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Conserved Histidine Residues of RCC1 Are Essential for Nucleotide Exchange on Ran[1]

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Charged amino acid residues of human RCC1 were converted to alanine and mutants which were unable to complement tsBN2 cells (a temperature-sensitive *rcc1*$^-$ mutant of the hamster BHK21 cell line) were selected. These RCC1 mutants were analyzed for the ability to inhibit premature chromatin condensation by microinjection into tsBN2 cells, and their steady-state kinetic parameters for guanine nucleotide exchange reaction were measured. Examined RCC1 mutants were unstable in tsBN2 cells at the restrictive temperature, yet they significantly inhibited premature chromatin condensation. Mutants located on the N-terminus of the RCC1 repeat showed an increased K_m, while their k_{cat} values were comparable to that of wild-type RCC1. In contrast, mutants containing the conserved histidine residues in the C-terminus of the RCC1 repeat showed a value of K_m similar to that of wild-type RCC1, while the k_{cat} values of these mutants were reduced, depending upon the RCC1 repeats on which the mutation was located. These steady-state kinetic parameters of mutants indicate that the N-terminus and the C-terminus of RCC1 repeats play different roles in guanine nucleotide exchange on Ran. The comparison of k_{cat} among the histidine mutants suggests that those histidine residues which are conserved in the RCC1 repeats and also through evolution comprise the catalytic site for the guanine nucleotide exchange reaction.

Key words: alanine scanning, mantGDP, Ran, RanGEF, RCC1.

RCC1 is an abundant chromosomal protein with a molecular mass of 45 kDa possessing seven internal repeats consisting of about 60 amino acid residues (*1, 2*). It was originally identified as a protein encoded by the human gene complementing defects of the temperature-sensitive (ts) mutant, tsBN2, of the BHK21 cell line derived from the golden hamster (*3, 4*), which shows either G_1 arrest, or premature chromatin condensation (PCC) from S phase onward (*5*). By use of anti-kinetochore autoimmune sera, human RCC1 was purified from HeLa cells (*6*). Genes homologous to RCC1 were cloned from *Drosophila* using a monoclonal antibody raised against a nuclear protein (*7*), and from *Saccharomyces cerevisiae* as a mutated gene of three independently isolated ts mutants, *srm1* (*8*), *prp20* (*9*), and *mtr1* (*10*), and also as a cell-cycle gene, *pim1*$^+$, from *S. pombe* (*11, 12*). All of these genes encode a protein possessing the putative RCC1 repeat. Human *RCC1* can complement both *srm1* and *prp20* mutations (*13*) and *vice versa* (*14, 15*). In addition, our recent results have shown that the *pim1*$^+$ gene complemented *srm1*, *prp20*, and *mtr1* mutations (Seino, unpublished results). Thus, RCC1 has been structurally and functionally well conserved through evolution.

RCC1 functions as a guanine nucleotide exchange factor (GEF) for Ran/TC4, a Ras-like nuclear small G protein (*16*) which is essential for the nuclear import of NLS-fused proteins (*17*) and also for mRNA export (*18*). In fact, *mtr1* has been isolated as a mutant defective in mRNA export (*10*) and the *rna1*, a ts mutant of Ran GTPase-activating protein (RanGAP), shows a defect in mRNA export similar to that of *prp20* which has been isolated as a mutant defective in mRNA splicing (*9, 19*). Furthermore, in tsBN2 cells, both nuclear import of proteins and mRNA export are defective at 39.5°C, the restrictive temperature (*20*), On the other hand, there have been several reports indicating that the RCC1-Ran pathway is involved in cell-cycle regulation. For instance, *srm1* causes G_1 arrest and *pim1*$^-$ is defective in chromosome decondensation (*8, 11, 12*). In human cells, Ran locked in a GTP form arrests the cell cycle in G2 (*21*). Furthermore, the finding that loss of RCC1 function causes the nuclear transport of cdc25C in tsBN2 cells (*22*) is in conflict with the finding that GTP-Ran is essential for nuclear transport (*17*), since GDP-Ran may accumulate through loss of RCC1 function.

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Abbreviations: GEF, guanine nucleotide exchanging factor; PCC, premature chromatin condensation.

Like the Ras superfamily, Ran may function as a biological switch, transferring upstream signals to downstream events (*23*). In this context, it is noteworthy that RCC1 is located on the chromatin. RCC1 may check the cell cycle progression on the chromatin, and transfer those signals to downstream events through Ran/TC4. If so, RCC1 may have at least two functional domains: one to act as a sensor for cell-cycle progression, and another to act as GEF on Ran/TC4. In order to investigate systematically the functional domain of RCC1, we introduced a mutation into RCC1 according to the alanine-scanning method (*24*). Prepared alanine mutants of RCC1 were examined for the ability to complement tsBN2-mutation and then assayed for GEF activity on Ran. We found that histidine residues of the RCC1 repeat which have been conserved through evolution and which lie among five RCC1 repeats, are important for the guanine nucleotide exchange activity of RCC1.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—The tsBN2 mutant of the BHK21/13 cell line from the golden hamster (*25*) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, and was maintained in a humidified atmosphere containing 10% CO_2 at 33.5°C. As the nonpermissive temperature, 39.5 or 40.5°C was used. Asynchronously growing cells were arrested at early S phase, as described (*5*).

In Vitro Mutagenesis of RCC1 cDNA—A 1.4 kbp *Pst*I fragment of pcD51 (*1*) was subcloned into the *Pst*I site of pUC118. At the 1st Met codon of the resultant plasmid, an *Nde*I site was introduced using the *in vitro* mutagenesis system (Amersham) and then a 1.3 kbp *Hgi*AI-*Pst*I fragment of the *RCC1*-coding region was subcloned into the *Pst*I site of a mammalian expression vector, pcD-SRα 296. A 2.9 kbp *Hin*dIII-*Sal*I fragment of pcD-SRα-296-RCC1^{wt} was recloned into the *Hin*dIII-*Sal*I site of pUC118, and then the indicated amino acid residues (shown by bold letters in Fig. 1) were changed to alanine by oligo-DNA-mediated site-directed mutagenesis (*26*). In order to express mutated *RCC1* in *Escherichia coli*, a 1.6 kbp *Nde*I-*Bam*HI fragment of *RCC1* cDNA was excised and subcloned into the *Nde*I-*Bam*HI site of pET3b.

