Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 319-324

PROPERTIES OF THE EXCHANGE RATE OF GUANINE NUCLEOTIDES TO THE NOVEL RAP-2B PROTEIN

Luis Molina y Vedia, Carol-Ann Ohmstede and Eduardo G. Lapetina

Division of Cell Biology, Burroughs Wellcome Co.,
Research Triangle Park, NC 27709

Received July 16, 1990

Rap-2B is a novel ras-related protein that is 89% identical to rap-2 at the amino acid level. Based on its amino acid sequence, it is anticipated that rap-2B binds guanine nucleotides. Here we show that purified, bacterially expressed rap-2B does bind both GTP and GDP in a Mg^{2+}-dependent fashion. The relative affinity of rap-2B for GTP is higher than that for GDP, both at low and high concentrations of Mg^{2+}. This contrasts with N-ras p21 and could be of functional significance. Moreover, a polyclonal antiserum was raised against the recombinant rap-2B protein purified from *E.coli* lysates. This antiserum recognized a major protein of Mr ~21000 on Western blots of platelet membrane proteins, and immunoprecipitates rap-2B complexed with GTP or GDP.

A platelet cDNA expression library (1) was screened with the monoclonal antibody M90 (2), which recognizes a specific epitope on *ras*-encoded p21 proteins (amino acids 107-130). DNA sequence analysis of one clone (EMBL/Gen Bank Database accession number X52987) revealed that it encoded the amino acid sequence of a novel protein closely related to rap-2 (3), which we named rap-2B (4,5). Rap-2B is 89% identical to rap-2 (3) at the amino acid level, with the most variability at the carboxyl terminus of the protein.

A high level of rap-2 expression was obtained with an E. coli expression system (5). The expressed protein binds GTP on blots, and it is not phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (5).

The biochemical properties, tissue distribution and functional role of rap-2B are unknown. In this report we describe the purification of expressed rap-2B protein, its GTPase activity, the effect of Mg^{2+} on the binding of GDP and GTP and its localization in platelet membranes.

MATERIALS AND METHODS

Purification of recombinant rap-2B: The construction of an E. coli strain containing rap-2 expression plasmids was previously described (4,5). Rap-2B protein was purified in Mg^{2+} containing buffers to preserve bound nucleotide as has previously been described for p21 N-ras (6), normal rho, Val-14 rho, and normal R ras (7). Cells from a 3 l culture were induced with 100 μM IPTG

0006-291X/90 $1.50

(Sigma) for 12 h, collected by centrifugation at 15,000 g and resuspended in 40 ml of buffer containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithioerythritol (DTE), 50 mM NaCl and 0.05 mM phenylmethylsulfonylfluoride (PMSF) (Buffer A). The cell suspension was frozen in liquid nitrogen, thawed, sonicated twice for 1 min and centrifuged at 12,000 g for 30 min at 4°C. The pellet was reextracted twice using the same procedure, and the supernatants were pooled, filtered through Whatman 1 Filter paper and applied to a column of DEAE-Trisacryl (IBF 2.5 x 12 cm) equilibrated in Buffer A. The column was washed with 200 ml of Buffer A and developed with a linear gradient of 0.05-0.5 M NaCl (150 ml each), and 4 ml fractions were collected. Fractions containing rap-2B (as detected by binding of [α^{32}P]GTP and by SDS-PAGE) were pooled and brought to 60% saturation with ammonium sulfate. The precipitate was collected by centrifugation at 10,000 g for 15 min, resuspended in 5 ml of Buffer B (Buffer A, containing 0.1 mM GDP and 200 mM NaCl) and dialyzed overnight against 500 ml of the same buffer. After filtration through Millex-HV filters (Millipore), a sample (2.5 ml) of the solution was applied to a column of sephacryl S-200 HR (2.5 x 150 cm) equilibrated with buffer B, which was previously calibrated with Blue Dextran and cytochrome C (Sigma). The fractions containing rap-2B were stored at 4°C. The procedure produced 36 mg of rap-2B, that was more than 95% pure as determined by SDS-PAGE using coomassie blue. Rap-2B (1 mg) was able to bind 30.5 nmoles of GTP in the presence of 0.5 µM Mg^{2+}, which is close to its theoretical capacity of 45 nmol assuming that one mole of GTP binds to one mole 21,000 m.w. protein.

