BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **218,** 54–60 (1996)
Article No. 0011

Phosphorylation of Rho GDI Stabilizes the Rho A-Rho GDI Complex in Neutrophil Cytosol

Nicolas Bourmeyster and Pierre V. Vignais[1]

Laboratoire de Biochimie (URA 1130 CNRS), Départment de Biologie Moléculaire et Structurale, CEA/Grenoble, 38054 Grenoble cedex 9, France

Received November 13, 1995

The GDP dissociation inhibitor Rho GDI from bovine neutrophil cytosol was purified in association with prenylated Rho A. Upon treatment of this complex with alkaline phosphatase, the Rho A and Rho GDI components were released to their free forms. Following migration in 2D-PAGE and specific immunodetection, the shape of the spot of Rho GDI was found to depend markedly on whether Rho GDI subjected to electrophoresis was present in a Rho A-Rho GDI complex or in a free form. In the first case Rho GDI focused as an elongated spot between pI 5.2 and pI 4.6 whereas in the later case it focused at a pI of 5.0-5.2 as a round spot. Activation of neutrophils by anaphylatoxin C5a in a [^{32}Pi] supplemented medium resulted in radiolabeling of Rho GDI. *In vitro* incubation of Rho GDI with a neutrophil homogenate in the presence of [γ^{32}P] ATP led also to radiolabeling of Rho GDI. Taken together these results suggest that Rho GDI in the Rho A-Rho GDI complex is phosphorylated and that the stability of the complex depends on the phosphorylation state of Rho GDI.

GDI (GDP dissociation inhibitors) are cytosolic proteins which inhibit GDP dissociation from small G proteins, and thereby the GDP exchange for GTP. Among the small G proteins, the Rab (1) and Rho/Rac subfamilies (2, 3) have been shown to bind specific GDIs. Rho-GDI, a protein of 27–29 kDa (2) is able to interact with Rho, Rac and CDC42Hs (3). A Rho A-Rho GDI complex was isolated and purified from bovine neutrophil cytosol (4). In this complex, GDP was found to be the only nucleotide bound to the Rho A component, and loss of bound GDP resulted in decreased stability of the complex (5). Phosphorylation of Rab GDI (6) and Ly GDI (7) or D4 (8), a Rho GDI related G protein abundant in lymphocytes, has been reported. In contrast, no phosphorylation was detected in three isoforms of Rho GDI expressed in human keratinocytes (9). In this paper, we described experiments which indicate that the Rho GDI component of the cytosolic Rho A-Rho GDI complex present in neutrophils is phosphorylated and that this phosphorylation is largely responsible for the stability of the complex.

EXPERIMENTAL PROCEDURES

Materials. The C3 ADP-ribosyltransferase from *Clostridium botulinum* was a kind gift from Dr. Patrice Boquet (Pasteur Institute, Paris). The Rho A-Rho GDI complex was purified from the cytosol of bovine neutrophils (4). Alkaline phosphatase was obtained from Boehringer, [^{32}P]NAD from NEN (USA), and [γ^{32}P]ATP and carrier-free ^{32}Pi from Amersham (England). All other reagents were of the highest grade commercially available. Bovine neutrophils were routinely isolated from 10 L of fresh blood (10).

Protein assays. Protein concentration was determined using the BCA protein assay (11). Bovine serum albumin was used as standard, and appropriate blanks for the medium were carried out.

Two-dimensional gel electrophoresis (2D-PAGE). The method of O'Farrell (12) consisting of IEF followed by SDS-PAGE was used with slight modifications (13). Proteins on nitrocellulose membranes were revealed by staining with colloidal gold (14).

Western blotting. The protein components of the Rho A-Rho GDI complex were resolved by 2D-PAGE. The resolved

[1] to whom correspondence should be addressed: DBMS/BCH, CEA/Grenoble, 17 rue des Martyrs, 38054 Grenoble cedex 9, France. Tel. (33) 76.88.30.36, Fax (33) 76.88.51.85.

Abbreviations: IEF: isoelectric focalisation; GDI:GDP dissociation inhibitor; PAGE: polyacrylamide gel electrophoresis; DTT: dithiothreitol; DMEM: Dulbecco minimum essential medium.

0006-291X/96 $12.00

proteins were electroblotted onto nitrocellulose. The nitrocellulose membrane was incubated with affinity purified rabbit antipeptide antibodies against the amino acid sequence 164-175 in Rho GDI (EAPKGMLARGSY), as described (5). The bound antibodies were detected with a goat antirabbit IgG coupled to peroxidase, and the bound peroxidase was revealed by luminescence using the ECL kit from Amersham.