Transformation of tsBN2 Cells—tsBN2 cells suspended in 400 μl of saline G (*5*) buffer at a concentration of 1×10^7 cells/ml were mixed with 2 μg of mutated *RCC1* cDNA carried on the pcD-SRα 296 vector and 0.2 μg of pSV2 neo DNA, exposed to a single electric pulse with a strength of 220 V and a capacitance of 330 μF using a Cell Electroporator™ (BRL) and then plated in the DMEM medium containing 10% fetal calf serum. After incubation at 33.5°C for 2 days, transfected cells were either cultured at 39.5°C, or else were given G418 (final concentration of 800 μg/ml) at 33.5°C. Two weeks later, cells were fixed and stained to enable the numbers of colonies to be counted, as described (*27*).

Purification of RCC1 Proteins—Wild-type and mutated *RCC1* cDNAs were cut from a vector, pcD-SRα296, introduced into a bacterial expression vector, pET3b, and then expressed in *E. coli* BL21(DE3) as described (*28*). *E. coli* cells producing RCC1 were suspended in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 4 mM EDTA, 1 mM DTT, 1 mM *p*-APMSF, treated with 0.4 mg/ml of lysozyme, and then sonicated in the presence of $MgCl_2$, CHAPS, and DNase I [final concentration, 15 mM, 1% (v/v), and 40 μg/ml, respectively). The cell lysate was centrifuged at 75,000 × *g* for 60 min and the supernatant was applied to a SP-Sepharose column (Pharmacia) equilibrated with buffer containing 25 mM HEPES-NaOH, pH 7.8, 1 mM DTT plus 150 mM NaCl. The column was washed, then RCC1 proteins were eluted with 500 mM NaCl. The fraction was dialyzed and applied to a MonoS HR5/5 FPLC column (Pharmacia). Fractions containing RCC1 were pooled, diluted with buffer containing 20 mM sodium phosphate, pH 7.8, 2 mM DTT, and then subjected to FPLC on a MonoQ HR5/5 column equilibrated with the same buffer plus 25 mM NaCl. Proteins were eluted from the MonoQ column using a linear gradient of 25-350 mM NaCl at the flow rate of 1 ml/min. Eluted proteins were concentrated to 3 mg/ml in a Millipore centrifugal concentrator (Ultra Free, CL), then frozen in liquid nitrogen and stored at −80°C.

Microinjection of RCC1—tsBN2 cells were plated at 1×10^5 cells on 18 × 18 mm cover glasses in 35-mm dishes and arrested with 2.5 mM hydroxyurea (HU) as described (*5*). RCC1 proteins diluted with PBS to the indicated concentration were injected into the cytosol with 0.2 mg/ml of nonspecific mouse IgG (Cappel), as described before (*28*). After microinjection, cells were incubated at 40.5°C for the indicated periods and then fixed with cold (−20°C) methanol for staining. The frequency of PCC inhibition was estimated as follows: {(Frequency of PCC in uninjected cells) − (Frequency of PCC in injected cells)}/(Frequency of PCC in uninjected cells) × 100.

Indirect Immunofluorescence—Microinjected cells were fixed and stained as described previously (*28*), with an anti-RCC1 antibody as the primary antibody (*2*), and a Texas-Red-conjugated goat anti-rabbit IgG antibody (Amersham) as the secondary antibody. Cells were then stained with an FITC-conjugated goat anti-mouse IgG antibody (TAGO, La Jolla, CA). DNA was visualized with 1 μg/ml of Hoechst 33342 (Calbiochem, San Diego, CA). As the primary antibody, anti RCC1 peptide antibody prepared by Ohtsubo *et al.* (*2*) and anti human RCC1 antibody (gift from Masafumi Nakamura) were used to detect the microinjected and the endogenous RCC1 protein, respectively. Photomicroscopy was performed using an Axiophot (Zeiss, Germany).

Analysis of Guanine Nucleotide-Exchange Reaction on Ran—Ran was purified according to the method described by Dasso *et al.* (*28*). A fluorescent GDP analogue, 2′,3′-bis-*O*-(*N*-methylanthraniloyl)guanosine diphosphate (mantGDP), was prepared as described (*29*) and was bound to Ran/TC4, as previously described (*28*). Ran/TC4-mantGDP was preincubated in reaction buffer containing 30 mM potassium phosphate, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, and GDP (two-hundred-fold excess of Ran/TC4-mantGDP in mol per mol ratio) at 25°C for 1 min, and the reaction was started by the addition of RCC1. The intensity of fluorescence emission at 450 nm, excited at 355 nm, was measured in the time drive mode of a fluorescence photometer, LS50B (Perkin-Elmer).

RESULTS

Systematic Mutagenesis of RCC1 Protein—Previously, we found that the N-terminus of RCC1 outside the RCC1 repeat is required for nuclear import of RCC1, but it is not essential for complementing tsBN2 mutation (*30*). In order to identify the functional domain of RCC1, therefore, we replaced the amino acid residues of the repeated domain with alanine, as described in "MATERIALS AND METHODS." As shown in Fig. 1, the charged amino acid residues that are likely to lie on the solvent-accessible surface of a protein were chosen as the targets for mutagenesis, in the expectation that such a mutation may abrogate the interactions of RCC1 with other factors.

For comparison, the mapped mutation sites of yeast RCC1 homologues are also shown in Fig. 1, comprising the sites of *S. cerevisiae mtr1-1, mtr1-2, srm1-1*, and *prp20-1* (*10, 31*) and of tsBN2 mutation (*4*). All of these mutations are temperature-sensitive, and except for the tsBN2 mutation, they are all located on the glycine residue conserved in the RCC1 repeat. In contrast to those mutations, the mutations introduced here may help to identify other functional aspects of RCC1 protein.

Transformation of tsBN2 cells by Mutated RCC1—Mutated human *RCC1* cDNAs were co-transfected into tsBN2 cells with pSV2 neo by an electric pulse method (*27*). The frequency of gene-transfer was estimated by counting the number of neo^r colonies. This method was used to normalize the ability of mutated *RCC1* cDNA to complement tsBN2 mutation. In Table I, the ts$^+$ transformation-efficiency of each mutant (the number of ts$^+$ colonies/the number of neo^r colonies) is given as a ratio to that of wild-type *RCC1*.