Binding of guanine nucleotides to rap-2B: Exchange reactions were carried out as previously described (6). Briefly for the "on" rates [α-^{32}P]GTP (2 µM) or [^{3}H]GDP (2 µM) was incubated with protein (0.5 µM) at 37°C in a solution containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$ and 50 mM NaCl. When required, the concentration of Mg^{2+} was reduced to 0.5 µM by the addition of 10 mM EDTA. The reaction was stopped on ice, and 50 µl of the reaction mixture was placed on nitrocellulose filters (Schleicher & Schuell BA85) and washed with 10 ml of a buffer containing Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT and 0.1 µg/ml albumin. Radioactive (^{3}H and ^{32}P) material bound to protein was determined by scintillation counting.

Determination of GTPase activity of rap-2B: The intrinsic GTPase activity of rap-2B was determined as described previously for p21 N-ras (8). Immuno precipation of the nucleotide-complexed protein was achieved using a rap-2B specific antibody.

Determination of N-terminus amino acid sequence of rap-2B: The amino acid sequence of rap-2B was determined after separation of bacterially expressed rap-2B SDS-PAGE and blotting onto PVDF filters (Millipore) using a modification of a method previously reported (9).

Antibody production: A rap-2B specific antiserum was generated in rabbits by injection of a purified preparation of the recombinant protein that had been expressed in *E.coli* (5). Antiserum specificity was evaluated by Western blotting using platelet membrane and cytosol as source of antigens.

RESULTS

Purification of the expressed rap-2B protein was performed using a method similar to that previously reported for ras and ras-related proteins, (6,7). The method included anion exchange chromatography and gel filtration chromatography (Fig. 1). No detergent or urea was used during this purification procedure. Amino acid sequence analysis of the amino terminus indicated exact correspondence of the first 20 amino acids of the purified protein with the sequence of rap-2B (4,5). The purity of rap-2B was determined by SDS-PAGE. The protein appeared as one band (Fig. 1) which

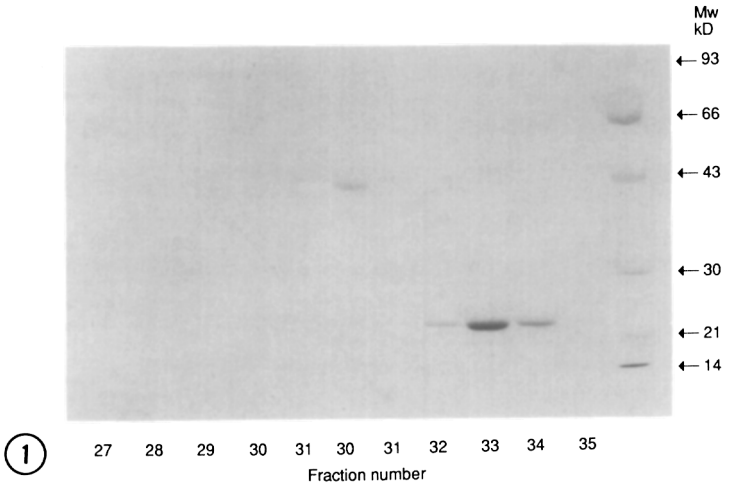
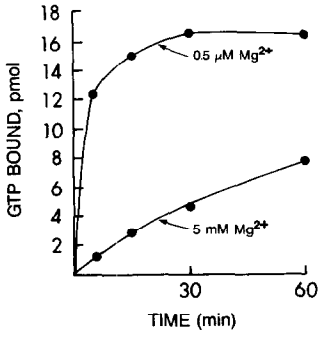
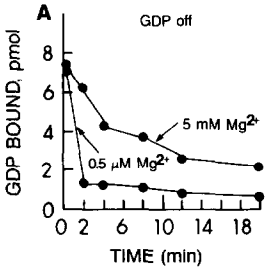


Fig. 1. Sephacryl-200 HR Column Chromatography. 10 µl of each fraction (10 ml) were analyzed by SDS-PAGE (10-20%) separation. Proteins were visualized by coomassie blue.

reacted with monoclonal antibody M90 (2) (not shown). The intrinsic GTPase activity of rap-2 was measured as was previously described (8) and it showed a k=0.002 min^{-1}. Under the same conditions p21 ras showed a value of 0.026 min^{-1}.

