Functional Expression in *Escherichia coli* of the Mitotic Regulator Proteins $p24^{ran}$ and $p45^{rcc1}$ and Fluorescence Measurements of Their Interaction

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ABSTRACT: The gene products for the mitotic regulator genes RCC1 and Ran, $p45^{rcc1}$ and $p24^{ran}$, were expressed in *Escherichia coli*, purified in large amounts, and characterized for their biochemical properties. $p24^{ran}$ binds guanine nucleotide as a 1:1 complex, which is only slowly released from the protein. $p45^{rcc1}$ catalyzes the exchange of nucleotide bound to the guanine nucleotide binding protein $p24^{ran}$ in the same way as the protein purified from HeLa cells. Likewise, the nucleotide dissociation from HeLa cell-derived $p24^{ran}$ protein is equally efficient with recombinant and nonrecombinant proteins. The recombinant proteins form a strong complex which contains no bound nucleotide. The kinetics of nucleotide exchange on $p24^{ran}$ in the presence or absence of $p45^{rcc1}$ can be conveniently monitored either by the direct tryptophan fluorescence of $p24^{ran}$ or by fluorescence energy tranfer measurements involving fluorescent nucleotides.

Entry into mitosis in the cell cycle of eucaryotic cells is usually controlled so as to ensure the completion of DNA synthesis in S phase. The dependence of M phase on S phase can be uncoupled by mutations in the RCC1 (regulator of chromosome condensation) gene. This gene or gene product has been found in hamster (Ushida et al., 1990), man (Kai et al., 1986; Ohtsubo et al., 1987; Bischoff et al., 1990), drosophila (Frasch, 1991), *Xenopus* (Nishitani et al., 1990), and fission yeast (Matsumoto & Beach, 1991) and budding yeast (Clark & Sprague, 1989). The protein has been found to be complexed to a nuclear ras-related protein with a molecular mass of 24 kDa, which had been cloned earlier as TC4 from a teratocarcinoma cell line on the basis of its amino acid homology to $p21^{ras}$ (Drivas et al., 1990). A fission yeast homologous gene has been cloned as spi-1 (suppressor of pim-1) on the basis of its ability to suppress (on a multicopy plasmid) a mutation in pim-1, the fission yeast homologue of RCC1. The human protein has been called $p24^{ran}$ (for *ras*-related *n*uclear protein) since it has been found localized in the nucleus of HeLa cells (Bischoff & Ponstingl, 1991a).

As was anticipated from its sequence homology to $p21^{ras}$, $p24^{ran}$ isolated from HeLa cells does indeed bind guanine nucleotides and has a GTPase activity (Bischoff & Ponstingl, 1991a). It has also been shown that the RCC1-encoded protein $p45^{rcc1}$ is the nucleotide exchange factor for $p24^{ran}$ whose function would be to promote the dissociation of protein-bound GDP and of its reloading with GTP (Bischoff & Ponstingl, 1991a). Presumably, again in analogy to other nucleotide binding proteins, the $p24^{ran}$–GTP complex would be the active species which functions as a switch in the control of the cell cycle.

In order to do detailed biochemical and structural investigations on the interaction of the two mitotic regulators, we have expressed $p24^{ran}$ and $p45^{rcc1}$ in *Escherichia coli* and investigated its biochemical properties.

MATERIALS AND METHODS

Cloning Procedures. PCR reactions with 30 cycles (60 s 92 °C, 60 s 60 °C, 90 s 72°C) were done under standard conditions using 150 ng of template DNA, 100 pmol of oligonucleotides, 200 μM dNTP's, and 4 units of Taq polymerase in PCR buffer (Boehringer). Fragments were isolated using Geneclean (Renner GmbH), digested with the corresponding restriction enzymes (see below), and ligated into the corresponding (see below) expression plasmids. The sequence of the inserts was verified by DNA sequencing using the T7 polymerase kit from Pharmacia. For expression of protein from PET plasmid, the *Escherichia coli* strain BL21-(DE3) was used, and for expression from the tac or trc promoter, the strain CK600K was used which is wild-type (CK600) K12 strain containing the plasmid pDMI,1 carrying the $lacI^{Q}$ gene and a kanamycin resistance gene (K) and compatible with pBR322-derived vectors (Certa et al., 1986). All other cloning methods are basically as described in Sambrook et al. (1989).

Ran Expression. For the construction of the $p24^{ran}$ expression plasmids the pUC-TC4 clone (Drivas et al., 1990) was the PCR template, using as forward and reverse primers 5′-GGCCATGGCTGCGCAGGGAGAGCC-3′ and 5′-GATGTGACTGCAGCAGTCTTATAT-3′ (restriction sites underlined, start codon boldface and underlined), respectively, which generates a 912 bp *Nco*I–*Pst*I fragment. This is ligated into the *Nco*I–*Pst*I cleaved vector ptrc99C (Amann et al., 1988) to give ptrcran. The coding sequence of the ran gene was cut out of ptrcran with *Nco*I and *Hin*dIII (blunt ended) and recloned into the expression vector pET3d, which had been opened with *Bam*HI (blunt ended) and *Nco*I (Studier et al., 1990). It was transformed into BL21(DE3) which contains the T7 polymerase under the control of the inducible lac promoter as a λ lysogen.