RCC1 mutants could be roughly divided into three groups based on their ability to complement tsBN2 mutation: (a) comparable to wild-type *RCC1* (>0.6), (b) able to complement tsBN2 mutation but with a low efficiency (0.3 to 0.1), and (c) incapable of complementing tsBN2 mutation (<0.1) (Table I). It is notable that, when one of the histidine residues near the C-terminus of the RCC1 repeat (Fig. 1) was replaced with an alanine residue, *RCC1* cDNA lost its ability to complement tsBN2 mutation. These histidine residues are conserved from yeast to humans among the RCC1-repeats (Fig. 7). In comparison with the mutation sites of *S. cerevisiae* RCC1 homologues, it is also notable that one of the non-complementing mutants, R101 is located near the sites of *srm1-1* and *mtr1-2*, which are not suppressed by overproduction of *S. cerevisiae* Ran homologue, GSP1/CNR1 (*10, 31*).

We chose non-complementing mutants in order to investigate further the functional domain of RCC1. In addition, the mutant D44 was selected to compare its activity with that of R101, since both are located near the N-terminus of the RCC1 repeat (Fig. 1).

Inhibition of PCC by Microinjection—Since it takes about two weeks to obtain clear ts$^+$ transformed colonies of tsBN2 cells, the ability of RCC1 mutants to complement tsBN2 cells may depend on their steady-state expression and the stability of mutated proteins in tsBN2 cells. On the other hand, tsBN2 cells arrested with hydroxyurea entered mitosis at the nonpermissive temperature within 4 h, resulting in premature chromatin condensation (PCC) (*5*). Such PCC was inhibited in a dose-dependent manner when wild-type RCC1 was injected into tsBN2 cells prior to an upward shift in the temperature (*32*). Compared to the case of complementation analysis, therefore, we could avoid ambiguity concerning the expression and stability of mutated proteins, by measuring the ability of RCC1 mutants to inhibit PCC after microinjection, so that the physiological activity of RCC1 mutants could be more precisely estimated.

In order to re-examine the physiological activity of RCC1 mutants by microinjection, RCC1 mutants which did not complement tsBN2 mutation were produced in *E. coli* and were highly purified, as shown in Fig. 2. The mutant D136

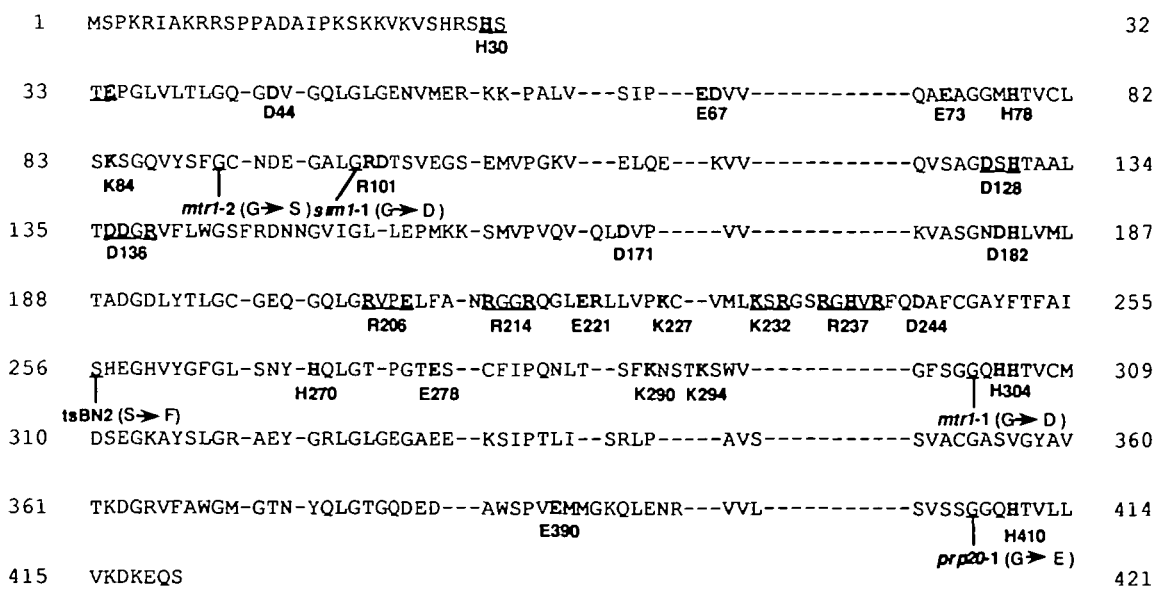

Fig. 1. **Designed mutations of RCC1 protein.** The primary structure of human RCC1 protein is aligned with the region of the putative seven RCC1 repeats. Bold letters indicate individual amino acids which were changed to alanine while the underlined letters indicate the clusters which were changed together. The mutation sites of RCC1 homologues which were mapped genetically, are also shown.

was not well expressed in *E. coli* and so could not be purified. All the other proteins were purified to better than 95% homogeneity. Throughout the purification steps, mutated proteins were purified in the same manner as wild-type proteins, indicating that these RCC1 mutants retain the basic chromatographic properties of wild-type RCC1. Consistently, these mutated RCC1 proteins formed a stable complex with Ran/TC4 in the presence of EDTA, *in vitro*, as previously described (data not shown) (*33*).

Purified RCC1 mutants were injected into tsBN2 cells arrested with hydroxyurea. Without microinjection, the number of cells showing PCC reached a plateau after incubation at 40.5°C for 5 h (Fig. 3c). Although a gradual increment in the number of cells showing PCC was observed even after incubation for 5 h, this may be due to the presence of unsynchronized cells in synchronized cultures, so that it does not reflect the real kinetics of PCC induction. Thus, we chose 5 h as the time point to estimate the frequency of cells showing PCC, which was measured in the manner previously described (*32*). Most of the RCC1 mutants which did not complement tsBN2 cells efficiently inhibited PCC, depending on the dose of injected proteins (Fig. 3a).

Injected cells were stained with human RCC1-specific antibody. Surprisingly, after incubation for 5 h at 40.5°C, the injected RCC1 mutants which did not complement tsBN2 mutation were not detectable in tsBN2 cells injected at the dose of 0.6 mg/ml of injection, at which concentration the mutated RCC1 proteins (except R101) efficiently inhibited PCC (Fig. 3b, comparison between 33.5 and 40.5°C). In contrast, D44, which complemented tsBN2 mutation albeit with low efficiency (Table I), was detected in injected cells at both temperatures after incubation for 5 h (Fig. 3b). All RCC1 mutants unable to complement tsBN2 mutation, therefore, were unstable at the restrictive temperature.

