBN2 cells are ts mutants derived from the BHK21 cell line (22), and the following cell lines are derivatives of tsBN2 cells: BN2-RV, a spontaneous

1. Abbreviations used in this paper: MPF, maturation promoting factor; PCC, premature chromosome condensation, ts, temperature sensitive.
Preparation of Antipeptide Antibodies

According to the sequence of RCC1 cDNA (25), five peptides (peptide 1, KSKKVKSVHRSHST; peptide 2, ENVMERKKPALVSI; peptide 3, KSRSGRHVRFODA; peptide 4, LGLGEGAEKSSPI; and peptide 5, HTVLVVKDKEQS) were synthesized by the solid-phase t-Boc procedure, using the Applied Biosystems, Inc. (Foster City, CA) model 430A automated synthesizer. Cysteine was added to the carboxy-terminal ends of all peptides to couple the carrier proteins.

Synthetic peptides were coupled to the carrier protein, keyhole limpet hemocyanin or BSA using N-(r-maleimidobutyryloxy)succinimide, and then were used to immunize rabbits (33).

Preparation and Fractionation of Nuclei

To prepare nuclei, cells were pelleted and washed twice in ice cold hypotonic buffer containing 10 mM Hepes, pH 8.0, 5 mM KCl, and 2 mM 2-mercaptoethanol, 3% (wt/vol) SDS, and 20% glycerol. Cellular proteins were electrophoresed in a 12.5% SDS-polyacrylamide slab gel (19), and analyzed by immunoblotting, as described previously (5), using the antibody against peptides and a Vectastain ABC kit. Protein concentration was determined by Bradford’s method (4).

Indirect Immunofluorescence Staining

Cells growing on glass coverslips were fixed in 3% (wt/vol) paraformaldehyde in PBS, permeabilized with 1% NP-40 in PBS containing glycine, and stained using the anti-peptide 1 and the rhodamine-conjugated goat anti–rabbit IgG (Tago Inc., Burlingame, CA), as described elsewhere (6). DNA was stained with Hoechst dye 33258 (1 μg/ml) (Hoechst Japan, Ltd., Saitama, Japan).

DNA-binding Assay

Cells labeled with [35S]methionine were lysed in buffer A (10 mM Tris- HCl, pH 8.0, 1 mM MgCl2, 0.5% NP-40, 0.45 M NaCl, 1 mM PMSF, and 5 μg/ml Pepstatin) (29). After dilution with buffer B (10 mM potassium phosphate, pH 6.2, 1 mM MgCl2, 0.5% NP-40, 1 mM DTT, 10% glycerol), the lysate was loaded onto the double-stranded calf thymus DNA-cellulose column. Bound proteins were eluted with buffer containing 10 mM Tris- HCl, pH 8.0, 1 mM DTT, and increasing concentrations of NaCl. The flowthrough and the eluted fractions were immunoprecipitated and analyzed by electrophoresis on 12.5% SDS–polyacrylamide gels. The radioactivity was detected by fluorography.

Preparation of Antibodies to Peptides Encoded by RCC1 cDNA

Using the cloned human RCC1 genomic DNA as a probe, two RCC1 cDNAs complementing the tsBN2 mutation were cloned (25). These clones have a different 5’ untranslated region, but share the open reading frame of 1,263 bp encoding a protein of 45 kD, which is estimated to be a product of the human RCC1 gene. According to the amino acid sequence of this putative RCC1 protein, five peptides were synthesized, as described in Materials and Methods, and the location of each is shown in Fig. 1. These peptides were conjugated with BSA or keyhole limpet hemocyanin, as a carrier protein, and then used as antigens to immunize the rabbits.

Identification of the Putative RCC1 Protein in Human Cells

Of the five synthetic peptide-specific antibodies, the anti-peptide 1 antibody recognized the protein with a molecular mass of 45 kD, detected in the lysate of HeLa cells by immunoblotting (Fig. 2). Other antibodies which recognized the synthetic peptides by Ouchterlony analysis did not specifically recognize any protein by immunoblotting (data not shown). The 45-kD mass is the same as that of the putative RCC1 protein estimated from the base sequence. This p45 protein was also found in two other cell lines, ST2-7 and BN2-pcD51, but not in BHK21 and tsBN2 cells (Fig. 2). Both ST2-7 and BN2-pcD51 cells contain the active human RCC1 gene (genomic and its cDNA, respectively) (15, 25). The presence of p45, therefore, is consistent with the presence of the active human RCC1 gene.

