Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes

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We have observed that stimulation of human natural killer cells with dibutyryl cAMP (Bt₂cAMP) reproduced the effects of ADP ribosylation of the GTP binding protein RhoA by *Clostridium botulinum* C3 transferase: both agents induced similar morphological changes, inhibited cell motility and blocked the cytolytic function. We demonstrate here that cAMP-dependent protein kinase A (PKA) phosphorylates RhoA in its C-terminal region, on serine residue 188. This phosphorylation does not affect the ability of recombinant RhoA to bind guanine nucleotides, nor does it modify its intrinsic GTPase activity. However, treatment of cells with Bt₂cAMP results in the translocation of membrane-associated RhoA towards the cytosol. Experiments using purified membrane preparations indicated that Rho-GDP dissociation inhibitor, which can complex phosphorylated RhoA in its GTP-bound state, was the effector of this translocation. Taken together, these data suggest that PKA phosphorylation of RhoA is a central event in mediating the cellular effects of cAMP, and support the existence of an alternative pathway for terminating RhoA signalling whereby GTP-bound RhoA, when phosphorylated, could be separated from its putative effector(s) independently of its GTP/GDP cycling.

Keywords: lymphocytes/phosphorylation/RhoA

Introduction

The mammalian Ras superfamily of small GTP binding proteins is divided into three major subgroups according to sequence homology and functional similarities (Hall, 1990; Valencia *et al.*, 1991): (i) the Ras proteins which represent major control check points in signal transduction initiated through tyrosine kinase-associated receptors (Lowy and Willumsen, 1993); (ii) proteins in the Rab subgroup which are involved in intracellular membrane traffic and thus regulate processes such as endocytosis and exocytosis (Chavrier *et al.*, 1990) and (iii) the Rho family which comprises RhoA, RhoB, RhoC, RhoG, Rac1, Rac2, TC10 and CDC42Hs and governs cell polarity and organization of the actin microfilament network (Chardin *et al.*, 1989; Nelson, 1992).

The actin cytoskeleton plays a role central to many, if not all, cell functions. Reorganization of actin filaments, controlled by actin polymerization–depolymerization events, is essential in adapting the cell shape and its motility to the microenvironment, and is observed particularly in response to growth factors or stimulation of cell surface receptors. It has been recognized recently that several proteins in the Rho subfamily are involved in these regulations (Downward, 1992). Most of what is known today on the function of Rho proteins was made possible by the use of C3 exoenzyme, a *Clostridium botulinum*-derived ADP-ribosyl transferase that covalently modifies the Asn residue in position 41, located at the border of the effector domain of Rho (Aktories *et al.*, 1988; Sekine *et al.*, 1989). This domain is thought to mediate molecular interactions of small G-proteins with their regulatory or effector proteins such as the GTPase activating proteins (GAP), whereas the GDP dissociation inhibitors (GDI) or GDP dissociation stimulators (GDS) presumably bind to the C-terminal portion of the molecules (Self *et al.*, 1993). Initial reports indicated that treatment of Vero cells with C3 transferase inhibited the function of Rho, and induced disassembly of the actin microfilament network (Chardin *et al.*, 1989). Rac and Rho proteins were then shown to act in concert in regulating membrane ruffling and actin stress fibers formation induced in fibroblasts by various growth factors such as platelet-derived growth factor, epidermal growth factor or insulin (Ridley and Hall, 1992; Ridley *et al.*, 1992). Rho proteins, through their effects on the actin cytoskeleton, have also been shown to be implicated in the control of some specialized functions of differentiated cells, such as platelet aggregation and mediator release (Morii *et al.*, 1992; Nemoto *et al.*, 1992) or leucocyte function antigen 1 (LFA1)-mediated adhesion in human B lymphocytes (Tominaga *et al.*, 1993).

We have reported previously that cell-mediated cytotoxicity, an essential function of T-lymphocytes and natural killer (NK) cells, was inhibited upon treatment of effector cells with C3 transferase. These experiments suggested that RhoA played an important role in controlling cytoskeleton rearrangements that are critical for the formation of conjugates between killer cells and their targets and/or for the polarized release of cytolytic granules (Lang *et al.*, 1992). These indeed represent two essential steps in the lytic process whereby killer and target cells first bind to each other through the interaction of cell adhesion molecules such as the CD2–LFA3 and LFA1–intercellular cell adhesion molecule (ICAM) pairs (Springer, 1989). Subsequent to binding, interaction of the cytoplasmic domain of these molecules with the cytoskeleton, primarily the β -integrin family, increases their affinity, thus strengthening adhesion, and triggers the release of granzymes- and perforin-containing lytic granules towards the

target (Ojcius *et al.*, 1991). It is of interest that intracellular accumulation of cAMP also inhibits lymphocyte-mediated cytotoxicity, as previously described (Goto *et al.*, 1983; Vitté-Mony *et al.*, 1990). In addition, treatment of human lymphocytes with cAMP analogues induced dramatic changes in cell morphology and motility that are similar to those obtained with C3 transferase. These observations led us to hypothesize that cAMP and C3 transferase may share a common pathway leading to functional inhibition of cytotoxic lymphocytes.