Fig. 2 examined the rate of association of [^{32}P]GTP with purified rap-2B. In interpreting this figure it is important to realize that rap-2B was purified in the presence of GDP to stabilize it and presumably existed as a rap-2B.GDP complex at the start of the experiment. The measured rates of association therefore actually measured the rate of exchange of labeled nucleotide for prebound GDP. The exchange rate for GTP was much more rapid





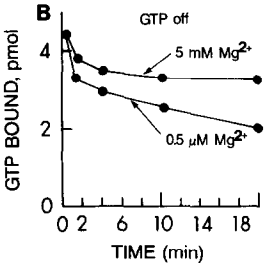


Fig. 2. Overall rate of exchange of [α-^{32}P]GTP with rap-2B at different concentrations of Mg^{2+}. Rap-2B-GDP (0.5 µM) was incubated with 2 µM [α-^{32}P]GTP as described in experimental procedures. The reactions were incubated in the presence of 5 mM Mg^{2+} or 0.5 µM Mg^{2+}.

Fig. 3. Rate of release of prebound [^{3}H]GDP (A) or prebound [α-^{32}P]GTP (B) by rap-2B. Binding of the nucleotides was carried out for 2 hours at 37°C, in the presence of 5 mM Mg using the same concentrations described in Fig. 3. Unlabeled GTP (2 mM) was added either with 10 mM EDTA (0.5 µM Mg^{2+}) or without EDTA (5 mM Mg^{2+}) at the indicated times and 50 µl samples were analyzed for the amount of radioactivity bound to protein.

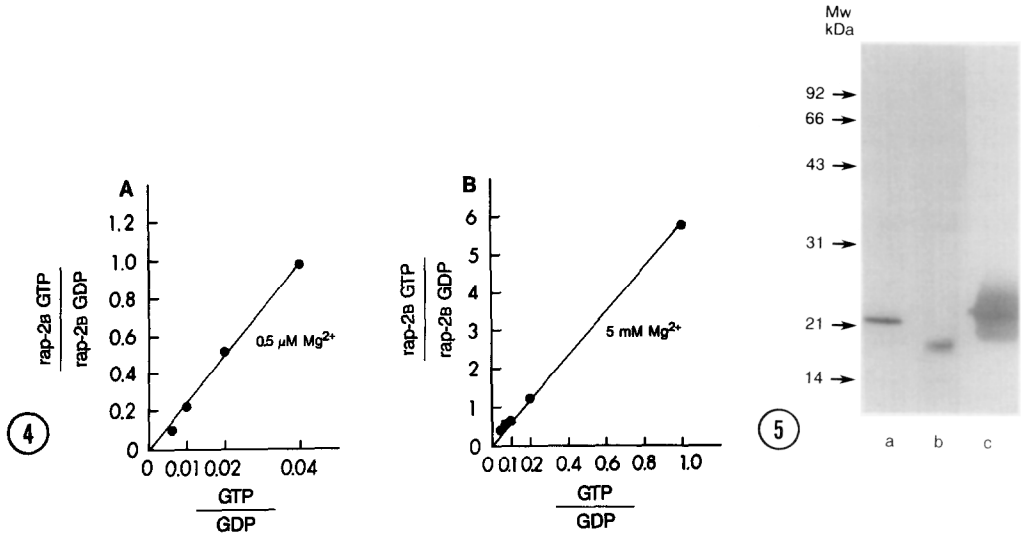


Fig. 4. Relative affinities of rap-2B for GTP and GDP. These reactions were carried out in 0.5 μM Mg^{2+} (A) or 5 mM Mg^{2+} (B). Rap-2B (0.5 μM) was incubated for 30 min at 37°C (A) or 2 hours (B) in a solution (50 μl) containing [α-^{32}P]GTP (2 μM) and variable concentrations of unlabeled GDP (2 to 300 μM). The relative association constant was calculated as previously reported (6).