RCC1 Expression. The human RCC1 clone pCD51 (Ohtsubo et al., 1987) was PCR amplified using as forward

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[1] Abbreviations: $p24^{ran}$, protein product of the human ran/TC4 gene; $p45^{rcc1}$, product of the human RCC1 (regulator of chromosome condensation) gene; mant-GDP, 2′,3′-bis-*O*-(*N*-methylanthraniloyl)guanosine diphosphate; GNB proteins, guanine nucleotide binding proteins; GAP, GTPase-activating protein.

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and reverse primers 5′-GGGAATTCTATGTCACCCAAGCGCATAGCTAAA-3′ and 5′-GGCTGCAGAGGATTCTGTTCTGCTGATGAGAAA-3′ (restriction sites underlined, start codon boldface and underlined). Since RCC1 cDNA contains an internal *Eco*RI restriction site, digestion with *Eco*RI and *Pst*I creates two fragments of 470 and 875 bp lengths, which are ligated stepwise into ptacras and digested with *Eco*RI–*Pst*I. The resulting construct was screened for expression of $p45^{rcc1}$ and the final clone verified by DNA sequencing.

Western Blots. The respective mixture of proteins was separated via SDS–polyacrylamide electrophoresis and blotted onto nitrocellulose filters in 25 mM Tris·HC,l pH 8.3, and 200 mM glycine with 200 mA for 1 h. The filter was blocked with a 2% solution of milk powder in PBS (150 mM NaCl, 25 mM KCl, and 10 mM sodium phosphate, pH 7.4) and then reacted with a polyclonal rabbit antibody directed against the HeLa cell-derived $p24^{ran}$–$p45^{rcc1}$ complex (a gift from H. Ponstingl and R. Bischoff) in PBS and 0.5% bovine serum albumin for 1 h at room temperature (RT). The detection was done by first reacting with a biotinylated anti-rabbit antibody in PBS/0.5% BSA (1 h, RT) with avidin peroxidase (Sigma, 1:400, 30 min, RT) and finally with 0.05% chloronaphthol/H_2O_2 in PBS.

Protein Purifications. (A) $p45^{rcc1}$. The expression clone for $p45^{rcc1}$ was grown in a 50-L fermenter (Braun) in LB medium at 37 °C to an OD_{580} value of 0.8, and the expression was induced with 0.5 mM IPTG for 15 h. Cells were harvested by centrifugation and kept frozen at −70 °C. Cells (26 g wet weight) were thawed in buffer A, 20 mM potassium phosphate (KP_i), pH 6.8, 1 mM DTE, 1 mM PMSF, 1 mM $MgCl_2$, and 50 mM NaCl. Cells were ruptured in a French press (SLM Aminco) with 1000 psi, the cell debris was centrifuged, and the clear supernatant was applied to a S-Sepharose (5.5 × 15 cm) equilibrated in buffer A. A gradient from 50 to 1000 mM NaCl was applied and RCC1 activity eluted between 400 and 500 mM NaCl. Pooled fractions were dialyzed against 20 mM potassium phosphate, pH 6.8, applied to a hydroxylapatite (Merck) FPLC column (2.6 × 10 cm), and eluted with a linear gradient (2 x 150 mL) from 20 to 500 mM KP_i. $p45^{rcc1}$ eluted between 150 and 200 mM KP_i. The active fractions were precipitated with 60% ammonium sulfate, and the precipitate was collected by centrifugation, dissolved in standard buffer (64 mM Tris·HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTE), and further purified on a AcA-54 (2.5 × 100 cm) gel filtration column in standard buffer with 0.1 M NaCl. Corresponding fractions containing purified $p45^{rcc1}$ were concentrated by pressure dialysis, shock frozen in liquid nitrogen, and stored at −70 °C. Under these conditions the catalytic activity of the protein is stable for at least six months.