In order to investigate further the correlation between the stability of RCC1 proteins and the PCC inhibition ability, we injected RCC1 mutants (R101, H410, and D44) into tsBN2 cells arrested with hydroxyurea, and then incubated the cells at 40.5°C. As shown in Fig. 3, c and d, the frequency of cells showing PCC was examined every hour and injected cells were stained every 30 min. As previously reported (*5*), after 1.5 h incubation at 40.5°C, endogenous RCC1 of tsBN2 cells became undetectable by immunofluorescence staining. R101 and H410 became undetectable after 1.5 and 2.5 h incubation at 40.5°C, respectively (Fig. 3d). On the other hand, while in tsBN2 cells without injection the frequency of cells showing PCC started to increase after 2 h incubation at 40.5°C (Fig. 3c) as reported (*5*), PCC induction was delayed in tsBN2 cells injected with R101 and H410 cells. After 5 h incubation, the frequency of cells showing PCC was very low in tsBN2 cells injected with H410, as shown in Fig. 3, a and c, while tsBN2 cells injected with R101 started to show PCC after 3 h incubation at 40.5°C. In tsBN2 cells injected with D44, which was stable at 40.5°C, no PCC appeared for at least 8 h at 40.5°C (Fig. 3c). Thus, PCC induction in injected cells was correlated with the loss of injected proteins.

Kinetic Analysis of Guanine Nucleotide-Release Activity—In order to examine the relationship between the structure of RCC1 and its GEF activity on Ran, we performed steady-state reaction analysis of GEF activity on

TABLE I. **Relative ability of mutated RCC1 to complement tsBN2 mutation.** Two micrograms of wild-type (wt) or mutated *RCC1* cDNA was transfected into 4×10^6 cells of tsBN2 with 0.2 μg of pSV2 neo DNA (see "MATERIALS AND METHODS"). The efficiency of ts^+ transformation was normalized as the number of colonies formed at the nonpermissive temperature (39.5°C) divided by the number of neomycin-resistant (neo^r) colonies, and the ratio of the efficiency obtained for RCC1 mutants to that of wild-type RCC1 is shown in this table. The numbers of ts^+ colonies were 1.1×10^4 in the case of wt and 4×10 in the case of H410 mutation, with no ts^+ colonies in the cases of R101, H78, D128, D136, and D182. The numbers of neo^r colonies were 3×10^2 (wt), 3.6×10^2 (H410), 3.5×10^2 (R101), 5.5×10^2 (H78), 5.3×10^2 (D128), 5.7×10^2 (D136), and 3.5×10^2 (D182).

Group	Mutated RCC1	Relative efficiency of ts^+ transformation
a)	WT	1.0
	H30	0.9
	E67	1.2
	E73	0.9
	K84	1.1
	D171	0.7
	R214	0.8
	E221	1.0
	K227	0.6
	K232	1.3
	R237	1.6
	H270	0.8
	E278	0.8
	K290	0.7
	K294	2.7
	E390	2.2
b)	D44	0.2
	E119	0.3
	R206	0.1
	D244	0.1
c)	H78	$<10^{-4}$
	R101	$<10^{-4}$
	D128	$<10^{-4}$
	D136	$<10^{-4}$
	D182	$<10^{-4}$
	H304	$<10^{-4}$
	H410	3×10^{-3}

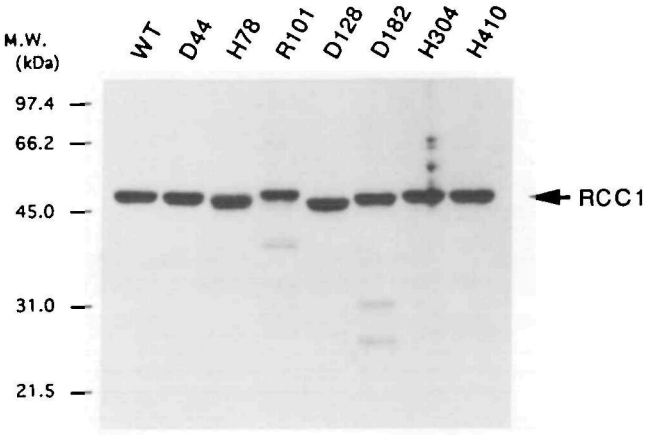

Fig. 2. **SDS-polyacrylamide gel electrophoretic analysis of purified RCC1 and its mutants.** About 1 μg of wild-type (wt) RCC1 and 1 μg of the indicated RCC1 mutants were electrophoresed on SDS-polyacrylamide (11%) gels and stained with Coomassie Brilliant Blue. The arrow indicates the position of wild-type RCC1 and its mutants. The amounts of RCC1 proteins in all the samples were estimated densitometrically using BSA as a standard.