Fig. 5. Identification of rap-2B in platelet membranes. Human platelets were lysed and the particulate (a) and cytosolic (b) fractions were prepared. Proteins from these fractions were separated by SDS-PAGE and Western blotted with the rap-2B antiserum (1:100 dilution). Antibody binding was detected by incubation of the blots with affinity-purified goat antibody $F(ab1)_2$ fragment to rabbit IgG labeled with ^{125}I. Lane c shows purified recombinant rap-2B protein. Due to the high amount of recombinant rap-2B loaded in lane c, the photographic exposure of this lane was much shorter than that for lanes a and b.

at 0.5 μM Mg^{2+} than at 5 mM Mg^{2+} (Fig. 2). The effect of Mg^{2+} on the exchange rate for GDP and GTP has previously been observed for N-ras p21 (6), rho and R-ras (7).

The half-lives of the rap-2B.GDP and rap-2B.GTP complexes were studied by preincubating rap-2B with radioactive GDP or GTP for 2 h and then incubating the complexes with 2 mM GTP in the presence of 0.5 μM Mg^{2+} or 5 mM Mg^{2+} for various times (Fig. 3A and 4B). Fig. 3A shows the effect of Mg^{2+} on the off-rate of GDP. The half-life for the GDP complex in the presence of 5 mM Mg^{2+} was about 12 min. However, at low Mg^{2+} concentration (0.5 μM), the half-life was less than 1 min. These rates are similar to those previously reported for N-ras p21 (6), rho and R-ras (7). However, when GTP is prebound, the effect of Mg^{2+} on dissociation is not as pronounced (Fig. 3B); this indicates that the rap-2B.GTP complex is more stable than the rap-2B.GDP complex at a low concentration of Mg^{2+}. The dissociation rate constant of GTP for ras, rho and normal R-ras at low Mg^{2+} has been reported to be less than 1 min (6,7). Our preparation provided a fast exchange rate for GDP to

GTP at low Mg^{2+}, but a very slow GTP to GTP exchange rate under the same conditions. The GTP "off" rate was also studied using [γ-^{32}P]GTP, and a similar stoichiometry was obtained after the 2 h exchange period. Therefore, it is valid to consider the GTP dissociation as a reaction, which is kinetically independent of GTP hydrolysis.

The relative affinities for GTP and GDP were further studied by incubating rap-2B with [α-^{32}P]GTP in the presence of variable concentrations of nonradioactive GDP until equilibrium was reached. At 0.5 μM Mg^{2+} the affinity of rap-2B for GTP is 25-fold greater than for GDP (Fig. 4A). When 5 mM Mg^{2+} is present the affinity of rap-2B for GTP is six times greater than that for GDP (Fig. 4B). In the presence of 5 mM Mg^{2+}, the affinities of N-ras p21 for GTP and GDP are the same (6).

It has been advanced that the concentration of Mg^{2+} plays a pivotal role in the conformation of N-ras p21. Removal of Mg^{2+} causes an open conformation of N-ras p21 that allows free exchange with exogenous nucleotides. The exchange rate is very slow at high Mg^{2+} concentration, corresponding to a closed conformation of the protein (6). We have observed that Mg^{2+} has a similar effect on guanine nucleotide exchange to rap-2B. However, the relative affinity of rap-2 for GTP in the open (low Mg^{2+}) and closed (high Mg^{2+}) conformation is higher than that for GDP.

The cellular distribution of rap-2B in human platelets was examined by immunoblotting particulate and cytosolic fractions and probing with the rap-2B antiserum (Fig. 5). All of the immunoreactive rap-2B protein Mr ~21000 was present in the particulate fraction. The immunoreactive bacterially expressed rap-2B protein is also shown in Fig. 5. The cytosolic fraction does not contain the 21 kDa form of rap-2B but the antiserum recognizes a protein with a lower molecular weight (Fig. 5).

DISCUSSION

Pizon et al. (3,10) initially isolated rap-1A, rap-1B and rap-2B while screening a human cDNA library from Raji human Burkitt lymphoma cells at low stringency with the Drosophila D*ras*3 gene. Their interest in the D*ras*3 gene product, and its potential similar human counterparts, was based on the observation that amino acid 61 of the D*ras*3 gene product is a threonine residue instead of the glutamine residue that is in normal *ras*. Der et al. (11) showed that almost every change at amino acid 61 in H-*ras* is associated with transformation.