(B) $p24^{ran}$. The expression strain for the ran protein was grown in a 50-L fermenter in LB medium at 37 °C to an OD_{580} of 1, induced with 10 μM IPTG for 15 h. Cells were harvested by centrifugation and kept frozen at −70 °C. Cells (40 g wet weight) were thawed in buffer B (20 mM KP_i, pH 6.6, 2 mM PMSF, 6.5 mM DTE, 2 mM $MgCl_2$), ruptured in a French press at 1000 psi. The cleared supernatant of the cell extract was treated with 1 mM GDP in the presence of 5 mM EDTA for 30 min at room temperature to ensure complete exchange of ran-bound nucleotides for GDP. The solution was applied to an S-Sepharose column (5.5 × 45 cm) and eluted with a linear gradient (2 × 1200 mL) of 0–1 M KCl in buffer B. $p24^{ran}$ eluted between 200 and 400 mM KCl. It was precipitated with 60% ammonium sulfate, and the precipitate collected, dissolved in standard buffer, and eluted from a AcA-54 (2.5 x 100 cm) gel filtration column in the presence of 10 μM GDP and 100 mM NaCl. The fractions containing $p24^{ran}$ were collected and applied to a (2.5 × 35 cm) Q-Sepharose column in standard buffer and eluted with a gradient from 0.1 to 1 M NaCl, with the protein eluting at 300 mM NaCl. Pure $p24^{ran}$ was collected, dialyzed against standard buffer, and concentrated by pressure dialysis. It was shock frozen in liquid nitrogen and stored at −70 °C for 9 months without appreciable loss of activity.

General Protein Methods. The concentration of $p45^{rcc1}$ was determined according to the method of Bradford (1976) using BSA as standard. The concentration of active $p24^{ran}$ was taken as equal to the concentration of protein-bound GDP which is determined by HPLC analysis on a C-18 HPLC column as described earlier (Feuerstein et al., 1987) directly after separating $p24^{ran}$ from excess nucleotide on a PD10 column. The concentrations of nonrecombinant $p24^{ran}$ and $p45^{rcc1}$–$p24^{ran}$ complex were determined (because of the small amounts available) by separating an aliquot on an SDS gel, staining it with Coomassie Blue, and quantifying the stained bands with a video scanning system (Fröbel Labortechnik, Lindau).

Kinetic Measurements. The recombinant proteins purified as described above were always stored, after shock-freezing in liquid nitrogen, in standard buffer: 64 mM Tris·HCl, pH 7.6, 5 mM dithiotreitol, and 1 mM sodium azide, in the presence or absence of 5 mM $MgCl_2$ at −70 °C as indicated. The nonrecombinant proteins $p24^{ran}$ and the complex $p24^{ran}$–$p45^{rcc1}$, purified as described before (Bischoff et al., 1990; Bischoff & Ponstingl 1991), were a gift from R. Bischoff and H. Ponstingl. Static and slow time fluorescence measurements were done using an SLM 8000 spectrophotometer. Excitation wavelength for the tryptophan of $p24^{ran}$ was 295 nm. Filter binding assays were done as previously described (Feuerstein et al., 1987; John et al., 1989). Nucleotide dissociation reactions, usually at 25 °C, with labeled nucleotides were measured using the filter binding assay basically as described before (Mistou et al., 1992). $p24^{ran}$ was loaded with [^{3}H]-GDP, excess radioactive nucleotide was removed by gel filtration on a small prepacked G-25 column (PD10, Pharmacia) in standard buffer with 5 mM $MgCl_2$, and the reaction was started by the addition of a large excess (500 μM) of unlabeled GDP and catalytic amounts of either $p45^{rcc1}$ alone or the complex $p24^{ran}$–$p45^{rcc1}$. Radioactive nucleotide bound to the protein was determined by nitrocellulose filter binding. Nucleotide exchange was also measured with fluorescent nucleotide as described in the text, by adding excess fluorescent mant-GDP [2′,3′-bis-*O*-(*N*-methylanthraniloyl)guanosine diphosphate] (John et al., 1989a) and following the decrease of fluorescence at 335 nm (excitation wavelength 295 nm). Fluorescent mant-GDP was a kind gift of R. S. Goody. Kinetic data were analyzed using a single-exponential function, with the program Grafit (Erithacus Software). In the case of the $p45^{rcc1}$-catalyzed dissociation reactions the single-exponential fit gave a reasonable good fit of the data although the exact kinetics remain to be formally verified.

RESULTS

Expression of $p24^{ran}$. For the expression of $p24^{ran}$, the human TC4 clone (Drivas et al., 1990), whose sequence has been revised by Matsumoto and Beach (1992), was cloned by PCR as a *Nco*I–*Hin*dII fragment into the expression vector ptrc99C, which uses the trc promoter (Amann et al., 1988). No expression was observed under conditions which induce the trc promoter (1 mM IPTG, data not shown). When the final construction was verified by DNA sequencing, we noticed a base change in the cDNA sequence which changes the derived