[^{32}P]ADP-ribosylation. The Rho A component of the Rho A-Rho GDI complex was [^{32}P]ADP-ribosylated in the presence of [^{32}P]NAD by C3 exoenzyme from *Clostridium botulinum* as described (15).

In vivo phosphorylation. Bovine neutrophils (3.10^8 cells) were first washed twice with a buffer consisting of 175 mM NaCl, 2.5 mM KCl and 10 mM Hepes pH 7.5 to remove traces of inorganic Pi. They were then incubated for 2 h at 37°C in a 10 ml of DMEM devoid of Pi and supplemented with 1 mCi [^{32}Pi] to allow basal protein phosphorylation to proceed. Then 100 nM C5a was added and neutrophil activation was let to proceed for 2–3 min at 37°C. This was followed by centrifugation of the cells at low speed and washing twice with a buffer consisting of 10 mM Tris pH 7.5, 150 μM NaCl, 1 mM EDTA and 1% Triton X-100 supplemented with 2 μM leupeptin, 1 μM pepstatin, 1 mM diisopropyl fluorophosphate, 10 mM NaF, 1 mM p-nitrophenyl pyrophosphate and 10 mM Pi. The lysate was centrifuged for 5 min at 12000g and the supernatant was subjected to 2D-PAGE. The radioactive spots were visualized using a PhosphorImager apparatus (Molecular Dynamics).

In vitro phosphorylation. A sample of 2 μg of Rho A/Rho GDI complex in 100 μL of medium consisting of 100 μM [γ^{32}P]ATP 10^7 dpm and 125 nM okadaic acid was incubated with 2 γg protein of a bovine neutrophil homogenate that had been previously centrifuged at 10.000g for 10 min at 4°C to remove unbroken cells, nuclei and a large proportion of granules. Incubation lasted for 1 h at 37°C and was terminated by addition of 50 μL of buffer consisting of 10 M urea, 2% Triton X-100, 5% ampholines (pH range 3.5–9.5) and 5% β-mercaptoethanol. The lysate was subjected to SDS-PAGE, and the [^{32}P]-phosphorylated proteins were revealed by autoradiography.

RESULTS

Effect of Alkaline Phosphatase on the Stability of the Rho A-Rho GDI Complex from Neutrophil Cytosol

Rho A can be differentiated from Rho GDI and the Rho A-Rho GDI complex by migration in 2D PAGE (4, 5). In fact Rho A migrates with an apparent mass of 24 kDa and a pI of 6.2 (16) whereas Rho-GDI has an apparent mass of 28 kDa and a pI of 5.0 (17). The Rho A-Rho GDI complex focused at the same pI as Rho GDI. In the experiment illustrated in Figure 1A the two components of the Rho A-Rho GDI complex purified from bovine neutrophil cytosol and ADP-ribosylated by [^{32}P]NAD in the presence of exoenzyme C3 from *Clostridium botulinum* were resolved by 2D-PAGE. Following IEF of the Rho A-Rho GDI complex in acrylamide gel loaded with ampholines and urea (first dimension), part of the [^{32}P] ADP-ribosylated Rho A was recovered undissociated from Rho GDI and visualized after SDS-PAGE (second dimension) as a radioactive spot corresponding to a mass of 24 kDa with a mean pI value of 5.0, whereas free [^{32}P]ADP-ribosylated Rho A focused at a pI of 6.2.

Following incubation of the [^{32}P]ADP- ribosylated Rho A-Rho GDI complex with alkaline phosphatase, the radioactive spot with a pI of 5.0 disappeared and all the radioactivity was recovered in a single spot with a pI of 6.2 and a mass of 24 kDa corresponding to free [^{32}P]ADP-ribosylated Rho A (Figure 1B). The splitting of the radioactive spot Rho A after SDS-PAGE may be due to an heterogeneous processing of its C-terminal region. The drastic effect of alkaline phosphatase on the migration of [^{32}P]ADP-ribosylated Rho A leads us to conclude that the strength of the association between Rho A and Rho GDI to form a complex depends on phosphorylation of amino acid residue(s) in the Rho A-Rho GDI complex.