RCC1 Protein Is Located in the Nuclei

HeLa cells were swollen in hypotonic solution and the intact nuclei were collected by gentle centrifugation. Proteins were extracted from both cytoplasmic and nuclear fractions, and analyzed by immunoblotting using anti–peptide 1. As shown in Fig. 3 A, p45 was present only in the nuclear fraction.

The nuclear location of the p45 was further confirmed by indirect immunofluorescence staining. HeLa cells and two other cell lines, tsBN2 and ST2-7, grown on glass coverslips were fixed, permeabilized, and exposed to anti–peptide 1. The presence of antibody was visualized by rhodamine-conjugated goat anti–rabbit IgG. The antibody to peptide 1 recognizes only the human RCC1 protein. Therefore, staining of the cells should be consistent with the presence of active human RCC1 protein. As shown in Fig. 4, the nuclei of both HeLa and ST2-7 were stained but the nuclei of tsBN2 cells were only stained faintly and were barely distinguishable from the cytoplasm. Since both HeLa and ST2-7 cells contain the active human RCC1 gene, these results are consistent with the presence of human RCC1 protein.

Subnuclear Localization of RCC1 Protein

To determine whether the RCC1 protein locates on the chro-
matrin or on the nuclear matrix, isolated nuclei were treated either with nuclease or salt.

The role of DNA in the nuclear association of p45 was first investigated. Nuclei prepared from HeLa cells were incubated in the presence of increasing amounts of DNase I. The release of p45 from the nuclei was monitored by immunoblotting with the anti-peptide 1 antibody. Fig. 5 shows that whereas p45 was completely retained by the nuclei after incubation in the absence of nuclease, even a mild nuclease digestion of DNA released ~80-90% of the total p45. This release of p45 correlates with the extraction of DNA from the nuclei (data not shown). RNA was not required to retain RCCI protein in the nuclei, since p45 was not released from the nuclei after digestion with RNase A (200 μg/ml) (data not shown).

**Figure 1.** Location of synthetic peptides on the open reading frame for putative RCC1 protein. Complete amino acid sequence of human RCC1 protein predicted from its cDNA is indicated (see reference 25). Regions of the protein selected for synthesis are underlined and numbered: (1) peptide 1 (amino acid residues 19-33); (2) peptide 2 (residues 52-65); (3) peptide 3 (residues 232-245); (4) peptide 4 (residues 326-339); and (5) peptide 5 (residues 410-421).

**Figure 2.** Identification of RCC1 protein by immunoblotting. Total cellular proteins were extracted from the following cell lines: tsBN2, BHK21, HeLa S3, BN2-RV, ST2-5, and BN2-pcDps1, as indicated. 60 μg of each extract was analyzed by immunoblotting, using anti-peptide 1 as the antibody. The position of p45 is indicated by the arrowhead and the position of molecular mass markers are shown at the left in kilodaltons.

**Figure 3.** Localization of RCC1 protein in the nuclei. HeLa S3 cells (5 × 10⁶ cells) were swollen in the presence of 0.5% NP-40, and the nuclei were separated from the cytoplasm by centrifugation. 60 μg of protein extracted from nuclei and cytoplasmic fractions were then electrophoresed and analyzed by immunoblotting using anti-peptide 1 as the antibody (A) or stained with Coomassie brilliant blue dye (B). As a control, 60 μg of total cellular protein was co-electrophoresed. In the case of Coomassie brilliant blue staining, 25 μg of each sample was used. Positions of the molecular mass markers are indicated on the left in kilodaltons.
Figure 4. Indirect immunofluorescence staining of cells. The cell lines tsBN2 (BN2), ST2-7 (ST2-7), or HeLa S3 (HeLa) were grown on glass coverslips, fixed, and stained using anti-peptide 1 and rhodamine-conjugated goat anti-rabbit IgG. Bar, 40 μm.
Approximately 10–20% of the total p45 remained associated with the nuclei after digestion with 100 μg/ml of DNase. At physiological salt concentrations, the DNA released from nuclease-treated nuclei is present in nucleoprotein complexes exhibiting characteristic features of chromatin structure, such as nucleosomal organization or histone HI-dependent higher levels of chromatin organization (38). The p45 proteins present in the nuclei after digestion with 100 μg/ml of DNase I may be attached to such a fragmented chromatin trapped in the nuclei or to the nuclear matrix (3). If the RCC1 protein which is present in the nuclei after DNase I digestion is indeed a component of the nuclear matrix, then the p45 would not be released, even in the presence of 2 M NaCl (3).