We report here that recombinant RhoA is a substrate for the cAMP-dependent protein kinase A (PKA), which phosphorylates the Ser188 of RhoA. Whereas phosphorylation of RhoA does not appear to affect its intrinsic biochemical properties, it allows Rho-GDI to bind with a high affinity to phosphorylated RhoA in its GTP-bound form. As a consequence, Rho-GDI was shown to extract GTP-bound RhoA from purified membranes *in vitro*, a finding that correlates with the membrane to cytosol translocation of RhoA observed in intact cells upon dibutyryl cAMP (Bt₂cAMP) treatment.

Results

Clostridium botulinum C3 transferase and Bt₂cAMP have similar effects on cell morphology and motility in normal human NK cells

During the course of parallel studies investigating the effects of cAMP analogues and of *C.botulinum* C3 transferase on the cytotoxic function of human NK cells, we observed that both agents induced dramatic changes in cell morphology. This was investigated further using time-lapse microcinematography, as described in Materials and methods. Normal human NK cells, that had been kept growing in medium supplemented with interleukin-2 (IL-2), displayed irregular, pear-like shapes and appeared polarized, with typical uropods as shown in Figure 1A. Treatment of NK cells by electroporation with 4 µg/ml C3 transferase induced a rapid and irreversible rounding up of the cells (Figure 1C) that was not observed in cells that had undergone control electroporation in the absence of C3 transferase (Figure 1B). Similar observations were made in parallel experiments where cells were treated with 500 µM of the permeant, non-hydrolysable analogue of cAMP, Bt₂cAMP (Figure 1D–F). However, in this case, shape changes appeared transient, with individual cells being round for a few minutes, then reassuming their original irregular shape, before rounding up again (arrows, Figure 1D–F). On the other hand, the use of time-lapse recording enabled us to observe that normal NK cells moved significantly in the Rose chamber, whereas C3 transferase-treated cells stayed completely immobile. In the case of Bt₂cAMP treatment, this immobility was coincidental with the cell appearing transiently round, and motility was recovered together with shape changes. Semi-quantitative analysis of motility indicated that inhibition of NK cell motility was of the same order of magnitude following treatment with C3 transferase or Bt₂cAMP (50–60% inhibition, data not shown).

Ser188 of RhoA is a phosphorylation target for PKA

Taken together with our earlier reports (Vitté-Mony *et al.*, 1990; Lang *et al.*, 1992), the morphological and motility

Fig. 1. Morphological effects of C3 transferase or Bt₂cAMP treatment of human NK cells. Human NK cells grown in IL-2 were cultured in a Rose chamber at 37°C on an inverted microscope equipped with Nomarski differential interference contrast. Images were collected with a 16 mm Arriflex movie camera. Pictures shown were taken after 60 min of observation following no treatment (A), electroporation alone (B) or electroporation in the presence of 4 µg/ml recombinant C3 transferase (C). Frames (D), (E) and (F) illustrate the reversibility of morphological changes (white arrows) when NK cells were cultured with 500 µM Bt₂cAMP for 30, 45 and 60 min respectively. Bar represents 10 µm.

data reported above led us to hypothesize that C3 transferase and Bt₂cAMP might share a common inhibitory pathway. Our previous finding that RhoA was the only detectable substrate for ADP ribosylation by C3 transferase in NK cells (Lang *et al.*, 1992) strongly suggested that this pathway might involve RhoA. We therefore investigated the possibility that cAMP may modify the function of RhoA, for instance by inducing its PKA-dependent phosphorylation. Amino acid sequence comparison between Rho proteins showed that RhoA was the only member of this family to possess a potential consensus K-K-K-S-G site for PKA phosphorylation (Taylor *et al.*, 1990), located at the very C-terminus of RhoA. To assess that this consensus site was indeed a target for PKA-mediated phosphorylation, a recombinant GST–RhoA fusion protein, purified by affinity chromatography on glutathione–Sepharose beads, was phosphorylated *in vitro* in the presence of the catalytic subunit of PKA, subjected

Fig. 2. *In vitro* phosphorylation of RhoA by PKA. GST fusion proteins were purified from *E. coli* lysates by adsorption onto glutathione-Sepharose beads and incubated in 25 μ l of phosphorylation buffer with 10 U of PKA catalytic subunit and [32 P]ATP, for 30 min at 30°C. Beads were then washed extensively, boiled for 2 min in sample buffer and proteins separated by SDS-PAGE, and blotted onto nitrocellulose. Following autoradiography (A and B), filters were analysed by Western blot using anti-GST antibodies (C), or the anti-RhoA 26C4 mAb (D). RhoA (B and D) was obtained following cleavage of the GST fusion protein with thrombin (10 U) for 30 min at 25°C. (E) and (F) show the analysis of two independent preparations of GST-RhoA proteins that have been mutated to contain an Ala residue instead of Ser in position 188 (S188A), as compared with the wild-type (WT) protein: (E) autoradiography following phosphorylation with PKA and [32 P]ATP; (F) Western blot with the anti-RhoA antibody. Arrows indicate the migration of the 49 and 27.5 kDa molecular weight markers.

to SDS-PAGE and electroblotted onto nitrocellulose. Autoradiography and control Western blots indicated that GST-RhoA was indeed an *in vitro* substrate for PKA, whereas GST-Rac1 or GST alone were not (Figure 2). Cleavage of the phosphorylated GST-RhoA protein with thrombin showed that radiolabelling occurred in the RhoA moiety of the fusion protein (Figure 2B), and phosphorylated amino acid analysis only showed the presence of phosphoserine (not shown).