In a complementary experiment, the purified Rho A-Rho GDI complex was subjected to a mild attack by alkaline phosphatase, so that only part of the Rho A-Rho GDI complex was dissociated. The mixture was chromatographed on a column of Mono Q HR5/5. The column was eluted with a NaCl gradient (0-200 mM) in a buffer consisting of 20 mM Tris buffer, 1 mM EGTA and 1 mM DTT, final pH 7.4. The Rho A-Rho GDI complex was eluted with 95-100 mM NaCl (4), and free Rho GDI was eluted later with 110-120 mM NaCl. Both eluted fractions were concentrated separately and subjected to a 2D-PAGE. The resolved proteins were electrotransferred onto nitrocellulose membranes. Proteins were revealed by staining with colloidal gold (Figures 2A, 2C), and

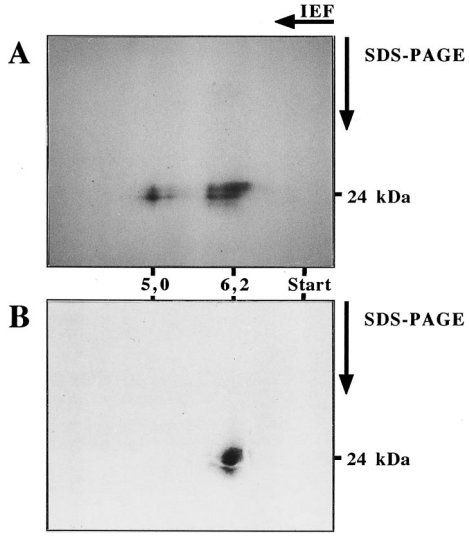

FIG. 1. Effect of alkaline phosphatase on the stability of the Rho A–Rho GDI complex. The Rho A component of the complex was [^{32}P]ADP-ribosylated by C3 exoenzyme. The figure shows the localization of radioactivity, using a PhosphorImager apparatus following 2D-PAGE of the Rho A–Rho GDI complex before treatment with alkaline phosphatase (A) and after treatment with alkaline phosphatase (B). In the latter case, a 100-ng protein sample of the [^{32}P]ADP- ribosylated Rho A–Rho GDI complex was incubated for 1 h at 37°C with 1 unit of alkaline phosphatase in 100 μl of 50 mM Tris, pH 8.5, and 0.1 mM EDTA.

Rho GDI was identified with specific Rho GDI antibodies (Figure 2B, 2D). The spot of immunodetected Rho GDI was virtually superimposable (Figure 2B) upon the gold-stained spot of Rho GDI (Figure 2A). The shape of this spot was characterized by a globular region corresponding to a pI of 5.0–5.2 and an elongated tail extending from a pI of 4.9 to a pI of 4.6 which appeared to consist of a few merged spots. In contrast with the Rho A-bound Rho GDI, free Rho GDI was revealed after gold staining (Figure 2C) and specific immunodetection (Figure 2D) as a rounded spot, focusing at pI 5.0–5.2 without any tail projecting towards acidic pHs.

In summary, migration of Rho GDI in the first dimension of 2D-PAGE, i.e in IEF, differed markedly whether the Rho A-Rho GDI complex was previously subjected or not to the action of alkaline phosphatase. In the absence of treatment by alkaline phosphatase, the Rho-GDI spot was characterized by an acidic tail. After treatment by alkaline phosphatase, the acidic tail disappeared, and the spot of Rho GDI adopted a rounded shape. Since alkaline phosphatase breaks phosphate ester bonds, it may be postulated that the acidic tail corresponds to a phosphorylated form of Rho GDI.

In vivo and in vitro Phosphorylation of Rho GDI

In vivo phosphorylation of Rho GDI was conducted as described under Experimental Procedures. Bovine neutrophils were first incubated with ^{32}Pi in a medium deprived of Pi for 2 h at 37°C to ensure a basal level of phosphorylation. This was followed by incubation with 100 nM C5a at 37°C for a further 2–3 min to activate neutrophils. The cells were then sedimented by centrifugation, washed twice with cold PBS and lysed with Triton X-100 in the presence of a cocktail of antiproteases. The Triton extract was subjected to 2D-PAGE. The resolved proteins were electrotransferred from the gel to a nitrocellulose sheet. Rho GDI was first identified with Rho-GDI antibodies, (cf. Experimental Procedures) (Figure 3B). Then, after washing, proteins were detected by gold staining (Figure 3A), and finally the [^{32}P] labeled proteins were visualized, using a PhosphoImager apparatus (Molecular Dynamics).