Isolated nuclei were incubated in the presence of increasing concentrations of NaCl. The release of p45 from the nuclei was monitored by immunoblotting with anti-peptide 1. As shown in Fig. 6, whereas no p45 was released from intact nuclei during incubation in buffer to which no salt had been added, a concentration of 300 mM NaCl was effective in extracting 80–90% of the total p45. But even in the presence of 400 mM NaCl, some p45 remained in the nuclei. To investigate whether these residual nuclear RCC1 proteins are located on the nuclear matrix, the nuclei were digested with DNase I (100 μg/ml) and then incubated in the presence of 2 M NaCl (Fig. 7). After treatment with 2 M NaCl, p45 was not detectable in the nuclei (Fig. 7 A, NM), in which fraction only nuclear matrix proteins remained (Fig. 7 B). Thus, RCC1 protein is located on the chromatin and not on the nuclear matrix.
RCC1 Proteins in the Mitotic Cell

Cells growing in culture dishes were fixed without hypotonic treatment, so that chromosomes of mitotic cells were condensed, as shown in Fig. 8 B (arrow points to mitotic cells). In all mitotic cells examined microscopically, RCC1 proteins were dispersed in the cytoplasm as shown in Fig. 8 A, rather than associated with chromosomes.

Binding of RCC1 Protein to DNA

The finding that RCC1 proteins were released from the nuclei, either by DNase I digestion or by low salt treatment, suggests that RCC1 protein binds to chromatin, as a result of its DNA-binding activity. To investigate the DNA-binding activity of RCC1 protein, the total cellular extract was subjected to chromatography on a DNA-cellulose column (Fig. 9).

Exponentially growing HeLa cells were labeled with [35S]methionine, and the total cellular proteins extracted as described in Materials and Methods. They were then charged onto a column of double-stranded calf thymus DNA-cellulose. The bound proteins were eluted with buffer containing increasing concentrations of NaCl. Flow-through and eluted fractions were immunoprecipitated, using the anti-peptide 1 antibody and analyzed by SDS-PAGE. There were no p45 proteins in the flow-through and washed fractions, hence all the p45 proteins were bound to the DNA-cellulose column. At the concentration of 300 mM NaCl, 80-90% of total p45 proteins were eluted from the DNA-cellulose column.

At the same concentration, two other proteins (>100 kD and 35 kD) were eluted. Since these proteins were immunoprecipitated by anti-peptide 1, these high and low molecular mass proteins may be present with p45 in a complex form.

Discussion

To identify the location of the RCC1 gene product in human cells, we prepared antibodies against peptides synthesized according to the putative human RCC1 protein. One of these antibodies specifically recognized the p45 protein in the extract of HeLa cells. Since the presence of p45 is consistent with the presence of the active human RCC1 gene and the molecular mass is the same as that estimated from the amino acid sequence of putative RCC1 protein, we concluded that the p45 is the human RCC1 protein.

Only the anti-peptide 1 antibody out of five synthetic peptide-specific antibodies recognized the RCC1 protein. The RCC1 protein has a domain with seven homologous repeats of 60 amino acids. Peptide 1 is located outside of this domain, while others are located inside the repeated domain. Therefore, the inability of peptide-specific antibodies to recognize RCC1 protein may reflect a particular structure of this protein, as deduced from the base sequence of human RCC1 cDNA (25).

We obtained evidence for the presence of RCC1 proteins in the nuclei. The nuclei of both HeLa and ST2-7 cells, but not tsBN2 cells were stained with anti-peptide 1. These observations are consistent with the presence of human RCC1 protein. Therefore, the possibility that the nuclei were stained artifactually by the anti-peptide 1 antibody can be ruled out.