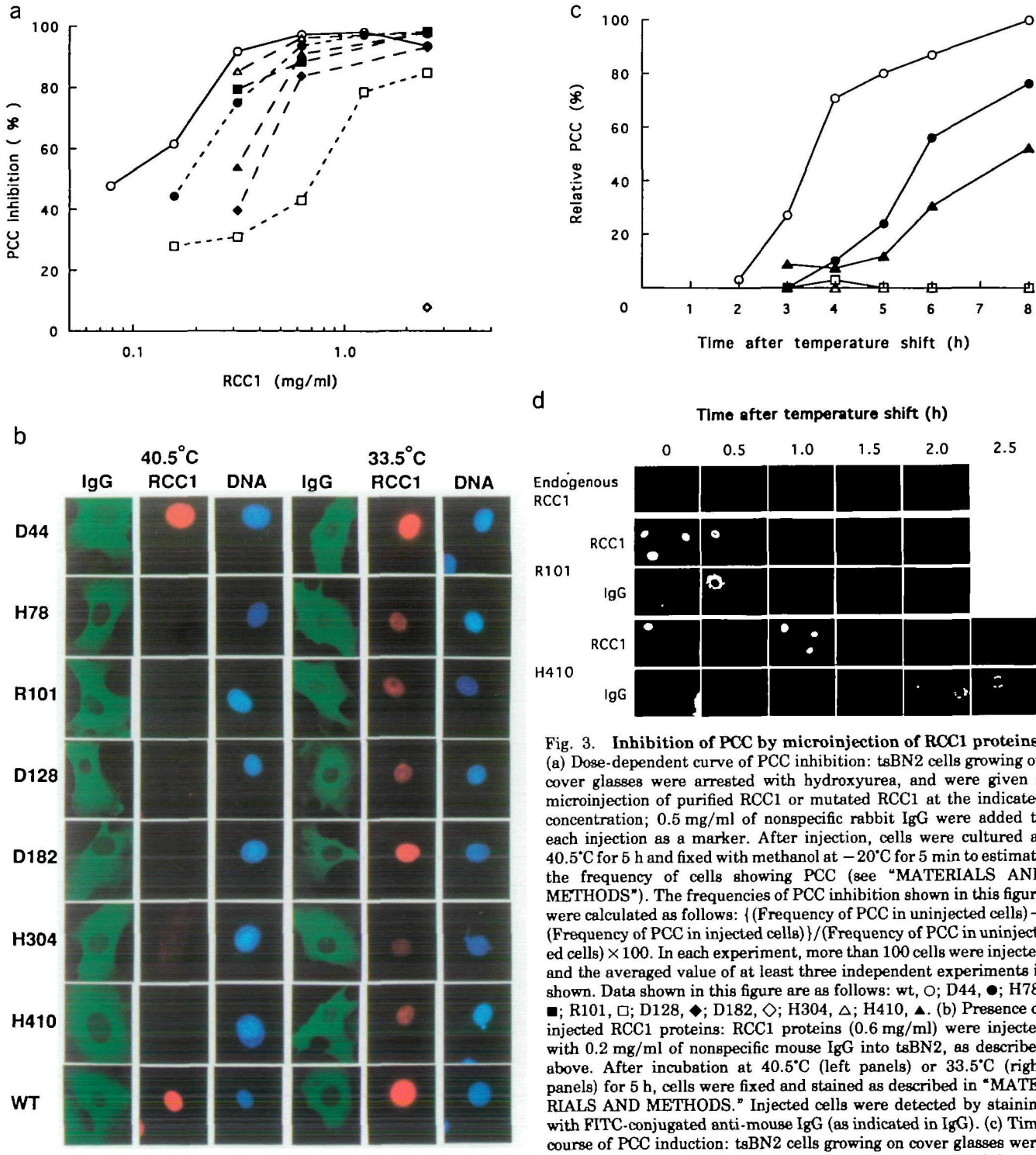

Fig. 3. **Inhibition of PCC by microinjection of RCC1 proteins.** (a) Dose-dependent curve of PCC inhibition: tsBN2 cells growing on cover glasses were arrested with hydroxyurea, and were given a microinjection of purified RCC1 or mutated RCC1 at the indicated concentration; 0.5 mg/ml of nonspecific rabbit IgG were added to each injection as a marker. After injection, cells were cultured at 40.5°C for 5 h and fixed with methanol at −20°C for 5 min to estimate the frequency of cells showing PCC (see "MATERIALS AND METHODS"). The frequencies of PCC inhibition shown in this figure were calculated as follows: {(Frequency of PCC in uninjected cells) − (Frequency of PCC in injected cells)}/(Frequency of PCC in uninjected cells) × 100. In each experiment, more than 100 cells were injected and the averaged value of at least three independent experiments is shown. Data shown in this figure are as follows: wt, ○; D44, ●; H78, ■; R101, □; D128, ◆; D182, ◇; H304, △; H410, ▲. (b) Presence of injected RCC1 proteins: RCC1 proteins (0.6 mg/ml) were injected with 0.2 mg/ml of nonspecific mouse IgG into tsBN2, as described above. After incubation at 40.5°C (left panels) or 33.5°C (right panels) for 5 h, cells were fixed and stained as described in "MATERIALS AND METHODS." Injected cells were detected by staining with FITC-conjugated anti-mouse IgG (as indicated in IgG). (c) Time course of PCC induction: tsBN2 cells growing on cover glasses were arrested with hydroxyurea, and were given a microinjection of RCC1-mutated proteins; wt (△), D44 (□), R101 (●), and H410 (▲), at a concentration of 0.6 mg/ml. As a control, one series of tsBN2 cultures was not injected (○). At the indicated time, cells were fixed and stained to enable cells showing PCC to be counted. (d) Disappearance of endogenous and injected RCC1 proteins: tsBN2 cells which were given a microinjection of RCC1-mutated proteins as in (c) were incubated for indicated periods at 40.5°C. Injected and an endogenous RCC1 was stained using anti human RCC1-peptide and anti human RCC1 polyclonal antibody, respectively as described in "MATERIALS AND METHODS."

Ran to calculate the kinetic parameters (K_m and k_{cat}) of each of the RCC1 mutants (Table II). Klebe *et al.* (*34*) have shown that fluorescent mant-GDP (mantGDP) can be used for measuring RCC1-mediated guanine nucleotide release from Ran/TC4. Using this method, they have found that the GEF activity of a bacterially produced RCC1 is comparable to that of RCC1 purified from HeLa cells. Since their method is suitable for measuring the time course of nucleotide release, we used Ran/TC4 labeled with the mantGDP as a substrate and examined the GEF activity of

TABLE II. **Michaelis-Menten kinetics parameters of the exchange of Ran-GDP with free GDP in the presence of RCC1 mutants.** All the constants were calculated based on the dissociation of mantGDP, as described in "MATERIALS AND METHODS."

	K_m (10^{-6} M)	k_{cat} (s^{-1})	k_{cat}/K_m (10^{-6} $M^{-1}\cdot s^{-1}$)
WT	2.2	19.1	8.5
D44	12.0	15.7	1.3
H78	2.5	8.7	3.5
R101	5.6	15.3	2.7
D128	3.1	0.7	0.2
H304	2.1	2.1	1.0
H410	1.9	9.9	5.1

selected RCC1 mutants. As the standard temperature for calculating the GEF activity of RCC1, we adopted 25°C degrees. Representative results of the reaction carried out by wild-type and mutated RCC1 proteins are shown in Fig. 4. D182 mutation caused a severe loss of GEF activity and so we could not estimate the kinetic parameters of this mutated protein.