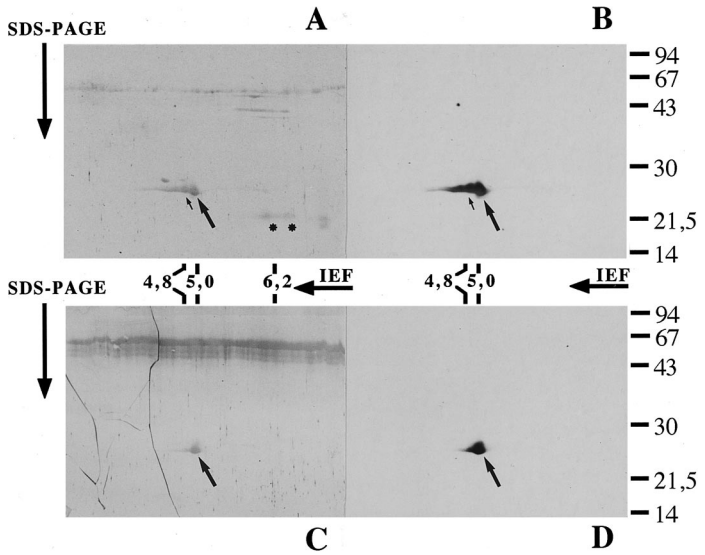

FIG. 2. Western blot analysis of the Rho GDI component of the Rho A–Rho GDI complex (A and B) and free Rho GDI (C and D). Purified Rho A–Rho GDI complex (A and B) and Rho GDI separated by chromatography from Rho A after treatment of the Rho A–Rho GDI complex by alkaline phosphatase were subjected to 2D-PAGE. After electrotransfer on nitrocellulose, Rho GDI protein was identified by specific antibodies. (A) and (C), Gold staining (Arrows refer to Rho GDI, and the star refers to Rho A); (B) and (D), Immunodetection of Rho GDI.

As in the preceding experiment the Rho-GDI protein of 28 kDa, immunoidentified by specific antibodies, focused at pI values ranging from 5.2 to 4.6 (3A and 3B). The [^{32}P] labeled spot corresponding to a 28 kDa protein with a pI of 5.2-5.0 was superimposable upon the less acidic portion of the immunodetected Rho-GDI spot. The localisation of the [^{32}P] radioactivity in the less acidic region of the Rho GDI spot might be explained by assuming that in intact neutrophils the Rho GDI component of the Rho A-Rho GDI complex is largely phosphorylated, which confers to the Rho GDI spot in IEF an elongated tail projecting towards acidic pHs. It is therefore presumed that *in vivo*, incorporation of ^{32}Pi can be achieved upon activation only in a minor fraction of dephosphorylated Rho-GDI, which corresponds to the less acidic region of the Rho GDI spot. In the absence of activation by C5a, incorporation of ^{32}Pi into Rho GDI was negligible. Cytochalasin B (5 μg/ml) added together with C5a slightly enhanced the extent of phosphorylation of Rho GDI (data not shown).

A complementary experiment consisting of the *in vitro* phosphorylation of Rho GDI is illustrated in Figure 4. An aliquot fraction of the purified Rho A-Rho GDI complex was mixed with an equal protein amount of bovine neutrophil homogenate devoid of nuclei, granules and unbroken cells, and was supposed to be a source of kinase(s) for Rho GDI. The mixture supplemented with [γ^{32}P]ATP was incubated at 37°C for 1 h. As in the preceding experiment, the immunodetected Rho GDI after 2D-PAGE and Western blotting (Figure 4A) appeared as an elongated spot comprising a globular region with a pI value of 4.8–5.0 and an acidic tail extending to a pI of 4.6. As in the *in vivo* experiment, the *in vitro* experiment revealed the presence of a radiolabeled spot coinciding with the immunodetected Rho GDI, with, however, this difference that in the present case, the incorporated radioactivity was predominantly localized in the more acidic portion of the Rho GDI spot. This may be explained in terms of a dephosphorylation-phosphorylation cycle at the level of Rho GDI due to the unmasking of the activity of endogenous phosphatase activity in the neutrophil homogenate. Endogenous phosphatase activity would contribute to release unlabeled bound phosphate from phosphorylated residue(s), thus making the dephosphorylated residues available for phosphorylation by added [γ^{32}P]ATP.

