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Inhibition by Anti-RCC1 Monoclonal Antibodies of RCC1-Stimulated Guanine Nucleotide Exchange on Ran GTPase[1]

Yoshiaki Azuma,* Takahisa Hachiya,† and Takeharu Nishimoto*,[2]

*Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Maidashi, Higashi-ku, Fukuoka 812-8582; and †Ina Research Laboratory, Medical & Biological Laboratories Co., Ltd., Ohara, Tera-Sawaoka, Ina, Nagano 396

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Nine monoclonal antibodies to RCC1, the guanine nucleotide-exchange factor on Ran GTPase, were obtained using recombinant RCC1 as the antigen. Epitopes of three monoclonal antibodies, which did not inhibit RCC1 function, were localized in the N-terminus outside the RCC1 repeat, while epitopes of the other 6 monoclonal antibodies were localized within the RCC1 repeat. Three of the latter 6 monoclonal antibodies, 2B6, 6C3, and 8D9, inhibited RCC1-stimulated nucleotide release. Two of them, 2B6 and 6C3, recognized the same amino acid residues in the N-terminus of the second RCC1 repeat, Tyr89, Ser90, Phe91, and Gly92, of which one, Gly92, is conserved in *Saccharomyces cerevisiae* and mutated in an *rcc1*$^-$ strain, *mtr1-2*. The monoclonal antibody 8D9 recognized two amino acid residues, Arg320 and Ala321, downstream of Gly319 in the N-terminus of the 6th RCC1 repeat, which corresponds to Gly92 in the second RCC1 repeat. The monoclonal antibodies which inhibited RCC1 function bound to RCC1 in homogenous solution and stained cellular RCC1. We propose that the N-terminus of the RCC1 repeat is exposed at the surface of RCC1 on the coated plate or in fixed cells, and is involved in the RCC1-stimulated nucleotide exchange on the Ran GTPase.

Key words: Ran, RCC1.

RCC1 is an abundant chromosomal protein of 45 kDa which carries out GDP/GTP exchange on Ran GTPase (*1, 2*). It comprises an N-terminal region of 40 amino acid residues and an internal repeated domain in which about 60 amino acid residues are repeated seven times (RCC1 repeat). Throughout the repeats, several amino acid residues are well conserved. RCC1 homologues have been isolated from a wide range of species, from yeast to mammals. Among them, only the RCC1 repeat is conserved (*2*). Therefore it seems likely that the RCC1 repeat is essential for RCC1-stimulated GDP/GTP exchange on Ran GTPase. In accordance with this notion, the RCC1-repeated domain alone can complement a temperature-sensitive *rcc1*$^-$ mutant, tsBN2, of the hamster BHK21 cell line (*3*).

The human *RCC1* gene has been isolated as a gene which rescues tsBN2 mutation (*4, 5*), and the *Saccharomyces cerevisiae* RCC1 homologue designated as Prp20p has been identified as a protein which is encoded by the gene whose defect causes receptorless mating (*srm1-1*) (*6*), nuclear accumulation of mRNA (*mtr1*) (*7*), or mRNA-splicing defect (*prp20-1*) (*8*). Consistent with the finding that the RCC1 repeat is essential for the nucleotide-exchange reaction, all of the *prp20*$^-$ mutations have been mapped within the RCC1 repeats. For instance, *srm1-1* and *mtr1-2* are located within the N-terminus of the second repeat, while *prp20-1* is located within the C-terminus of the 7th repeat (*7, 9*). Overexpression of the *S. cerevisiae* Ran homologue Gsp1p GTPase, can suppress *prp20-1*, but not *srm1-1* or *mtr1-2* (*7, 9*). In contrast, the mutation of the *DED1* gene, *ded1-21*, results in the opposite suppression (*10*). Such an allele-specific suppression of *prp20*$^-$ may reflect the complexity of the RCC1 repeat. We previously found, using alanine-scanning mutagenesis, that the N-terminal half of the RCC1 repeat plays a different role in the GDP/GTP exchange activity of RCC1, from the C-terminal half of the RCC1 repeat (*11*). The alanine mutants of RCC1, Asp44Ala and Arg101Ala, which are located within the N-terminal of the RCC1 repeat, have a high k_m but a normal k_{cat} for the steady-state RCC1-stimulated nucleotide-exchange reaction on Ran GTPase. In contrast, the alanine mutants of histidine residues, which are conserved through the RCC1 repeats and which are located at the C-terminal end of the RCC1 repeat (*2*), show opposite results, that is, these mutants have a normal k_m, but a low k_{cat}.

