Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 174-179

ROLE OF BOUND GDP IN THE STABILITY OF THE RHO A-RHO GDI COMPLEX PURIFIED FROM NEUTROPHIL CYTOSOL

Nicolas BOURMEYSTER[1]*, Patrice BOQUET[2] and Pierre V. VIGNAIS[1]*

[1]Laboratoire de Biochimie (URA 1130/CNRS), Département de Biologie Moléculaire et Structurale, CEA/Centre d'Etudes Nucléaires de Grenoble, 38054 Grenoble cedex 9, France

[2]Unité des Antigènes Bactériens, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris, France

Received September 19, 1994

Summary. The rho A-rho GDI complex purified from bovine neutrophil cytosol was found to contain GDP as the only bound nucleotide at a ratio of 1 mol of GDP per mol of complex. The rho GDI component of the complex (pI 4.8-5.0, apparent molecular mass 28-29 kDa) and the rho A component (pI scattered between 5.0-6.2, apparent molecular mass 24 kDa) were resolved by 2D gel electrophoresis. Upon dephosphorylation of bound GDP by apyrase, the rho A component of the complex was prone to proteolytic cleavage. The integrity of rho A in the presence of apyrase was preserved by addition of excess GTP. These data suggest that rho A liganded by GDP in the rho A-rho GDI complex is maintained in a conformation that escapes action of proteases.

Like all G proteins, rho proteins are present in cells in two interconvertible forms, one bound to GDP and the other to GTP (1). The GDP/GTP exchange is under the control of two regulatory enzymes, one of which inhibits the exchange (GDI) (2) and the other acts as a stimulator (GDS) (3). A GDI protein of 27-29 kDa apparently specific for the proteins of the rho family and referred to as rho-GDI (4) was reported to interact specifically *in vitro* with both the GDP- and GTP-bound forms of rho (5, 6). Association between rho proteins and rho GDI probably occurs through hydrophobic interactions between an hydrophobic sequence of rho GDI and the geranylgeranyl tail of processed rho. We recently described the purification of a native rho A-rho GDI complex from the cytosolic fraction of a bovine neutrophil homogenate (7). We have investigated the nature of the bound guanine nucleotide

*To whom correspondence should be addressed: DBMS/BCH, CEN-G, 17 rue des Martyrs, 38054 Grenoble cedex 9, France. Fax No: (33) 76.88.51.85.

Abbreviations: **TCA**: Trichloracetic acid ; **rho-GDI**: GDP dissociation inhibitor for proteins of the rho family ; **GDS**: GDP dissociation stimulator ; **HPLC**: high performance liquid chromatography ; **SDS-PAGE**: Na dodecyl sulfate polyacrylamide gel electrophoresis ; **IEF**: isoelectric focalisation ; **C3 exoenzyme**: C3 ADP-ribosyltransferase from *Clostridium botulinum* ; **CAAX**: peptide sequence at the C terminus of monomeric G proteins, where C is cysteine, A's are two aliphatic amino acids and X is any amino acid.

0006-291X/94 $5.00

in the native rho A-rho GDI complex and have also examined the effect of the enzymatic dephosphorylation of the bound nucleotide on the stability of the complex. The results indicate that rho A in the native rho A-rho GDI complex is bound to GDP and that bound GDP stabilizes rho A in a conformation not susceptible to proteolytic attack. Furthermore, rho A is present in the rho A-rho GDI complex under a number of forms that differ by their pI's in IEF and that probably arise by post-translational modifications of the C terminal CAAX motif.

EXPERIMENTAL PROCEDURES

Materials. The C3 ADP-ribosyltransferase was purified from *Escherichia coli* transformed by a plasmid carrying the cloned gene of C3 (8). The rho A-rho GDI complex was purified in the native form from the cytosol of bovine neutrophils as described (7). Apyrase (grade III) was from Sigma. [^{32}P]NAD was obtained from NEN (USA). All other chemicals were of the highest grade commercially available.

Protein assays. Protein concentration was determined by the method of Bradford (9). Two dimensional gel electrophoresis consisting of IEF followed by SDS-PAGE was carried out by the method of O'Farrell (10) with some modifications (7).

[^{32}P]ADP-ribosylation. The rho A component of the rho A-rho GDI complex was ADP-ribosylated by C3 exoenzyme from *Clostridium botulinum*, as described (11).