The initial velocities of reactions were calculated by linear fitting based on the relative fluorescence intensity obtained between zero time and the time when 10% of the initial mantGDP fluorescence was lost after the addition of RCC1. Using either a constant or an increasing concentration of Ran, the rate constants of guanine nucleotide release were plotted in a double reciprocal Lineweaver-Burk plot (Fig. 5). All the data could be fitted to the linearized equation, and so they were used to calculate the Michaelis-Menten parameters (Table II). In all cases, the correlation coefficient to the Michaelis-Menten equation was greater than 0.97 (Fig. 5, insert).

In the case of wild-type RCC1, the K_m was 2.2 μM while the k_{cat} was 19.1 s^{-1}. Although K_m was similar, k_{cat} was higher than reported earlier based on a non-linear fitting of the data (*35*). These results support a recent detailed kinetic analysis of the RCC1-catalyzed GEF reaction, where the rate-limiting step was found to have a rate constant of 19-21 s^{-1}, depending on the nucleotide (*36*). The K_m was increased in both D44 and R101, with mutations located near the N-terminus of the RCC1 repeat (Fig. 1), while the k_{cat} of these mutants was comparable with that of wild-type RCC1 (Fig. 6). On the other hand, all the mutations containing one of the conserved histidine residues located near the C-terminus of the RCC1 repeat (Fig. 1) showed no change in K_m values compared to wild-type RCC1, but their k_{cat} values were significantly reduced by the mutation. These findings may suggest that the mutation in the N-terminus of the RCC1 repeat affects the association of RCC1 with Ran/TC4 and that histidine residues near the C-terminus of the RCC1 repeat are required for the dissociation of the ternary complex of Ran/TC4-GDP/GTP•RCC1, which is the rate-limiting step for RCC1-stimulated guanine nucleotide release (*36*).

We noticed that H78 and H410, which were located on the first and the seventh RCC1 repeats (Figs. 1 and 7), respectively, had comparable k_{cat} values, about 40 to 50% of that of wild-type RCC1, and another pair of mutants, D128 and H304 which were located on the second and the fifth RCC1 repeats, respectively, had similar k_{cat} values of less than 10% of that of wild-type RCC1. Furthermore, D182, which was located on the third RCC1 repeat, had

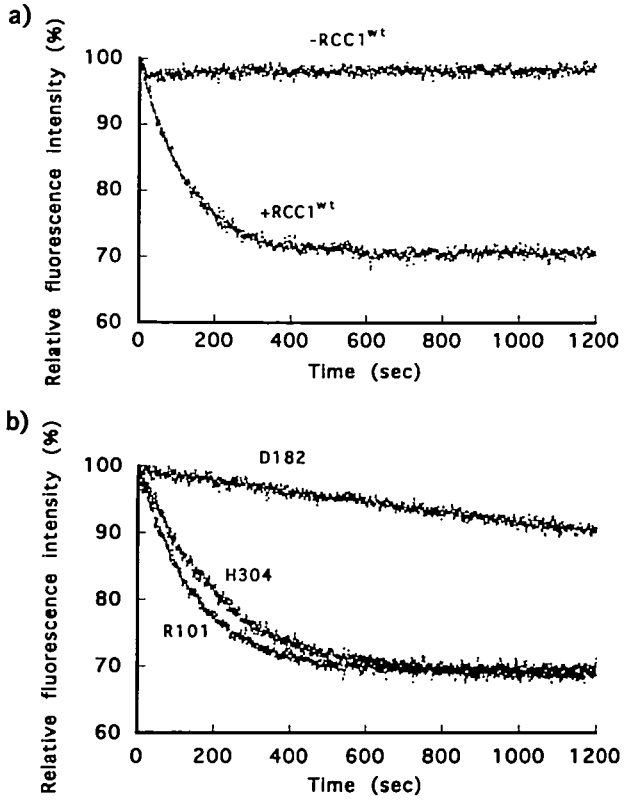

Fig. 4. **Kinetics of the GDP (mantGDP) dissociation from Ran/TC4.** Purified RCC1 proteins were mixed with various concentrations of mantGDP-Ran/TC4 and the reaction mixtures were incubated at 25°C. The intensity of fluorescence emission at 450 nm, excited at 355 nm, was measured every second (see "MATERIALS AND METHODS"). (a) with (+) or without (−) 5 nM wild-type RCC1. (b) 5 nM, R101; 25 nM, D182; and 25 nM, H304, as an enzyme, and 4 μM Ran/TC4-mantGDP as a substrate in the presence of a two-hundred-fold excess of free GDP with respect to the concentration of mantGDP-Ran/TC4 used.

very low GEF activity, if any (Fig. 4). Thus, the GEF activity of these histidine mutants seems to be reduced in a stepwise manner from both ends; H78 and H410 to the center, D182.

DISCUSSION

In this work, we prepared a series of alanine mutants of RCC1 and assayed them for the ability to complement tsBN2 mutation. Mutants which did not complement tsBN2 mutation were further assayed for the ability to inhibit PCC induction of tsBN2 cells and for stability, by microinjection into tsBN2 cells. On the other hand, the GEF activity of these RCC1 mutants was analyzed by equilibrium kinetic measurements using fluorescent nucleotide. Since Ran and RCC1 interact with each other in homogenous solution, the GEF activity of RCC1 on Ran could be considered as a general enzyme reaction (*36*).

All RCC1 mutants which did not complement tsBN2 mutation turned out to be unstable at the restrictive temperature. In accordance with this, D44, which complemented tsBN2 mutation, was stable at 40.5°C, although its GEF activity (ratio of k_{cat}/K_m) is quite low compared to those of other noncomplementing RCC1 mutants. Thus,