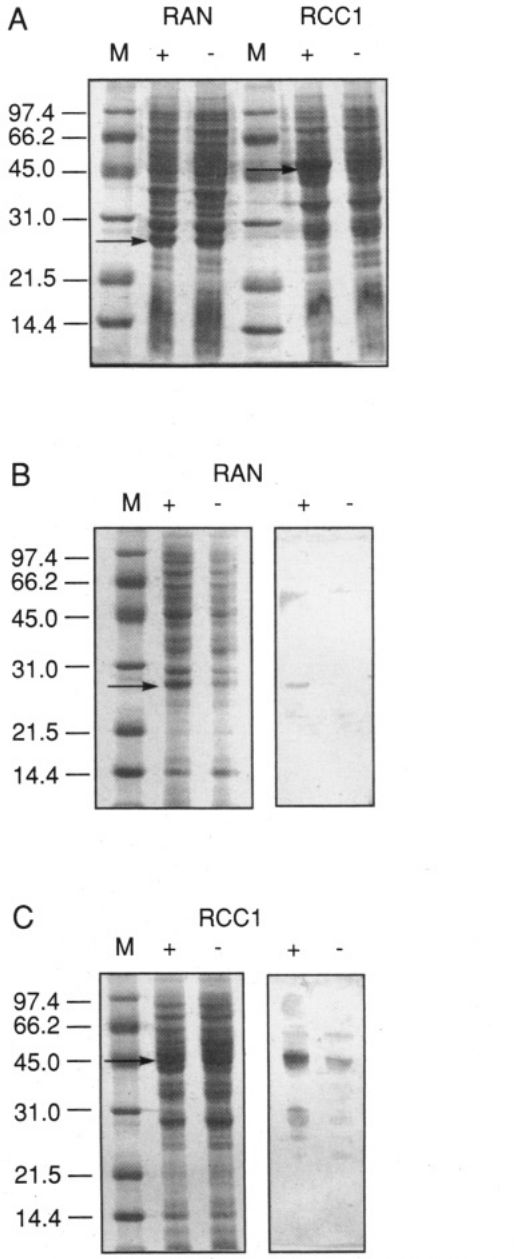

FIGURE 1: Expression of p24ran and p45rcc1 in *E. coli*. (A) SDS gel electrophoresis of total bacterial extracts from cells containing the p24ran expression plasmids pET-ran or ptrc-RCC1 in the presence or absence of 0.5 mM IPTG. (B, C) SDS gel electrophoretic analysis of soluble protein from pET-ran (B) or ptrc-RCC1 (C) with or without 0.01/0.5 mM IPTG, either stained with Coomassie Blue (left) or detected immunologically with an anti-ran, anti-RCC1 polyclonal antibody (a gift from H. Ponstingl) as described in the Materials and Methods. Lane M contains marker proteins with the indicated molecular masses.

protein sequence of p24ran from Ser-129 (codon AGT) to Arg-129 (codon AGG). The same amino acid is found in the fission yeast protein spi-1. To exclude a PCR artifact, the original TC4 clone was sequenced and found also to contain AGG codon 129. The same sequence has also recently been found by Ren et al. (1993).

We constructed a vector, pET-ran, where p24ran is expressed from the T7phi10 promoter using the plasmid pET-3d (Studier et al., 1990). T7 polymerase, which is under the control of the lacUV5 promoter, is supplied in trans by using the *E. coli* strain BL21(DE3) (Studier et al., 1990). After induction with IPTG, large amounts of a protein are visible in the Coomassie-stained SDS gel of a crude cell extract (Figure 1). This protein has an apparent molecular mass of 24 kDa as determined with marker proteins, which is very close to its

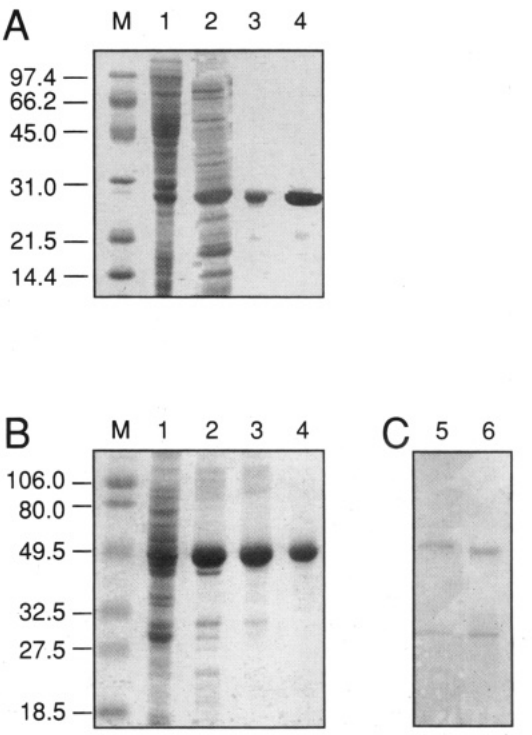

FIGURE 2: Purification of soluble recombinant proteins from bacterial extracts. (A) Purification of p24ran with crude soluble extract (lane 1), pooled fractions after S-Sepharose (2), AcA-54 gel filtration (3), and Q-Sepharose (4). (B) Purification of p45rcc1 with soluble extract (1), fractions after S-Sepharose (2), hydroxylapatite (3), and AcA-54 (4). Lane M contains marker proteins with the indicated molecular masses. (C) HeLa cell-purified (5) and recombinant (6) p24ran–p45rcc1 complex.