DNA plays an important role in locating RCC1 proteins in the nuclei, since almost all the RCC1 proteins were released by the mild digestion of DNA with DNase I. Also, almost all the RCC1 proteins were released from the nuclei by digestion with 300 mM of NaCl. At the same concentration of NaCl, RCC1 proteins bound to DNA-cellulose columns were effectively eluted. Thus, the RCC1 protein is associated with DNA in a salt labile fashion. Some RCC1 protein was released from nuclei after treatment of 0.1 M NaCl, and a considerable amount was released with 0.2 M NaCl. Presumably, some of the RCC1 protein was free from chromatin in the nucleoplasm. After sequential treatment with DNase I and 2 M NaCl, no RCC1 protein remained in the nuclei. Therefore, the RCC1 protein is not a component of the nu-
clear matrix. This is further supported by the finding that in mitotic cells, RCC1 proteins were dispersed in the cytoplasm. Hence, RCC1 protein did not specifically bind to the mitotic chromosomes.

In addition to the p45, two other proteins were eluted from the DNA-cellulose column using the same concentrations of NaCl. Since these proteins were immunoprecipitated by anti-peptide 1, which is specific for human RCC1 protein, it may be that these two other proteins form a complex with RCC1 protein in vivo. These proteins are either essential for RCC1 protein binding to DNA or bind DNA with the aid of RCC1 protein.

We cloned both BHK and tsBN2 RCC1 cDNA, using the human RCC1 cDNA as a probe, and confirmed that the wild-
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The sequence of both these cDNA, we found that the RCC1 gene has a point mutation in tsBN2 cells (Uchida, S., T. Sekiguchi, M. Ohtsubo, H. Nishitani, and T. Nishimoto, manuscript in preparation). Based on these results, we concluded that the RCC1 gene was indeed the ts mutated gene (tsBN2 gene) in tsBN2 cells. The phenotype of tsBN2 cells shows that it has a ts defect in the negative regulator for onset of chromosome condensation. Thus, the RCC1 gene is probably one of the negative regulators which inhibit the condensation of chromosomes until the G2 phase (25, 26).

Induction of PCC in tsBN2 cells is completely inhibited by cycloheximide and partially so by actinomycin D. Therefore, we considered that the induction of PCC is regulated mainly in a posttranscriptional fashion (24). However, the present results suggest that RCC1 protein functions in the nuclei. Thus, in somatic cells, there is the possibility that RCC1 protein regulates the onset of chromosome condensation at the transcriptional or RNA-processing level, rather than translational level.

It has recently been shown that purified MPF from Xenopus and H1 histone kinase from starfish both contain a protein closely related to p34cdc2 encoded by the fission yeast cell cycle control gene, cdc2 (1, 11, 17). The cdc2 gene encodes a protein kinase potentially regulated by phosphorylation (8, 18, 34). Consistently, in fission yeast, upstream regulators of cdc2 encode putative protein kinases (weel ÷ niml+), thereby suggesting that activation of MPF depends on protein phosphorylation cascades (31, 32). The cdc2 gene is also regulated by another mitotic control gene, cdc13, which encodes a protein with a sequence closely related to that of the invertebrate cyclins (13, 35). Though the role of cyclins in MPF activation is unknown, translation of cyclin mRNA is essential for MPF activation in the frog Xenopus (21, 37).

The RCC1 protein has no sequence homology with protein kinases or cyclins. Since induction of PCC in tsBN2 cells requires a new protein synthesis and RCC1 protein localizes on the chromatin, this protein may act as an upstream regulator of MPF by playing a role in repressing the synthesis of cyclins or other activator proteins of MPF, at the level of transcription, mRNA maturation, or both. In Aspergillus, a positive regulator of mitosis, nimA, shows the mitosis-specific expression of mRNA levels (27). Like tsBN2 cells, nimA overexpression causes mitotic events even if cells are blocked at the S phase (28). Thus, RCC1 protein may regulate the mRNA levels of the nimA-like gene in mammalian cells. In any case, RCC1 protein is a first nuclear DNA-binding protein which is involved in MPF activation and the regulation of onset of chromosome condensation. Functional characterizations of this protein are expected to elucidate the early mechanisms involved in the activation of MPF.

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