Anti rho GDI antibodies. A peptide corresponding to the amino acid sequence 164-175 in rho GDI (EAPKGMLARGSY) was synthesized and coupled by the C terminal tyrosine to limpet haemocyanin in the presence of bisdiazotized benzidine. The purified conjugate containing 15 to 20 peptides per haemocyanin was injected into rabbits according to a described immunization schedule (12). The specific antibodies were then purified by affinity chromatography on a peptide-coupled affigel 10 column.

Western blotting. The protein components of the rho A-rho GDI complex were resolved by IEF followed by SDS-PAGE. The proteins were then electroblotted onto nitrocellulose. The nitrocellulose paper was incubated with the anti-GDI antibodies. The bound antibodies were detected with a goat antirabbit IgG coupled to peroxidase, and the bound peroxidase was revealed by a luminescence method using the ECL kit from Amersham.

Identification and quantification of the guanine nucleotides bound to the native rho A-rho GDI complex. Bound guanine nucleotides were extracted from the rho A-rho GDI complex with 10% TCA at 2-4°C for 1 to 2 h. The denatured protein was sedimented by a 10 min centrifugation at 10000g. Nucleotides in the TCA extract were resolved by HPLC using a column of Partisil SAX-10 (100 mm x 8 mm) equilibrated with 1 mM phosphate buffer pH 4.0. The column was eluted with a linear gradient of phosphate buffer pH 4.0 from 1 mM to 750 mM at a flow rate of 2 ml/min. Elution of nucleotides was monitored at 252 nm. The amount of guanine nucleotides was determined by peak integration and calculated by reference to standard samples.

RESULTS

Nature and stoichiometry of the bound guanine nucleotide in the native rho A-rho GDI complex from bovine neutrophil cytosol

The TCA extract of 0.8 nmole (40 μg) of the native rho A-rho GDI complex was subjected to HPLC using a Partisil-SAX-10 column. Elution of the column revealed only one peak with a retention time (10.0 min) identical to that of a standard solution of GDP (Figure 1A). The amount of GDP present in this peak was estimated at 0.6 nmole by peak

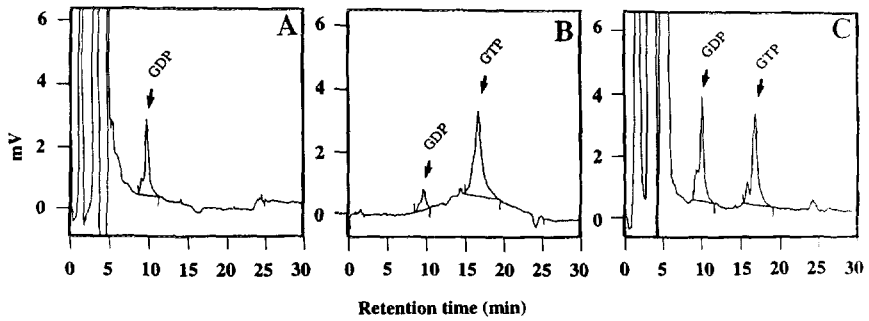

Figure 1. HPLC analysis of nucleotides present in a TCA extract of the native rho A-rho GDI complex from bovine neutrophil cytosol. HPLC was carried out with a Partisil 10SAX column (cf. Experimental Procedures). **A**, Analysis of nucleotides present in a TCA extract from 0.8 nmole of native rho A-rho GDI complex (the eluted peak corresponds to 0.6 nmole of GDP) ; **B**, resolution of GDP and GTP in a commercial sample of 1.4 nmole of GTP (HPLC analysis gave values of 1.2 nmole of GTP and 0.2 nmole of GDP) ; **C**, analysis of nucleotides in a TCA extract containing a mixture of 1.4 nmole of commercial GTP (1.2 nmole of GTP plus 0.2 nmole of GDP) and nucleotides released from 0.8 nmole of rho A-rho GDI complex by TCA treatment.

migration, which corresponded to a molar ratio of GDP to rho A-rho GDI of 0.75. Since two proteins of the rho family, rac and CDC 42, loaded with GTP have been reported to combine with rho GDI *in vitro* (5) (13) (14), we decided to explore the possibility that a putatively bound GTP in the rho A-rho GDI complex could have been degraded into GDP during TCA extraction. An aliquot of commercial GTP analyzed by HPLC and found to consist of 1.2 nmole of GTP (retention time 17.1 min) and 0.2 nmole of contaminant GDP (Figure 1B) was added to another 0.8 nmole sample of native rho A-rho GDI complex. The mixture was treated by TCA, and the TCA extract was subjected to HPLC (Figure 1C). The eluted amount of GDP was 0.9 nmole and that of GTP was 1.1 nmole. These experimental values were very close to the theoritical ones, 0.8 nmole GDP and 1.2 nmole GTP. Clearly, TCA extraction had no deleterious effect on the added GTP. This experiment was repeated twice with samples of rho A-rho GDI complex purified from different batches of bovine neutrophil cytosol. GDP was found to be the only nucleotide present in the extracts, and the molar ratios of GDP to rho A-rho GDI were 0.75 and 0.80. Taken together, these results allow us to conclude that GDP is the only nucleotide present in the native rho A-rho GDI complex at a ratio of one mole of GDP bound to rho A per mole of complex.