To confirm our previous results from a different aspect, we prepared a series of mouse mABs against *Escherichia coli*-produced recombinant RCC1 and examined the relation between their epitopes and their inhibitory activities on the RCC1-stimulated nucleotide-releasing reaction. In accordance with our previous findings, it was suggested that the N-terminal sites of the RCC1 repeat were exposed at the surface of RCC1 and were involved in the RCC1-stimulated GDP/GTP exchange on Ran. Interestingly, we found no histidine residues, which were previously proposed to be essential for the RCC1-stimulated nucleotide exchange

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[2] To whom correspondence should be addressed. Phone: +81-92-642-6175, Fax: +81-92-642-6183

Abbreviation: mAB, monoclonal antibody.

(*11*), among the epitopes of the mABs obtained.

MATERIALS AND METHODS

Analysis of Guanine Nucleotide-Exchange Reaction on Ran—The guanine nucleotide-exchange activity of RCC1 was assayed as described previously (*12*).

Deletion of RCC1 cDNA—pUC118-SRα RCC1 (*11*) was digested with the enzymes *Nde*I and *Pst*I, and the resulting 1.3 kbp *Nde*I-*Pst*I fragment containing human RCC1 cDNA was inserted into pUC28, resulting in pUC28-RCC1. pUC28-RCC1 was digested with the enzymes *Nco*I, and *Pst*I or *Eco*RI. The resulting 1.4 kbp *Nco*I-*Pst*I fragment was partially digested with *Nla*IV or *Rsa*I, and then inserted into the *Nco*I/*Sma*I sites of pGEX-CS (*13*), resulting in Δ121, Δ145, Δ216, Δ248, and Δ356. An additional 0.9 kbp of the *Nco*I-*Eco*RI fragment of pUC28-RCC1 was inserted into the *Nco*I/*Eco*RI sites of pGEX CS, resulting in Δ290.

Other deleted proteins of human RCC1 were prepared from cDNAs amplified with PCR, using the appropriate oligonucleotides as primers. The nucleotide sequences of all of the obtained human RCC1 cDNA-deletion fragments were determined.

Purification of ΔRCC1 Proteins—pGEX-ΔRCC1 plasmids were transformed into *E. coli* BL21(DE3). *E. coli* cells producing ΔRCC1 were suspended in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM DTT, 10% glycerol, 2% CHAPS, and 1 mM *p*-APMSF, and then sonicated. The cell lysate was centrifuged at 70,000 $\times g$ for 30 min and the supernatant was mixed with Glutathione-Sepharose 4B beads. After rotation at 4°C for 1 h, the beads were spun down and washed 4 times with lysis buffer, and then adsorbed proteins were eluted with 10 mM reduced glutathione. The concentration of proteins in the obtained ΔRCC1 fractions was determined densitometrically.

Biosensor Analysis—Real-time interaction analysis of the binding between RCC1 and mABs was performed with a BIAcore biosensor instrument (Pharmacia Biosensor AB) (*14*). CM5 sensor chips and amine coupling kits were obtained from Pharmacia Biosensor AB. The anti-mouse Fcγ antibody (Pharmacia) was immobilized on the CM5 sensor chip as recommended by the manufacturer, and then 10 μg/ml of RCC1-mAB was injected to be trapped on the sensor chip through the anti-mouse Fcγ antibody.

Binding experiments were performed through the injection of 100 nM of purified recombinant RCC1 in buffer containing 25 mM Hepes, 100 mM NaCl, and 3 mM potassium phosphate (pH 7.6). Evaluation and calculation of the binding parameters were carried out according to the manual Bia evaluation software type 1 model provided by Pharmacia Biosensor AB.

RESULTS

Preparation of Mouse Monoclonal Antibodies to RCC1—BALB/c mice were immunized with 20 μg of purified recombinant RCC1 and Freund's complete adjuvant every week. After this had been carried out 5 times, the splenocytes were fused with P3U1 mouse myeloma cells by using polyethylene glycol. Antibodies were screened by enzyme-linked immunoassay using recombinant RCC1. Purified IgG of monoclonal antibodies (mABs) was prepared by using a protein A affinity column. Finally 9 hybrid clones producing mAB against RCC1 were obtained.