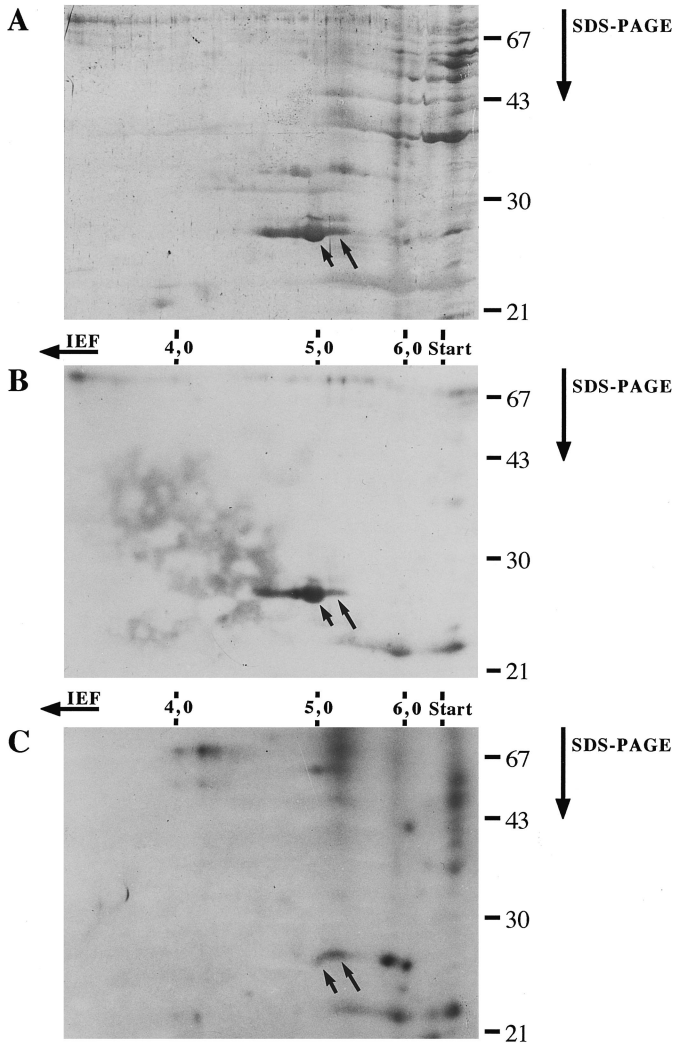

FIG. 3. Metabolic labeling of Rho GDI. Experimental conditions are detailed under Experimental Procedures. After incubation with [^{32}Pi], a cell extract was subjected to 2D-PAGE. Following protein electrotransfer on a nitrocellulose sheet, Rho GDI was identified by specific antibodies (B), then proteins were revealed by gold staining (A) and finally radioactivity was localized using a PhosphorImager apparatus (C). The Rho GDI spot in A appears more intense than it should, due to the presence of the antibody.

DISCUSSION

Bovine neutrophil cytosol contain relatively large amounts of the Rho A-Rho GDI complex (4). Two sets of results reported in this paper indicate that, in the Rho A-Rho GDI complex, Rho GDI is present in a phosphorylated form and that the strength of the association of Rho GDI with Rho A depends in part of the phosphorylation state of Rho GDI. 1-A molecular species identified as Rho GDI was shown to be phosphorylated not only *in vitro*, but also in activated neutrophils, 2- The Rho A-Rho GDI complex which focused in a pI range from 4.6 to 5.2 in IEF dissociated with the release of free Rho A and Rho GDI upon incubation with alkaline phosphatase.

The cytosolic Rho A-Rho GDI complex in which Rho A is liganded by GDP (5) is maintained in a non active conformation. On the other hand, free prenylated Rho A is anchored to membranes by its prenylated tail, and following replacement of bound GDP by GTP, it assumes an active

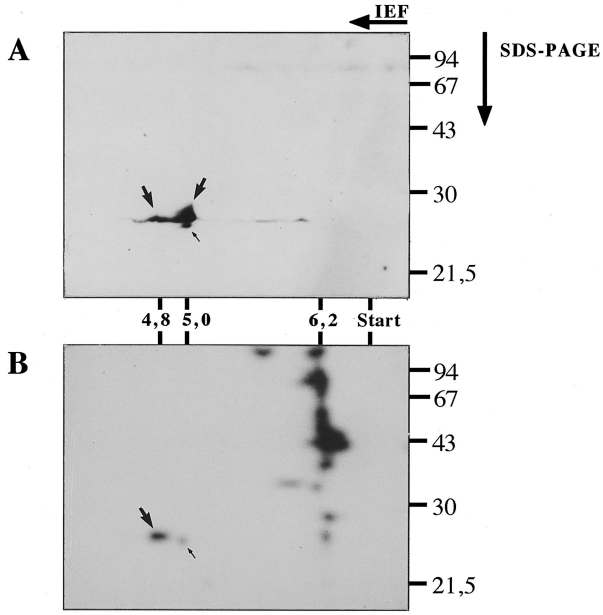

FIG. 4. *In vitro* phosphorylation of Rho GDI. Experimental conditions are described under Experimental Procedures. (A) Identification of Rho-GDI by specific antibodies, (B) Radiodetection of incorporated [^{32}Pi].