Cleavage of bound GDP makes the rho A component of the native rho A-rho GDI complex susceptible to proteolytic attack

To identify the rho A component of the rho A-rho GDI complex after electrophoretic migration, the complex was incubated with [^{32}P]NAD and the C3 exoenzyme from *Clostridium botulinum*, which resulted in the [^{32}P]ADP-ribosylation of rho A. Identification of rho A was also performed with a monoclonal antibody (gift of Dr J. Bertoglio). Rho GDI was revealed by immunoreaction with anti rho GDI antibodies.

Following IEF of the [^{32}P]ADP-ribosylated rho A-rho GDI complex in an acrylamide gel loaded with ampholines but devoid of urea, both rho A and rho GDI were found to be

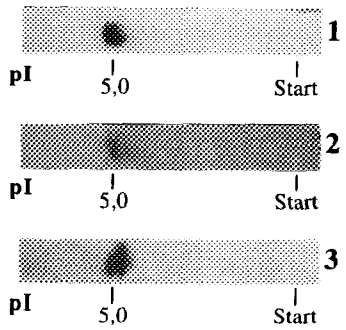

Figure 2. Isoelectric focusing of the [^{32}P]ADP-ribosylated rho A-rho GDI complex (2 µg) in 10% acrylamide gels loaded with a mixture of 2% ampholines pH 3-10 and ph 4-6. The gels did not contain urea. *(1)* autoradiograph ; *(2)* Immunoblot carried out with a monoclonal anti rho A antibody ; *(3)* Immunoblot carried out with polyclonal anti rho GDI antibodies. The double spot revealed in the gels might reflect the presence of a modified form of rho GDI.

located at the same place in the gel corresponding to a pI of 4.8-5.0 (Figure 2 - *1, 2, 3*). ADP-ribosylation did not modify the electrophoretic properties of rho A (Figure 2 - ***1 and 2***).

When 8 M urea was added to the acrylamide gel loaded with ampholines, the components of the rho A-rho GDI complex migrated separately. Figure 3 (A and B) shows the localization of rho GDI revealed by Western blot using specific antibodies as well as that of [^{32}P]ADP-ribosylated rho A detected by autoradiography after a 2D gel electrophoresis (IEF/urea followed by SDS-PAGE). All rho GDI focused at a pI of 4.8-5.0 accompanied by a minor fraction of rho A. The two proteins were separated by SDS-PAGE, on the basis of their apparent molecular mass of 29 kDa and 24 kDa, respectively (Figure 3 A and B). A major fraction of rho A focused at a pI of 6.2 while the remaining rho A was resolved in a discrete number of bands with pI's scattered between 5.4 and 5.8, sometimes confluent.

To test the role played by bound GDP, the [^{32}P]ADP-ribosylated rho A-rho GDI complex was treated by apyrase, an enzyme which cleaves pyrophosphate bonds. The autoradiograph in Figure 3C shows that, following degradation of bound GDP by apyrase, the electrophoretic migration was strikingly modified. The pI's of the [^{32}P] labeled spots were all shifted to values comprised between 3.5 and 4.2, and their molecular masses were scattered between 19 kDa and 14 kDa, indicating that peptide bonds in the [^{32}P]ADP-ribosylated rho A had been cleaved by protease contaminants probably present in the apyrase sample. In contrast, the migration of the rho GDI component of the complex was not modified (not shown). When a large excess of GTP (100 µM) was added together with apyrase, the 2D electrophoretic migration pattern (Figure 3D) remained essentially similar to that of the control (Figure 3B), and a smaller amount of rho A cleavage products could be detected. Selective degradation of rho A in the rho A-rho GDI complex treated by apyrase was amplified by traces of trypsin added after incubation with apyrase ; it was prevented by GTP added before apyrase (not shown). We therefore conclude that added GTP offered as a substrate to apyrase prevents the enzymatic cleavage of the bound GDP in the rho A-rho GDI complex. Taken together these results lead us to postulate that bound GDP in the rho A-rho GDI complex stabilizes a conformation of rho A which escapes proteolytic attack.