Table I: Preparation of Human Recombinant p24ran

	total protein (mg)	active protein[a] (mg)
crude extract	1440	nd[b]
S-Sepharose eluate	444	43.2
ammonium sulfate precipitate	104	14.2
AcA-54 eluate	38	13.9
Q-Sepharose eluate	16	8.6

[a] The activity is determined by incooperation of ^{3}H labeled GDP; see materials and methods. [b] nd: the amount of active ran in the crude extract cannot be reliably determined.

calculated mass of 24 338 Da. Under conditions of full induction of the T7phi10 promoter, mostly insoluble protein is produced. Under carefully controlled induction conditions (10 μM IPTG, 3 h, 37 °C) the greater part of the protein is found in the soluble extract (Figure 1 B). The Western blot in Figure 1B shows that the protein can be recognized by a ran-specific antibody directed against the p24ran–p45rcc1 complex isolated from HeLa cells (Bischoff et al., 1990; Bischoff & Ponstingl, 1991b).

p24ran can be purified from the crude soluble extract by a three-step purification procedure involving gradient elution (0–1 M KCl) from S-Sepharose at pH 6.6, gel filtration on AcA-54, and gradient elution (0–1 M KCl) from Q-Sepharose at pH 7.6, as shown in Figure 2A and summarized in Table I. Final yield of pure (>95%) p24ran is 16 mg, which corresponds to 1 mg of protein/g of cell paste.

Almost all guanine nucleotide binding (GNB) proteins bind guanine nucleotides with high affinity. Correspondingly, they are usually isolated as a stoichiometric protein–nucleotide complex. p24ran was analyzed for its nucleotide content by HPLC analysis on a C-18 reverse-phase column as described before (Feuerstein et al., 1987). Figure 3 shows that it contains

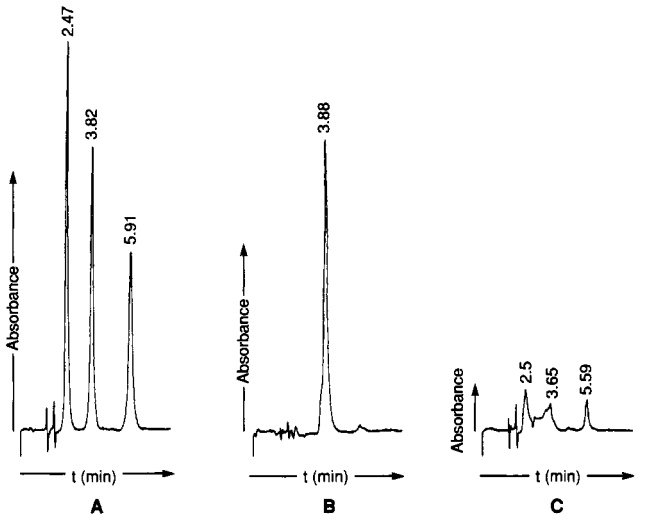

FIGURE 3: HPLC analysis of nucleotide content of purified proteins. Protein-bound nucleotides were analyzed by elution of protein samples from a C-18 reverse-phase column described before (Feuerstein et al., 1987). (A) Calibration sample containing an equimolar mixture (100 μM) of GMP, GDP, and GTP. (B) Protein sample containing 440 pmol of $p24^{ran}$ as determined by Bradford (calculation of the peak area gives 472 pmol of GDP). (C) 440 pmol of the $p24^{ran}$–$p45^{rcc1}$ complex (peak area for GDP < 20pmol).

only GDP and no GTP or deoxy-GDP as for $p21^{ras}$ (Feuerstein et al., 1987), since for the chromatography on S-Sepharose it was necessary to uniformly load the protein with GDP. Quantitative analysis of the chromatogram shows the ratio of GDP to protein as determined by the method of Bradford is close to 1:1.

On the other hand, we consistently find that only 50–60% of the protein is able to bind radioactive nucleotide as determined by nitrocellulose filter binding. We do not know the reason for this discrepancy. Judging from various experiments which involve the binding of $p24^{ran}$ to nitrocellulose filters, we assume that this GNB protein is not binding quantitatively to or denaturing on the filters similar to what we have found for truncated $p21^{ras}$ (amino acids 1–166). Only 20–40% of that protein was found to be active on the basis of the nitrocellulose filter assay even though it contained bound GDP in a 1:1 ratio (John et al., 1989b; Wittinghofer & Pai, 1991). It appears that this property of truncated $p21^{ras}$, and possibly that of full-length $p24^{ran}$, is due to its lower stability because the melting temperature of truncated $p21^{ras}$ is 11 °C lower than that of wild-type $p21^{ras}$ with 189 amino acids (Franken et al., 1993). The observation that truncated ras, ral, and rap1A proteins cannot be renatured on nitrocellulose filter after blotting as measured by binding of radioactive GDP, whereas full-length protein can (F.-J. Klinz, unpublished observation), is a further indication that proteins are more or less denatured on nitrocellulose filters. Since $p24^{ran}$ appears to be less stable than $p21^{ras}$ under various conditions (C. Klebe, unpublished observations), its concentration cannot be reliably measured by the nitrocellulose filter binding assay. The concentration of $p24^{ran}$ was thus taken as the amount of GDP bound to the protein as determined by HPLC.