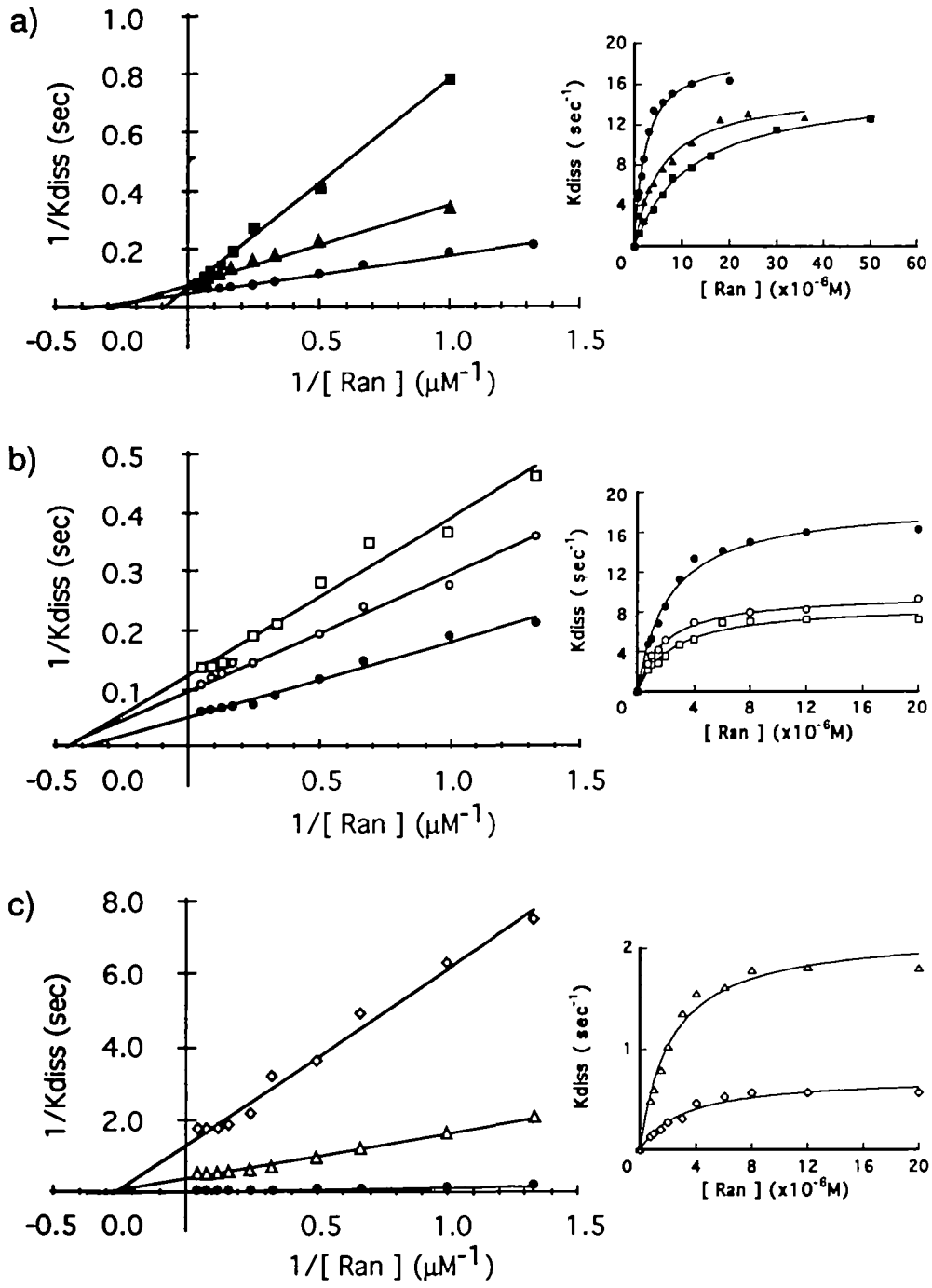

Fig. 5. **Lineweaver-Burk plots for guanine nucleotide-exchange reaction carried out by wild-type and mutated RCC1 proteins.** The initial velocity of reaction was estimated by linear fitting between zero-time and the time when 10% of the reaction had been completed for each reaction, and this was then divided by the concentration of RCC1 to estimate the dissociation coefficient (K_{diss}). These coefficients were then plotted based on the double reciprocal Lineweaver-Burk plot. In the insert, calculated K_{diss} values were plotted against the substrate concentration and fitted to the Michaelis-Menten equation to give the K_m and k_{cat}. These linear and curvilinear fittings were achieved using the Kaleida Graph program on a Macintosh computer. The concentration of RCC1 protein in each reaction was 5 nM for wild-type RCC1, and RCC1 mutants: D44, H78, R101, and H410, and 25 nM for RCC1 mutants: D128 and H304. (a) wt: wild-type RCC1, ●; D44, ■; R101, ▲. (b) wt: wild-type RCC1, ●; H78, □; H410, ○. (c) wt: wild-type RCC1, ●; D128, ◇; H304, △. WT is not shown in the [s] *versus* K_{diss} graph of the insert because of its high value compared to the value of D128 and H304.

stability of the mutated RCC1 proteins is essential to complement tsBN2 mutation. In addition, GEF activity is required. The lower complementation efficiency of D44 may reflect the requirement for GEF activity on Ran to complement tsBN2 mutation. The GEF activity of other RCC1 mutants which complement tsBN2 mutation with low efficiency (group b in Table I) remains to be investigated to confirm the relationship between the ability to complement tsBN2 mutation and the GEF activity on Ran.

The stability of RCC1 mutants is apparently correlated with PCC induction, since after prolonged incubation at 40.5°C, PCC was induced in injected cells depending on the stability of the RCC1 mutants. It is remarkable, however, that after 5 h incubation, when injected RCC1 mutants were no longer detectable, injected tsBN2 cells did not show PCC. H78 and H304 inhibited PCC with a similar or slightly higher efficiency compared to D44, which is stable at 40.5°C. Thus, the frequency of PCC examined after 5 h incubation does not solely depend on the stability of mutants. This is also true in the case of R101, which showed delayed PCC induction (Fig. 3c) while both R101 and the endogenous RCC1 became undetectable after 1.5 h incubation at 40.5°C. Probably the delay of PCC induction in tsBN2 cells injected with R101 reflects a residual enzyme