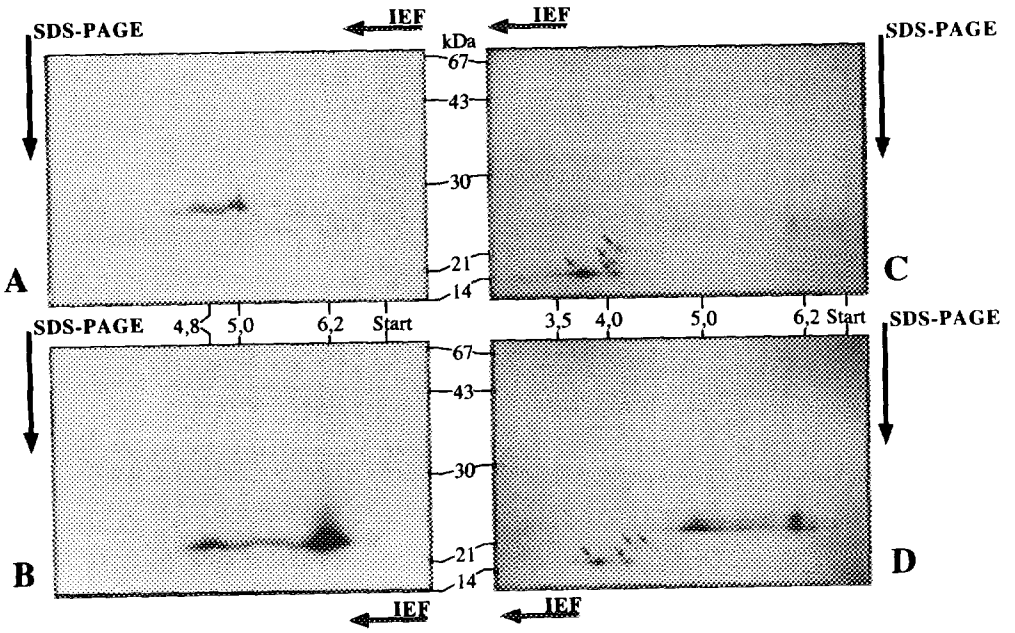

Figure 3. 2D-PAGE electrophoresis of the [^{32}P]ADP-ribosylated rho A-rho GDI complex. Isoelectric focusing was carried out in an acrylamide gel containing ampholines (cf legend of Figure 1) and supplemented with 8 M urea (1st dimension). This was followed by SDS-PAGE (2nd dimension). Panels A and B correspond to the native rho A-rho GDI complex (2 µg). **A** is a Western blot of the 2D gel corresponding to the immunodetection of rho GDI by specific antipeptide antibodies ; **B** is an autoradiograph of the 2D gel showing one major spot of [^{32}P]ADP-ribosylated rho A at pH 6.2 and two minor bands at pH 4.8-5.0 and at pH 5.4-5.8 ; **C**, the [^{32}P]ADP-ribosylated rho A-rho GDI complex (2 µg) was incubated with 2 units of potato apyrase for 10 min at 30°C and then subjected to 2 D electrophoresis. The figure shows the autoradiograph of the 2D gel ; **D**, same experiment as in C, except that GTP was added at the final concentration of 100 µM in the incubation medium together with apyrase.

DISCUSSION

The experiments described in this paper demonstrate that the rho A component of the native rho A-rho GDI complex is liganded exclusively by GDP. Bound GDP appears to be an essential parameter for the stability of the rho A-rho GDI complex, as shown by an increased susceptibility of unliganded rho A to proteolytic attack. In non denaturating conditions (absence of urea in the gel), the two components of the rho A-rho GDI complex focused at a pI of 4.8-5.0, suggesting that the surface charges of rho GDI contribute to a large extent to the pI of the whole complex.