conformation. It is presumed that cycling of Rho GDI between membrane and cytosol is governed by subtle regulatory mechanisms. One of them might involve the participation of lipids and most particularly the dissociation of the complex by specific phosphoinositides (4). Another mechanism suggested by the present study is probably related to the activity of specific kinase(s) and phosphatase(s) which control the phosphorylation state of Rho GDI. In fact, phosphorylation of Rho GDI might contribute to trap efficiently Rho A by its prenyl tail into a hydrophobic sequence of Rho GDI. Conversely, dephosphorylation of phosphorylated Rho-GDI would release bound Rho GDI from Rho A. These considerations may apply as well to the association of Rho-GDI with other small G proteins of the Rho family, and particularly to Rac which is required for oxidase activation of neutrophils (18).

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, Dr. Patrice Boquet for a generous gift of *Clostridium botulinum.* C3 exoenzyme, and Mrs Jeannine Bournet for excellent secretarial assistance.

REFERENCES

1. Matsui, Y., Kikuchi, A., Araki, S., Hata, Y., Kondo, J., Teranishi, Y., and Takai, Y. (1990) *Mol. Cell Biol.* **10,** 4116–4122.
2. Ueda, T., Kikucki, A., Ohga, N., Yamamoto, J., and Takai, Y. (1990) *J. Biol. Chem.* **265,** 9373–9380.
3. Hiraoka, K., Kaibuchi, K., Ando, S., Musha, T., Takaishi, K., Mizuno, T., Asada, M., Menard, L., Tomhave, E., Didsbury, J. R., Snyderman, R., and Takai, Y. (1992) *Biochem. Biophys. Res. Commun.* **182,** 921–930.
4. Bourmeyster, N., Stasia, M.-J., Garin, J., Gagnon, J., Boquet, P., and Vignais, P. V. (1992) *Biochemistry* **31,** 12863–12869.
5. Bourmeyster, N., Boquet, P., and Vignais, P. V. (1994) *Biochem. Biophys. Res. Commun.* **205,** 174–179.
6. Steele-Mortimer, O., Gruenberg, J., and Clague, M. J. (1993) *FEBS Lett.* **329,** 313–318.
7. Scherle, P., Behrens, T., and Staudt, L. M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7568–7572.
8. Lelias, J.-M., Adra, C. N., Wulf, G. M., Guillemot, J.-C., Khagad, M., Caput, D., and Lim, B. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 1479–1483.

9. Leffers, H., Nielsen, M. S., Andersen, A. H., Honoré, B., Madsen, P., Vandekerckhove, J., and Celis, J. E. (1993) *Exp. Cell. Res.* **209,** 165–174.
10. Morel, F., Doussière, J., Stasia, M. J., and Vignais, P. V. (1985) *Eur. J. Biochem.* **152,** 669–679.
11. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Gocke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal.Biochem.* **150,** 76–85.
12. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021.
13. Stasia, M.-J., Dianoux, A.-C., and Vignais, P. V. (1989) *J. Biol. Chem.* **28,** 9659–9667.
14. Yamaguchi, K., and Asakawa, H. (1988) *Anal. Biochem.* **172,** 104–107.
15. Chardin, R., Boquet, P., Madaule, P., Popoff, M. R., Rubin, E. J., and Gill, D. M. (1989) *Embo J.* **8,** 1087–1092.
16. Lang, P., Gesbert, F., Thiberge, J.-M., Troalen, F., Dutarte, H., Chavrier, P., and Bertoglio, J. (1993) *Biochem. Biophys. Res. Commun.* **196,** 1522–1528.
17. Fukumoto, Y., Kaibuchi, K., Hori, Y., Fujioka, H., Araki, S., Ueda, T., Kikuchi, A., and Takai, Y. (1990) *Oncogene* **5,** 1321–1328.
18. Bokoch, G. M. (1995) *Blood* **86,** 1649–1660.