RCC1-Deletion Mapping of Epitopes for RCC1-mABs—In order to determine the epitopes of these mABs, we prepared a series of RCC1 cDNA fragments deleted from the C-terminal end and fused to GST at the N-terminal end (Fig. 1). *E. coli*-produced GST-fused RCC1 fragments were partially purified using the Glutathione beads, resolved on SDS-PAGE and electroblotted onto PVDF membrane. These membrane filters were probed with the obtained mABs. Three mABs, 2F3, 3D11, and 8B11, recognized all of the RCC1 fragments from d40 to d85, indicating that their epitopes were localized within the N-terminal peptide (amino acid residues 1-40), outside the RCC1 repeat. The other 6 mABs were found to have their epitopes within the RCC1 repeat. Four of them, 1H3, 2B6,

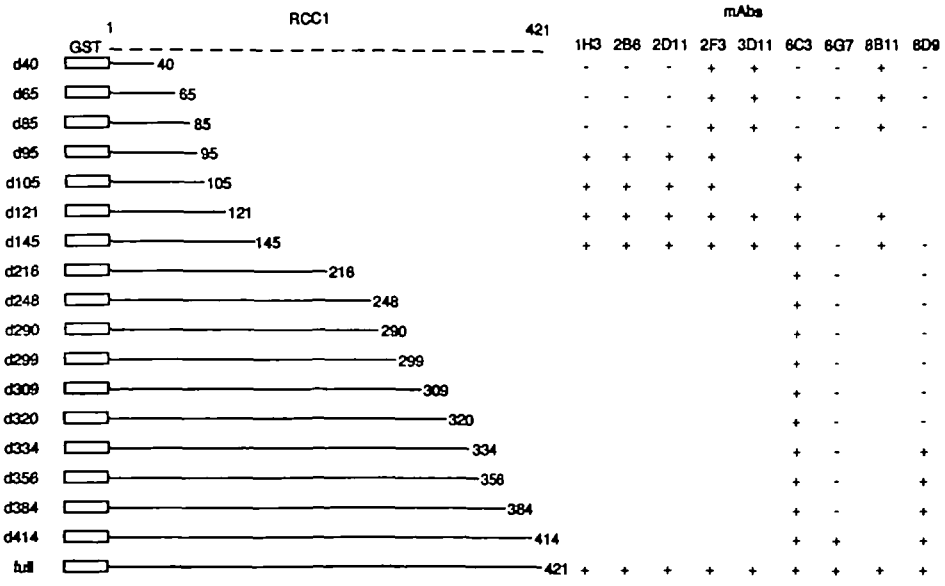

Fig. 1. **RCC1 fragments recognized by RCC1-mABs.** GST-fused *RCC1* cDNA fragments deleted from the C-terminus were prepared, expressed in *E. coli* and purified on GST columns as described in "MATERIALS AND METHODS." One ng of RCC1 fragments was subjected to SDS-PAGE in 11% gel and transferred onto PVDF membranes. Membranes containing a series of blotted RCC1 fragments were probed with the indicated mABs. The number of amino acid residues at the C-terminal end of the RCC1 fragment is indicated. Depending on the length, the RCC1 fragment was either recognized by mABs (+) or not recognized (−).

2D11, and 6C3, recognized peptides covering from the 85th residue to the C-terminal end. Thus, their epitopes were localized within the region ranging from 85 to 95 amino acid residues of RCC1. In such a way, the epitopes of the mABs 8D9 and 6G7 were localized within the region from 321 to 334 amino acid residues and from 385 to 414 amino acid residues of RCC1, respectively.

Inhibition of RCC1-Stimulated GDP Release from GDP-Ran by RCC1-mABs—In parallel, we determined the activities of RCC1-mABs to inhibit RCC1-stimulated nucleotide release from GDP-Ran. *E. coli*-produced RCC1 was highly purified as described (*11*) and mixed with an increasing amount of mABs. The mixtures were incubated with [^{3}H]GDP-preloaded Ran.