Isoelectric heterogeneity of rho proteins was previously mentioned (15) ; however the analyzed sample was a crude extract of adrenal glands which could have contained multiple molecular species of C3 substrate. Our study, carried out with a homogeneous rho A-rho GDI complex, demonstrates that isoelectric heterogeneity is a genuine property of rho A associated with rho GDI. A small fraction of rho A comigrated with rho GDI with a pI of 4.8-5.0, pointing to the presence of a minor amount of undissociated rho A-rho GDI complex despite the presence of urea in the gel. The bands of rho A with pI's of 6.2 and 5.4-5.8 were likely to correspond to modified forms of rho A arising during the post-translational processing at the

C terminus. Processing of rho proteins occurs in two steps, which consist in the addition of a geranylgeranyl tail to the cysteinyl residue of the C terminal CAAX box, followed by proteolytic cleavage of the C terminal peptide AAX and methylation of the free carboxyl group of cysteine. In contrast to prenylation which is not readily reversible, carboxymethylation is characterized by a rapid turnover (16), which is consistent with the fact that the extent of carboxymethylation of ras-related proteins in neutrophils is rapidly increased in response to chemoattractants (17). On the other hand, whereas geranylgeranylation and AAX release from the CAAX motif of rho is required for interaction with rho GDI, methylesterification of the C-terminal cysteine is not required (5). In brain extracts, rho GDI was found to be associated with a soluble pool of prenylated, but unmethylated CDC 42 Hs, a small G protein of the rho family (18, 19). In the case of the neutrophil [^{32}P]ADP-ribosylated rho A-rho GDI complex, the detection of labeled bands with pI's scattered between 6.2 and 5.4 might reflect the presence in the complex of at least two forms, methylated and unmethylated, of prenylated rho A, each form assuming a given conformation characterized by a specific pI.

Acknowledgments. This work was supported in part by grants from the "Centre de la Recherche Scientifique (URA 1130)", from the "Association pour la Recherche sur le Cancer" and from the "Université Joseph Fourier, Faculté de Médecine de Grenoble". The authors thank Alexandra Fuchs for critically reading the manuscript, Drs Jacques Bertoglio and Paul Lang for the gift of a monoclonal rho A antibody, and Mrs Jeannine Bournet for her excellent secretarial assistance.

REFERENCES

1. Hall, A. (1990) Sciences **249**, 635-640.
2. Ohga, N., Kikuchi, A., Ueda, T., Yamamoto J. and Takai, Y. (1989) Biochem. Biophys. Res. Commun. **163**, 1523-1533.
3. Yamamoto, T., Kaibuchi, K., Mizuno, T., Hiroyoshi, M., Shirataki, H. and Takai, Y. (1990) J. Biol. Chem. **265**, 16626-16634.
4. Ueda, T., Kikuchi, A., Ohga, N., Yamamoto, J. and Takai, Y. (1990) J. Biol. Chem. **265**, 9373-9380.
5. Hancock, J. F. and Hall, A. (1993) Embo J. **12**, 1915-1921.
6. Sasaki, T., Kato, M. and Takai, Y. (1993) J. Biol. Chem. **268**, 23959-23963.
7. Bourmeyster, N., Stasia, M.-J., Garin, J., Gagnon, J., Boquet, P. and Vignais, P. V. (1992) Biochemistry **31**, 12863-12869.
8. Popoff, M. R., Hauser, D., Boquet, P., Eklund, M. W. and Gill, D. M. (1991) Infect. Immun. **59**, 3673-3679.
9. Bradford, M. M. (1976) Anal. Biochem. **72**, 248-254.
10. O'Farrel, P. H. (1975) J. Biol. Chem. **250**, 4007-4021.
11. Chardin, R., Boquet, P., Madaule, P., Popoff, M. R., Rubin, E. J. and Gill, D. M. (1989) Embo J. **8**, 1087-1092.
12. Brandolin, G., Boulay, F., Dalbon, P. and Vignais, P. V. (1989) Biochemistry **28**, 1093-1100.
13. Chuang, T.-H., Bohl, B. P. and Bokoch, G. M. (1993) J. Biol. Chem. **268**, 26206-26211.
14. Hart, M. J., Maru, Y., Leonard, D., Witte, O. N., Evans, T. and Cerione, R. A. (1992) Science **258**, 812-815.
15. Narumiya, S., Morii, N., Ohno, K., Ohashi, Y. and Fujiwara, M. (1988) Biochem. Biophys. Res. Commun. **150**, 1122-1130.
16. Chelsky, D., Ruskin, B. and Koshland, D. E. (1985) Biochemistry **24**, 6651-6658.
17. Philips, M. R., Pillinger, M. H., Stand, R., Volker, C., Rosenfeld, M. G., Weissman, G. and Stock, J. B. (1993) Biochem. Biophys. Res. Commun. **259**, 977-980.
18. Backlund, P. S. (1992) J. Biol. Chem. **267**, 18432-18439.
19. Backlund, P. S. (1993) Biochem. Biophys. Res. Commun. **196**, 534-542.