We have found a new member of the rap family of proteins, rap-2B, which is 89% identical to rap-2 (3). Rap-2B is a membrane-bound protein in human platelets. Rap-2B is 61% identical to rap-1A and rap-1B (3,10) and 46% homologous to K-*ras*. The highest homology for rap-2B and K-*ras* was in the regions that contain residues involved in binding guanine nucleotides. Also

conserved between K-*ras* and rap-2B are amino acids 32-42, the effector domain, which are implicated in the interaction with GAP. Rap-2B also contains the carboxyl-terminal CAAX motif thought to be determinant for processing and membrane anchoring of the *ras* proteins (12-15).

The nucleotide exchange of rap-2B is slow in the presence of a high concentration of Mg^{2+}, and it is very rapid in the presence of a low concentration of Mg^{2+}. The preference of rap-2B for GTP and the stability of the rap-2B.GTP complex might be important in the possible biological actions of this protein. It was proposed that in vivo formation of p21-ras.GTP is favored after activation of the exchange reaction (GDP for GTP) by an activated receptor. Since the intracellular millimolar (16) concentrations of GTP and GDP are believed to be in the ratio 25:1 (17), we expect the relative affinities of rap-2B for GTP or GDP to govern which nucleotide is bound to rap-2B.

If rap-2B is proven to have antioncogenic activity, as has been shown for Krev-1 (18) [Krev-1 is identical to rap-1A, (3)], it might be due to its high relative affinity for GTP and the stability of the rap-2B.GTP complex.

REFERENCES

1. Wicki, A.N., Walz, A., Gerber-Huber, S., Wenger, R.H., Vornhagen, R. & Clemetson, K.J. (1989) *Thromb. Haemostasis,* **61**, 448-453.
2. Lacal, J.C., and Aaronson, S.A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5400-5404.
3. Pizon, V., Chardin, P., Lerosey, I., Olofsson, B., and Tavitian, A. (1988) *Oncogene* **3**, 201-204.
4. Farrell, F.X., Ohmstede, C.-A., Reep, B.R. and Lapetina, E.G. (1990) *Nucl. Acid. Res.*, in press.
5. Ohmstede, C.A., Farrell, F.X., Reep, B.R., Clemetson, K.J., and Lapetina, E.G. (1990) *Proc. Natl. Acad. Sci.*, in press.
6. Hall, A., and Self, A.J. (1986) *J. Biol. Chem.* **261**, 10963-10965.
7. Garrett, M.D., Self, A.J., van Oers, C., and Hall, A. (1989) *J. Biol. Chem.* **264**, 10-13.
8. Adari, H., Howy, D.R., Willumsen B.M., Der, C.J., and McCormick F., (1988) *Science* 240, 518-521.
9. Aebersold, R.H., Leavitt, J., Saavedra, R.A., Hood, L.E., and Kent, S.B.H. (1987) *Proc. Natl. Acad. Sci.* **84**, 6970-6974.
10. Pizon, V., Lerosey, I., Chardin, P. and Tavitian, A. (1988) *Nucl. Acid. Res.* **16**, 7719.
11. Der, C.J., Finkel, T. & Cooper, G.M. (1986) *Cell* **44**, 167-176.
12. Hancock, J.F., Magee, A.I., Childs, J.E. and Marshall, C.J. (1989) *Cell* **57**, 1167-1177.
13. Gutierrez, L., Magee, A.I., Marshall, C.J. and Hancock, J.F. (1989) The *EMBO J.* **8**, 1093-1098.
14. Clarke, S., Vogel, J.P., Deschenes, R.J., and Stock, J. (1988) *Proc. Natl. Acad. Sci.* **85**, 4643-4647.
15. Gutierrez, L., Magee, A.I., Marshall, C.J. and Hancock, J.F. (1989) The *EMBO J.* **8**, 1093-1098.
16. Goodrich, G.A. and Burrell, H.R. (1982) *Anal. Biochem.*, **127**, 395-401.
17. Proud, C.G. (1986) *Trends Biochem. Sci.* **11**, 73-77.
18. Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y., and Noda, M. (1989) *Cell* **856** 77-84.