The three mABs, 2B6, 6C3, and 8D9, which recognized the N-terminal area of the 2nd and the 6th RCC1 repeats, significantly inhibited RCC1-stimulated GDP release (Fig. 2). The mAB 8D9 almost completely inhibited GDP release from GDP-Ran at a high dose. On the other hand, the inhibitory activities of both mABs 2B6 and 6C3 reached a plateau at a molar ratio (antibody/RCC1) of more than 10: 1. The mABs which recognized peptides outside the RCC1 repeat had no inhibitory effect on the RCC1-stimulated GDP-releasing reaction.

Amino Acid Mapping of the Epitopes—Of the four mABs which recognize the same region of RCC1 (amino acid residues; 85 to 95), two mABs inhibited the function of RCC1, while the other two did not. In order to determine the amino acid residues recognized by these mABs, we prepared a series of peptides in which a single amino acid residue from amino acids 86 to 95 was changed to alanine, as shown in Fig. 3A. The prepared peptides were spotted on the membrane and probed with the indicated mABs. Surprisingly, none of these four mABs recognized Phe91Ala and Gly92Ala peptides. In addition, the mABs 2B6 and 6C3, both of which inhibited the function of RCC1, did not recognize Tyr89Ala, although the other mABs, 1H3 and 2D11, recognized this peptide. Furthermore, the mAB 1H3 did not recognize Asp95Ala peptide. It was thus suggested that the amino acid residues Tyr89, Phe91, and Gly92 are important for the RCC1-stimulated GDP release from GDP-Ran.

As a control, a series of peptides consisting of 10 amino acid residues covering the region of RCC1 from 1 to 40 or from 320 to 341 (Fig. 3, B and C) was prepared and spotted on the membrane. The mAB 3D11 recognized the 4th and 5th peptides of the region (1 to 43), indicating that the amino acid sequence of Ala-Asp-Ala-Ile-Pro-Lys, which was overlapped by these two peptides, contained the epitope of the mAB 3D11. On the other hand, the mAB 8D9, which inhibited the RCC1 function, recognized only the first peptide of the region (320 to 341 amino acid), indicating that its epitope involves the two amino acid residues Arg320 and Ala321.

RCC1-Binding Ability of mABs—The mABs may inhibit the RCC1 function depending on their abilities to bind to

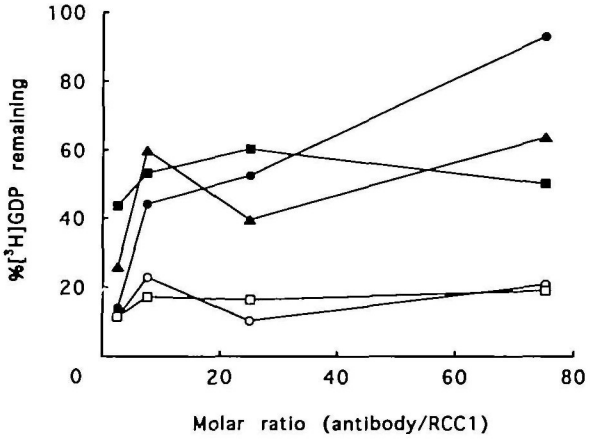

Fig. 2. **Inhibition of RCC1-stimulated GDP release by mABs.** *E. coli*-produced and purified RCC1 (0.1 pmol) was mixed with the indicated amount (molar ratio) of mABs. After overnight incubation at 4°C, [^{3}H]GDP-bound Ran (25 pmol) was added and the mixtures were incubated at 30°C for 5 min. The reaction mixture was filtered through a nitrocellulose filter (0.45 μm, BA85, Schleicher & Schuell) and the radioactivity remaining with Ran on the filter was counted with a liquid scintillation counter. ○ 3D11; □ 8B11; ● 8D9; ■ 6C3; ▲ 2B6.