Expression of $p45^{rcc1}$. The starting cDNA for the expression of RCC1 was pCD51 (Ohtsubo et al., 1987). It was used as template to generate by PCR an *Eco*RI–*Pst*I fragment which was cloned into the plasmid ptacras, which has been used to express large amounts of soluble $p21^{ras}$ (Tucker et al., 1986). The resulting plasmid ptac-RCC1 produces, upon induction with IPTG, a new protein band in the polyacrylamide gel electrophoresis of total cellular extract as shown in Figure 1. The protein with an apparent molecular mass of 45 kDa is recognized by a polyclonal antibody against $p24^{ran}$–$p45^{rcc1}$ complex from HeLa cells (Bischoff et al., 1990). The protein is apparently soluble in *E. coli*, even under high induction conditions (500 μM IPTG, 37 °C, Figure 1). It is purified from the soluble extract with a three-column purification procedure which involves chromatography on S-Sepharose at pH 6.8 (gradient from 0.05 to 1 M KCl), chromatography on hydroxylapatite (gradient of 20–500 mM potassium phosphate), and gel filtration on AcA-54 (Figure 2B) as summarized in Table II. Approximately 5 mg of pure $p45^{rcc1}$ is obtained from 1 g of wet *E. coli* paste.

Table II: Preparation of Human Recombinant $p45^{rcc1}$

	total protein (mg)	tot act. (units)	sp act. (units/mg)
crude extract	2250	840 000	373
S-Sepharose eluate	300	231 000	770
hydroxylapatite eluate	257	184 800	719
AcA-54 eluate	200	162 533	812

[a] One unit of activity is defined as the amount of RCC1 that catalyzes the GDP exchange reaction of 1 nmol of $p24^{ran}$/min under standard conditions (1 μM $p24^{ran}$, 25 °C).

Exchange Factor Activity. In order to be able to compare the activity of different GTPase-activating proteins (GAPs) and to judge different preparations of the same GAP, we have introduced a definition for the unit of activity for ras-GAP (p120-GAP and neurofibromin) and have defined standard conditions for measuring it (Gideon et al., 1992; Wiesmüller et al., 1992). We also propose here a unit definition for the activity of the guanine nucleotide release factors (GRF). In analogy to ras-GAP the activity of the ran-GRF is measured using 1 μM $p24^{ran}$ complexed to GDP or the fluorescent analogue mant-GDP (John et al., 1990a), at 25 °C in standard buffer, in the presence of excess Mg^{2+}. One unit of activity is the amount of GRF protein which catalyzes the dissociation of 1 nmol of GDP from the guanine nucleotide binding (GNB) protein per minute, in this case, $p24^{ran}$ in a particular buffer. The rate of nucleotide release from 1 μM $p24^{ran}$ is linearly dependent on the $p45^{rcc1}$ concentration up to 30 nM, and this concentration range is always used for the activity measurements. $p45^{rcc1}$ under those conditions has a specific activity of 812 units/mg. The results of the purification shown in Table II seem to indicate either that the activity of ran-GRF is not reliably measured in the crude bacterial extract or that the protein losses its activity during the first chromatographic step. At the moment we have no indication that the protein is unstable under the conditions of the purification.

Figure 2C shows that recombinant $p45^{rcc1}$ runs slightly ahead of authentic $p45^{rcc1}$, which could be due to either different molecular weights or different posttranslational modifications of the proteins. The N-terminal amino acid sequence of the recombinant protein was determined to be XXSPPADAIPKS, where X could not be identified, but should be R according to the sequence. This means that at least the first eight amino acids which are coded for by the RCC1 cDNA, MSPKRIAK, are removed during growth of the bacteria and/or purification of the protein. This explains nicely the different mobility of the recombinant protein in SDS gel electrophoresis.

Preparation of the $p24^{ran}$–p45 Complex. Since we are interested in the structural analysis of the complex between the GNB protein $p24^{ran}$ and its GRF, we have also prepared the complex between these two proteins. We use this complex also to compare the activity of recombinant $p45^{rcc1}$ with the same protein from HeLa cells (see below), because $p45^{rcc1}$ from these cells was sufficiently soluble only as such a complex