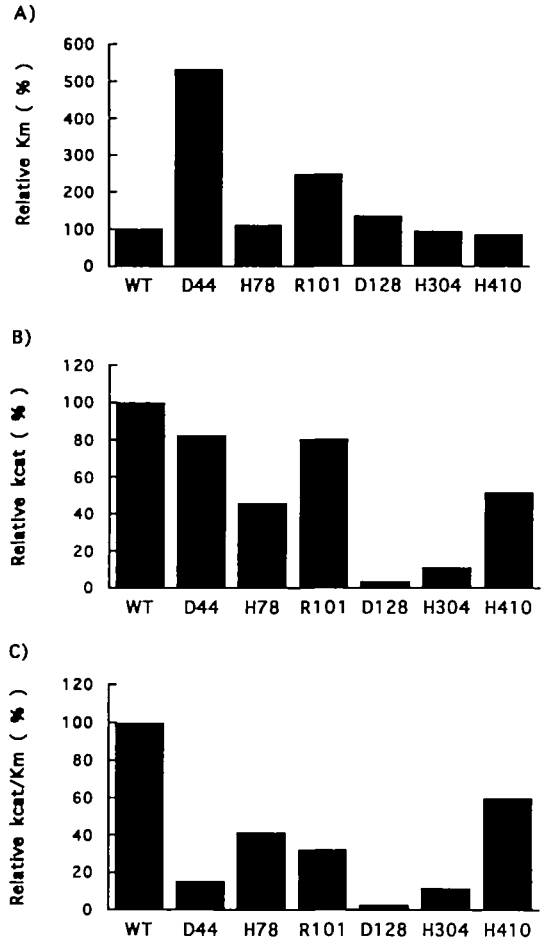

Fig. 6. **Comparison of steady-state kinetic parameters for wild-type and mutated RCC1 proteins.** The obtained kinetic parameters (K_m, k_{cat}, and K_m/k_{cat}) were normalized against the values of wild-type RCC1 protein. The relative K_m values of GEF reaction of each of the wild-type and mutated RCC1 proteins are presented in (A), the relative k_{cat} values of GEF reaction are presented in (B), and the relative K_m/k_{cat} values are shown in (C).

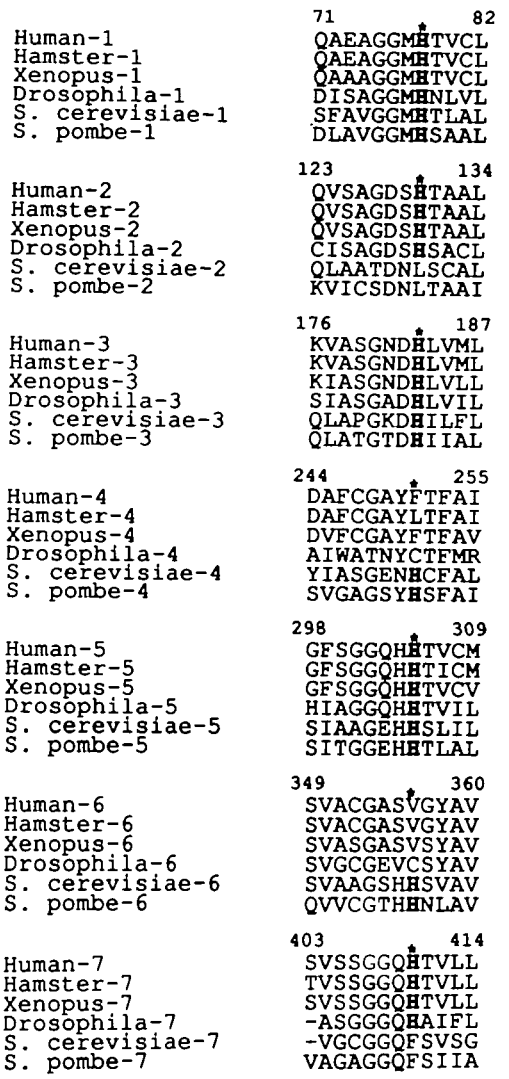

Fig. 7. **Histidines conserved in the RCC1 repeat through evolution.** Bold letters indicate conserved histidine residues located in the C-terminus of the RCC1 repeat. The number corresponds to the position of amino acid residue in human RCC1 protein. The sequences of RCC1 homologues are referred to as follows: human (*1*), hamster (*4*), *Xenopus* (*27*), *Drosophila* (*7*), *S. cerevisiae* (*8*), and *S. pombe* (*11*).

activity of R101. The GEF activity on Ran was rapidly abolished in the extracts of tsBN2 cells at 39.5°C (*37*) while a considerable amount of the GEF activity of R101 remains in our *in vitro* assay at 40.5°C (Azuma, unpublished data).

Among the examined RCC1 mutants, the GEF activities of D128, H304, and D44 are very low compared to those of the other mutants. Even so, at the dose of 0.6 mg/ml of injection, D128 inhibited PCC in 80% of injected tsBN2 cells. Thus, the GEF activity required to inhibit PCC induction seems to be low. The minimum requirement of GEF activity to inhibit PCC induction could be the GEF activity of D44, which is stable and inhibits PCC for a long period. The exact relationship between the GEF activity of RCC1 and the ability to inhibit PCC induction remains to be further investigated by using stable mutants.

Except for R101, mutants which cannot complement tsBN2 mutation were located on one of five histidine residues near the C-terminus of the RCC1 repeat. These histidine residues have been well conserved through evolution and lie among five RCC1 repeats of each species, as shown in Fig. 7. While all these histidine mutants have a K_m similar to that of wild-type RCC1, their k_{cat} values are low compared to that of wild-type RCC1 and vary depending upon the site of mutation. The k_{cat} is considered to represent the overall reaction rate. In the case of the RCC1-Ran/TC4 pathway, the rate-limiting step is most likely the dissociation of the ternary complex (*36*). The lower value of k_{cat}, therefore, may suggest that these histidine residues are required for dissociation of GDP from the ternary complex Ran/TC4-GDP/GTP·RCC1. We noticed that the k_{cat} of histidine mutants decreases depending on the position of mutation among the RCC1 repeats. Such a stepwise reduction of the GEF activity may suggest that these five histidine residues co-operate for the GEF reaction of RCC1 on Ran by comprising the catalytic site for dissociation of the ternary complex Ran/TC4-GDP/GTP·RCC1.

In contrast to the histidine mutants, the mutations located near the N-terminus of the RCC1 repeat, D44 and R101, have a higher K_m compared to that of wild-type RCC1, while their k_{cat} values are similar to that of wild-type RCC1. The high K_m value may reflect weak binding ability of these mutants to Ran, indicating that the N-terminus of the RCC1 repeat is involved in binding to Ran. Considering the kinetic parameters of histidine mutants located within the C-terminus of the RCC1 repeats and those of D44 and R101 located within the N-terminus of the RCC1 repeats, we suggest that the two ends of the RCC1 repeat play different roles in exchanging a guanine nucleotide on Ran. We are currently investigating the kinetic parameters of GEF activity of other RCC1 mutants which complemented the tsBN2 mutation, in order to confirm the existence of a functional difference between the N-terminus and the C-terminus of the RCC1 repeats.

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