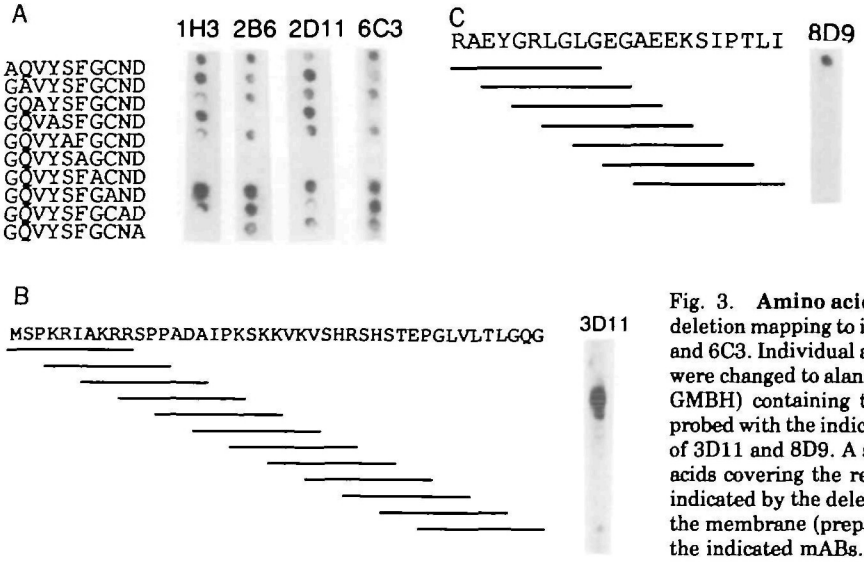

Fig. 3. **Amino acid residues recognized by mABs.** (A) Amino acid deletion mapping to identify the epitopes of the mABs, 1H3, 2B6, 2D11, and 6C3. Individual amino acid residues in the RCC1 peptides (86 to 95) were changed to alanine. The membrane (prepared by JERINI BioTools GMBH) containing the peptide aligned and spotted as indicated was probed with the indicated mAB. (B, C) Peptide mapping for the epitopes of 3D11 and 8D9. A series of overlapping peptides containing 10 amino acids covering the region (1 to 43) (B) and (320 to 341) (C) of RCC1 indicated by the deletion analysis was prepared as indicated, spotted on the membrane (prepared by JERINI BioTools GMBH) and probed with the indicated mABs.

RCC1. In order to address this issue, we examined the abilities of the mABs to immunoprecipitate RCC1. As expected, the mABs 2B6 and 6C3, which inhibited RCC1 function, could efficiently precipitate RCC1, whereas the mABs 1H3 and 2D11, which did not inhibit RCC1 function, did not precipitate RCC1 (Fig. 4). Thus, the inhibitory activities of mABs against RCC1 are correlated with their binding abilities to RCC1 in homogenous solution.

In order to confirm this, we examined the RCC1-binding ability of other RCC1-mABs. Two mABs, 3D11 and 8B11, efficiently precipitated RCC1, although neither of them inhibited RCC1 function (Fig. 2). In contrast, the mAB 8D9, which inhibited RCC1 function in a dose-dependent manner, did not precipitate RCC1 (Fig. 4). Taking these results together, in contrast to the case of the mABs 2B6 and 6C3, the RCC1-binding abilities of mABs did not seem to be necessary for inhibiting RCC1 function. In order to investigate this issue further, we analyzed the protein-

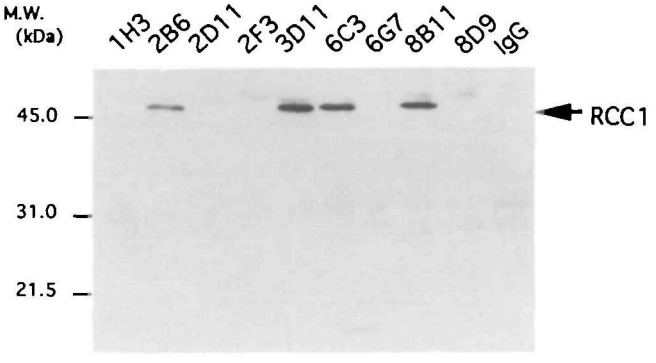

Fig. 4. **Immunoprecipitation of RCC1 by mABs.** *E. coli*-produced and highly purified RCC1 (20 ng) was mixed with 2 μg of mABs in PBS containing 0.1% BSA and 2 mM DTT (final volume 50 μl). After rotation at 4°C for 2 h, protein-G Sepharose beads (Pharmacia) (25 μl bed) were added. After rotation at 4°C for an additional hour, the beads were spun down and washed 5 times with PBS. Proteins adsorbed onto the beads were subjected to SDS-PAGE in 11% gel, transferred onto a PVDF membrane and assayed for the presence of RCC1 using the polyclonal anti-human RCC1 antibodies.