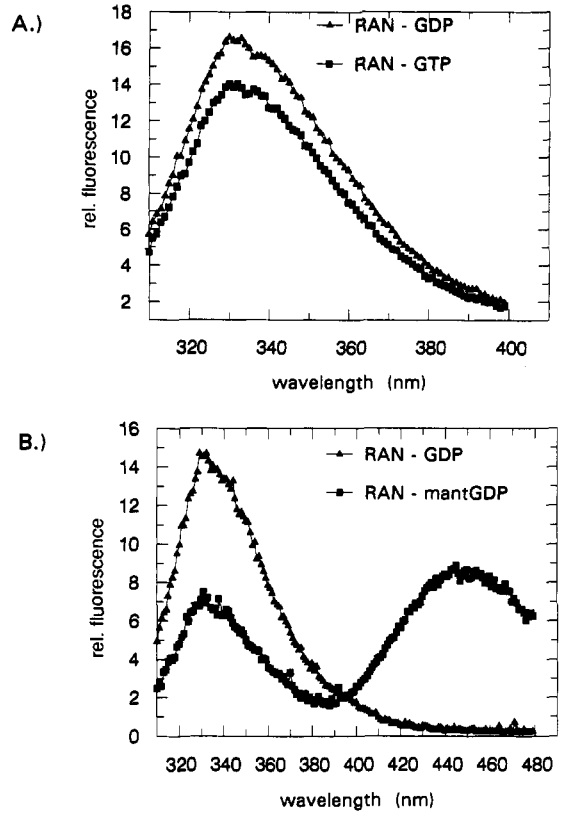

FIGURE 4: Fluorescence emission spectra of p24ran complexes. (A) Spectra of p24ran in the GDP- or GTP-bound form, excitation wavelength 295 nm. The protein–nucleotide complexes were prepared as described in Materials and Methods. (B) 1 μM p24ran–GDP before and after reaction with an excess (25 μM) of fluorescent GDP analogue mant-GDP. The reaction was initiated with 5 mM EDTA.

(Bischoff & Ponstingl, 1991a,b). To do this, we have mixed equimolar amounts of these two proteins, added 20 mM EDTA, and eluted the mixture from an AcA-54 gel filtration column in standard buffer containing 1 mM EDTA. The SDS–polyacrylamide gel analysis of the complex (Figure 2) shows equimolar amounts of the two proteins. The analysis of the nucleotide content by HPLC shows (Figure 3C) that the complex does not contain any nucleotide, supporting earlier observations with the nonrecombinant proteins (Bischoff & Ponstingl, 1991) and observations about the p21ras–SDC25 interaction (Mistou et al., 1992) and about EF-Tu, which also makes a nucleotide-free complex with its elongation factor Ts (Miller & Weissbach, 1969).

Fluorescence Measurements of the p24ran–p45rccl Interaction. Since p24ran contains three tryptophans, we wanted to see whether or not the fluorescence spectrum of the protein can be used to monitor changes upon binding of different ligands. Figure 4 shows the fluorescence emission spectrum (excitation wavelength 295 nm) of the GDP- versus the GTP-liganded protein. Both spectra have a maximum at 335 nm, but the p24ran–GDP complex has a higher fluorescence yield. The difference is 20% and can potentially be used to monitor the GTPase reaction. The rate of the GTPase reaction under spectrophotometer conditions is however much slower than the rate of denaturation and could thus not be measured under these conditions.

Another much more pronounced decrease of the fluorescence emission of the protein is observed upon addition of a large excess of fluorescent mant-GDP, which has been used before in the analysis of the p21ras–nucleotide interaction (John et al., 1990; Neal et al., 1990). The time course of the fluorescence change is fast with EDTA and slow in the presence

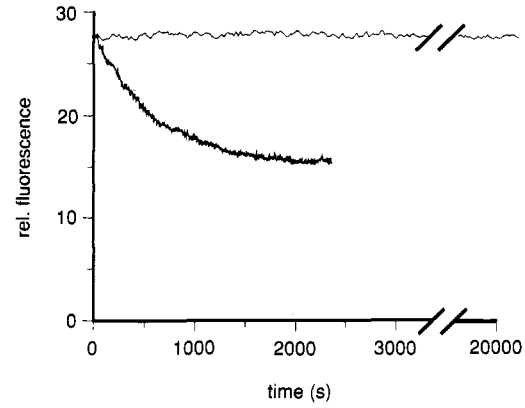

FIGURE 5: Kinetics of the GDP dissociation from p24ran. 1.5 μM p24ran–GDP complex was incubated at 25 °C with 25 μM mant-GDP in standard buffer either without or with 5 nM p45rccl, and the change in fluorescence emission at 335 nm (excitation 295 nm) was followed with time.

of Mg^{2+}, which means that it monitors the release of bound GDP and the binding of mant-GDP. The new fluorescence emission peak at 445 nm is thus due to fluorescence resonance energy transfer between tryptophan and the mant group on GDP. The large decrease (50%) of the fluorescence emission at 335 nm can thus be used to monitor the kinetics of dissociation in the presence and absence of the exchange factor (Figure 5). The dissociation rate of p24ran-bound GDP is very slow, 1.0×10^{-5} s^{-1} with 5 mM Mg^{2+} at 37 °C and 3.8×10^{-3} s^{-1} in the absence of Mg^{2+} (5 mM EDTA). The 460-fold increase in the dissociation rate due to removal of Mg^{2+} is similar to what has been found for p21ras (Tucker et al., 1986; Hall & Self, 1986) and p24ral (Frech et al., 1990). The dissociation reaction can be stimulated more than a thousandfold in the presence of 20 nM p45rccl. This indicates that one possible mode of action of GRF proteins, which would be to remove Mg^{2+} from the nucleotide binding site and thereby increase the dissociation reaction, cannot be the only explanation for the large increase in rate.