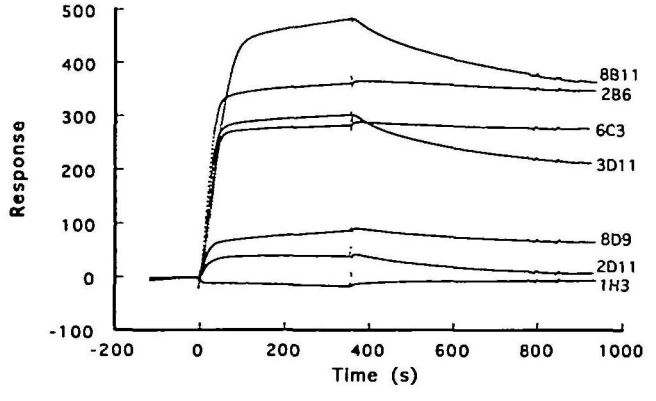

Fig. 5. **Real-time interaction analysis.** Anti-mouse Fcγ antibody was immobilized on the sensor chip and then 10 μg/ml of mAB was trapped on the sensor chip through the anti-mouse Fcγ antibody. Purified recombinant RCC1 (100 nM) was then injected and the binding was calculated as described in "MATERIALS AND METHODS."

protein interaction between mAbs and RCC1 by real-time analysis (*14*). The mABs were immobilized on sensor tips and the binding of RCC1 to these mABs was calculated by BIA evaluation software. As shown in Fig. 5, all of the mABs which immunoprecipitated RCC1, interacted with RCC1. Interestingly, the mAB 8D9 interacted with RCC1, while its response at the equilibrium phase was very low. However, the affinity constants of the mAB 8D9, which were calculated from resonance curves, were comparable

TABLE I. **Kinetic rate constants, affinity constants, and equilibrium response of mABs.** Association (k_a) and dissociation (k_d) rate constants were calculated by the BIA evaluation program as described in "MATERIALS AND METHODS." Req is the equilibrium response obtained from BIA evaluation curve fitting for 100 nM RCC1 used as an analyte.

	k_a ($M^{-1}\cdot s^{-1}$)	k_d (s^{-1})	K_A (M^{-1})	Req
2B6	3.0×10^5	1.1×10^{-4}	2.7×10^9	337
3D11	2.5×10^5	8.4×10^{-4}	3.0×10^8	337
6C3	4.6×10^5	1.3×10^{-4}	3.5×10^9	150
8B11	8.7×10^4	6.4×10^{-4}	1.4×10^8	663
8D9	8.3×10^5	9.9×10^{-4}	8.4×10^8	39.4

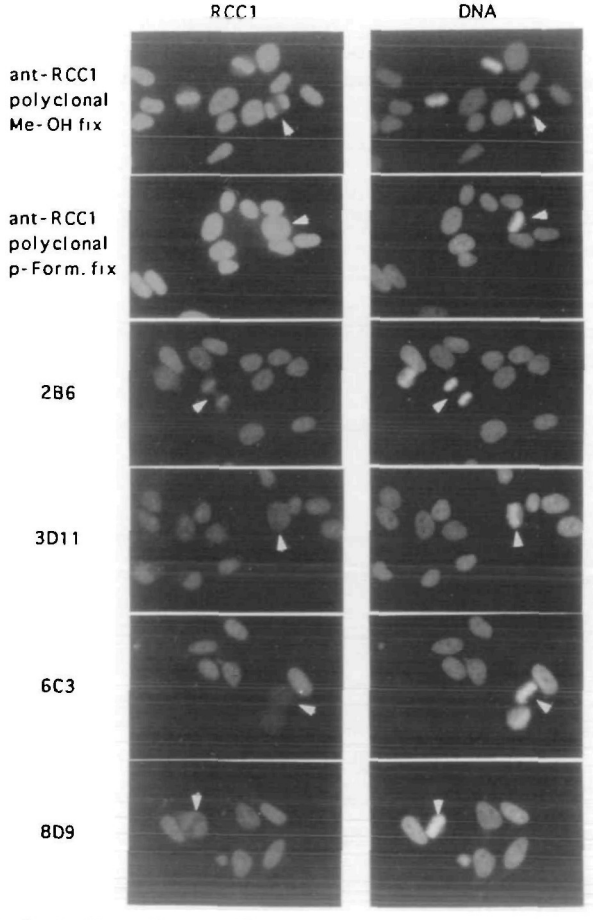

Fig. 6. **Indirect immunofluorescence staining of HeLa cells.** HeLa cells exponentially growing on a coverslip were fixed with cold methanol (Me-OH fix) or by *p*-formaldehyde (p-Form and other samples). Fixed cells were stained with the primary antibodies indicated on the left side of the panel, and then with either rhodamine-conjugated anti-rabbit goat-IgG (anti-RCC1) or FITC-conjugated anti-mouse goat-IgG as described previously (*11*). DNA was visualized with Hoechst 33342 dye.

with those of the mABs, 2B6 and 6C3, which inhibited RCC1 function (Table I). The mAB 8D9 was, therefore, unable to precipitate RCC1 due to its low equilibrium response (Req) value to RCC1. Thus, all of the mABs which inhibited RCC1 function could potentially interact with RCC1 in homogenous solution.

Staining of Cellular RCC1 with mABs—In order to address the question of whether the obtained mABs to *E. coli*-produced recombinant RCC1 recognize endogenous RCC1, exponentially growing HeLa cells were fixed and stained with the mABs to RCC1, then with the secondary antibody as indicated (Fig. 6). All of the mABs to RCC1 which can bind to recombinant RCC1 stained the nucleus of HeLa cells, as previously reported (*11*). Representative results are shown in Fig. 6. The two mABs 1H3 and 2D11, which neither immunoprecipitate RCC1 nor bind to RCC1 in Biosensor Analysis, did not stain the endogenous RCC1 (data not shown). The nuclear staining patterns with the mABs to RCC1 were similar to those obtained with the anti-human RCC1 polyclonal antibodies (Fig. 6 compares anti-RCC1 polyclonal and mABs). In all cases, only the nucleus was stained in interphase cells.

In the case of mitotic cells, the anti-human RCC1 polyclonal antibodies differentially stained RCC1 depending on the fixation method. In formaldehyde-fixed cells, RCC1 in the cytosol of mitotic cells was stained, but in methanol-fixed cells, RCC1 bound to condensed chromosomes was stained. In contrast to polyclonal RCC1-antibodies, RCC1 bound to the condensed chromosome was recognized by the mAB 2B6, but not mABs 3D11, 6C3, and 8D9 (Fig. 6, arrowhead), irrespective of fixation methods.

DISCUSSION

The regions of RCC1 recognized by the mABs are underlined in the amino acid sequence of RCC1 (Fig. 7). It is remarkable that four out of 6 independently isolated mABs recognized the same amino acid residues. Although the two mABs 2B6 and 6C3 recognized the same amino acid residues, they are not identical, since the RCC1-binding abilities of these mABs are not the same (Fig. 5) and furthermore, the staining pattern of endogenous RCC1 differs between 2B6 and 6C3 (Fig. 6). Thus, the fact that four independently isolated mABs recognized the same amino acid residues suggests that firstly, the region around these amino acid residues is exposed at the surface of RCC1. Since one of them, mAB 2D11, does not recognize *E. coli*-produced recombinant RCC1 in homogenous solution or endogenous RCC1, it is, however, unclear whether the amino acid residues Phe91 and Gly92, which were recognized by mAB 2D11, are exposed at the surface of the three-dimensional RCC1 structure. Secondly, the repeated isolation of mABs which recognize the same amino acid residues indicates that our isolation of mABs against *E. coli*-produced recombinant RCC1 was saturated.