Crossover Activities. It has been shown for p21ras and other small GNB proteins that they contain motifs such as CAAX in the case of p21ras or CAC in the case of p21rab at the C-terminal end of the polypeptide chain, where A is an aliphatic and X any amino acid. These motifs are responsible for extensive posttranslational modifications of the C-terminus of these proteins, which are in turn responsible for the membrane attachment and biological activity of these proteins (Valencia et al., 1991). p24ran and p45rccl are nuclear proteins, which apparently lack any of the known motifs for posttranslational processing. They have both been found to be blocked at the N-terminus (Bischoff & Ponstingl, 1990, 1991b), and it has been suggested that p45rccl may be modified in cell cycle-dependent manner. Therefore, it was of interest to compare the interaction of p24ran with its GRF for the recombinant and the authentic proteins, especially since it has been suggested that C-terminal modifications are responsible for efficient interaction of GNB proteins with their exchange factors (Takai et al., 1992; Orita et al., 1993). Figure 6 A shows that the stimulation of GDP dissociation from recombinant p24ran–[^{3}H]GDP with identical amounts of either recombinant or *E. coli*-produced p45rccl is indistinguishable. Likewise, the stimulation of GDP dissociation with recombinant p45rccl from p24ran purified from either HeLa or *E. coli* cells is very similar (Figure 6B). Therefore, if there are any posttranslational modifications in addition to the modifications of the N-termini of these proteins, they are not required for efficient interaction between p24ran and p45rccl.

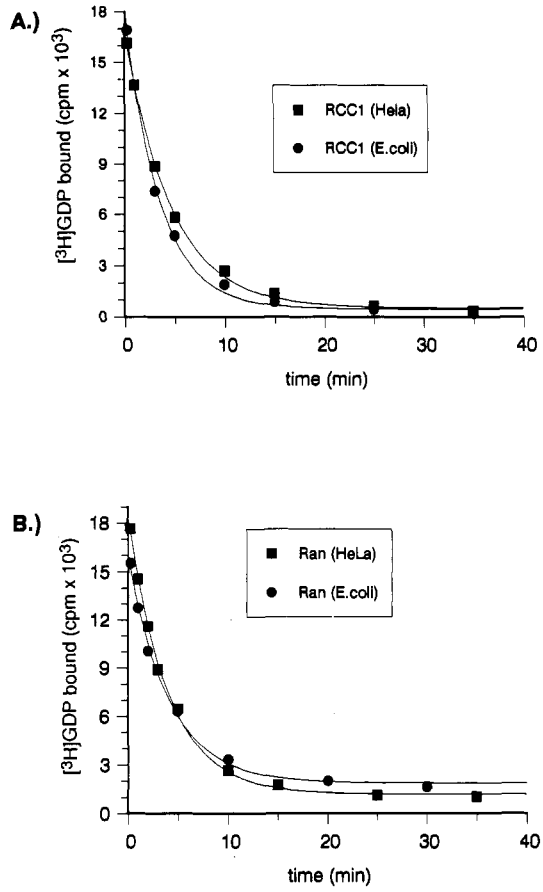

FIGURE 6: Crossover activities of recombinant against nonrecombinant proteins. In (A), 1 μM recombinant $p24^{ran}$–[^{3}H]GDP complex was incubated with 7.5 nM HeLa cell or *E. coli*-derived $p45^{rcc1}$ (applied as an equimolar complex with $p24^{ran}$) in the presence of standard buffer plus 20 mM $MgCl_2$. In (B), 200 nM $p24^{ran}$ from either HeLa cells or *E. coli* complexed to [α-^{32}P]GTP was incubated with 1 nM recombinant RCC1 in the presence of 50 μM GTP and 0.1% Prionex in standard buffer.

CONCLUSION

We have shown here that recombinant $p24^{ran}$ and $p45^{rcc1}$ can be efficiently produced in *E. coli* and that their interaction is indistinguishable from that of the native proteins. We have also shown that the kinetics of the $p45^{rcc1}$-mediated release of nucleotides bound to $p24^{ran}$ can be easily monitored spectroscopically either via the direct tryptophan fluorescence of $p24^{ran}$ or via the fluorescence energy transfer from one or more of the tryptophans of $p24^{ran}$ to fluorescent guanosine nucleotides. Therefore, the system presented here should prove a useful tool to study the kinetic mechanism of the interaction of GNB proteins with guanine nucleotide release factors (GRF) or guanine dissociation stimulators (GDS), which has so far not been studied in great detail.

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