Consistent with the finding that the mABs 2B6 and 6C3 inhibit the RCC1 function, Gly92 is mutated to Ser in *S. cerevisiae rcc1⁻ mtr1-2* (*7*). The Gly92 appears throughout the seven RCC1 repeats (*5*) and has been conserved through evolution (*2*). The amino acid sequence of Arg-Ala on the 6th RCC1-repeat which is recognized by the other RCC1-inhibitory mAB 8D9, is also localized next to Gly319, corresponding to Gly92 on the second RCC1 repeat. It is noteworthy that the mABs, 2B6 and 6C3, both of which efficiently bind and precipitate RCC1, can not inhibit more than 60% of the RCC1 function even at high dose, while the mAB 8D9, which binds very weakly to RCC1, almost completely inhibits the RCC1 function at the high dose. These results may reflect structural and functional differences of these regions. Probably, Ran GTPase binds to the sites of RCC1 recognized by the two mABs, 2B6 and 6C3, and the mABs are thereby competing for the binding site with Ran GTPase. In contrast, Ran may not bind, or else may bind only weakly to the site recognized by the mAB 8D9. Based on this assumption, we speculate that the Gly92 region of RCC1 recognized by the mABs 2B6 and 6C3 is involved in binding to Ran. Consistent with this notion, the alanine mutant, Arg109Ala, has a high k_m value for the RCC1-stimulated nucleotide-exchange reaction (*11*).

While the mABs 3D11 and 8B11 do not inhibit RCC1-stimulated nucleotide exchange, they bind and immunoprecipitate RCC1 efficiently. This result is consistent with our previous finding that the N-terminal region outside the RCC1 repeat functions as a nuclear location signal of RCC1, but is not required for complementing tsBN2 mutation (*3*). We assume that the RCC1 repeat comprises the central core of RCC1 and that both the ends outside the repeat are free.

Previously, we found that the histidine residues con-

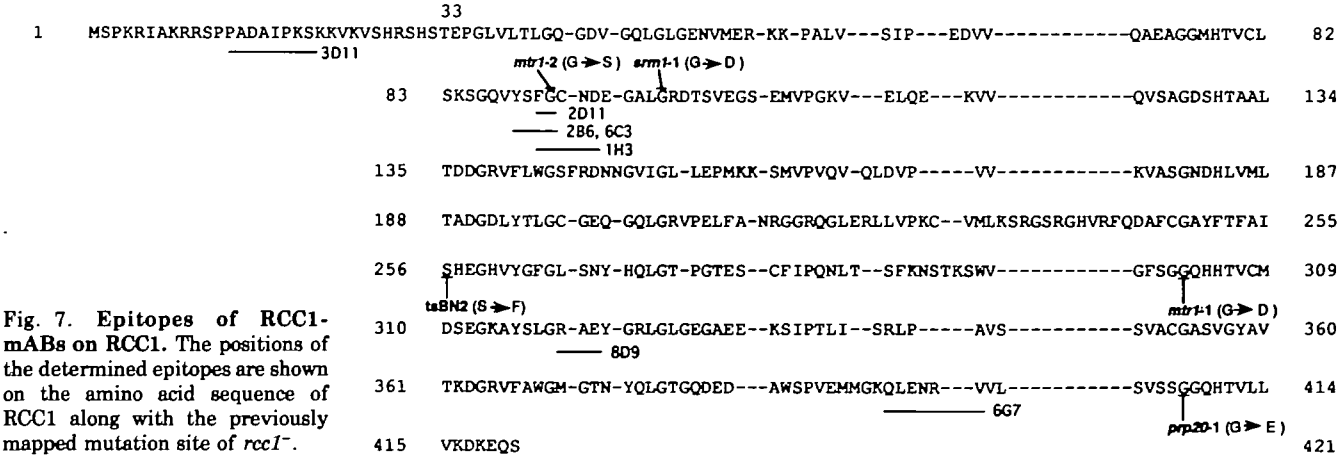

Fig. 7. **Epitopes of RCC1-mABs on RCC1.** The positions of the determined epitopes are shown on the amino acid sequence of RCC1 along with the previously mapped mutation site of *rcc1⁻*.

served in the C-terminal region of the RCC1 repeat are essential for RCC1-stimulated nucleotide exchange. However, none of the residues of these histidines are recognized as epitopes by RCC1 mAB, indicating that these histidine residues are not exposed on the surface of the RCC1 repeat. Probably, the Ran GTPase first binds to the epitopes of the mABs, 2B6 and 6C3, located within the N-terminus of the RCC1 repeat, while subsequently, the three-dimensional structure of RCC1 changes, exposing the histidine residues to react with Ran. To verify this theory, it will be necessary to determine the three-dimensional structure of RCC1.

The English used in this manuscript was revised by Miss K. Miller (Royal English Language Centre, Fukuoka).

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