To identify further which serine residue(s) was phosphorylated by PKA, peptides resulting from CNBr cleavage of phosphorylated GST-RhoA were separated by HPLC and sequenced. These experiments indicated that the radiolabel was recovered essentially in a short peptide with the sequence A-T-R-A-A-L-Q-A-R-x-G-K-K-K-x-G-x, that resulted from cleavage at Met173 and includes Ser188 (data not shown). On the basis of these results, mutagenesis experiments were performed to produce RhoA proteins bearing an Ala residue instead of Ser in position 188. As shown in Figure 2E and F, substitution of Ser188 by Ala completely removed the ability of RhoA to be phosphorylated by PKA, whereas it was still recognized by a monoclonal antibody (mAb) (26C4) specific for RhoA (Lang *et al.*, 1993).

PKA phosphorylation of recombinant RhoA does not affect guanine nucleotide binding or hydrolysis

In a search for functional consequences of RhoA phosphorylation, we first investigated possible modifications of the ability of recombinant RhoA to bind and hydrolyse GTP. GST-RhoA proteins were phosphorylated as

Fig. 3. RhoA phosphorylation does not affect its ability to bind and hydrolyse GTP. (A) GST-RhoA fusion proteins (200 ng) bound to glutathione-Sepharose beads were phosphorylated with PKA and ATP as in Figure 2. Phosphorylated RhoA or non-phosphorylated control were incubated for 30 min at room temperature in the presence of 6 nM [α - 32 P]GTP with appropriate MgCl₂ buffer concentrations (as indicated), with or without a 100-fold excess concentration of cold GTP or GDP. Following extensive washes, radioactivity bound to the beads was measured by liquid scintillation. (B) GST-RhoA proteins, either phosphorylated or not, were pre-loaded with [α - 32 P]GTP as above, washed and transferred to 37°C for the indicated periods of time. Following elution of the bound nucleotide, its nature was analysed by thin layer chromatography. Results are expressed as the ratios of nucleotide converted to GDP over GTP. GTP hydrolysis in control samples incubated at 4°C for 30 min never exceeded 5%.

described above, except that non-radioactive ATP was used. In parallel experiments, the stoichiometry of the phosphorylation reaction was measured, and it was found that between 0.7 and 0.9 pmol of phosphate was transferred to 100 ng of the 49 kDa GST-RhoA (2 pmol). Thus, 35–45% of GST-RhoA proteins were phosphorylated. GST-RhoA fusion proteins that had been phosphorylated and adsorbed to glutathione-Sepharose beads were then incubated with [α - 32 P]GTP under low MgCl₂ buffer conditions that allowed loading of the nucleotide (Garrett *et al.*, 1989). In these experiments, phosphorylated GST-RhoA showed no alteration of its binding capacity for either GTP or GDP, as compared with untreated controls (Figure 3A). In contrast to other Ras-related proteins, recombinant proteins of the Rho subgroup display a low but measurable GTPase activity *in vitro* in the absence of GAP (Garrett

Fig. 4. *In vivo* and *in vitro* phosphorylation of RhoA is prevented by its association with GDI. (A) YT cells were pre-loaded with 0.5 mCi/ml ortho- ^{32}P in phosphate-free medium for 3 h, then stimulated for 45 min, in the presence of 0.1 μM okadaic acid, with 0.5 mM Bt_2cAMP or 50 μM forskolin + 100 mM IBMX. Cell lysates (10^7 cell equivalent) were immunoprecipitated with 5 μg of 26C4 or CD3 antibodies and protein G-Sepharose beads and analysed by SDS-PAGE and Western blot. Time of exposure for autoradiography was 24 h. The arrowhead points to the RhoA signal. The signals from the immunoprecipitating antibody light (Ig-L) chains, as well as the RhoA signal, are marked with arrows on the Western blot analysis. (B) Cytosol (C) and cell membrane (M) fractions were prepared by ultracentrifugation of YT cell freeze-thaw lysates. Each fraction (200 μg proteins in 50 μl) was incubated separately in phosphorylation buffer with 60 U of PKA and [^{32}P]ATP. The reaction was stopped by boiling in 0.5% SDS. Following a 1:5 dilution in phosphorylation buffer, samples were immunoprecipitated with either the 26C4 anti-RhoA antibody, 26C4, in the presence of 100 μM of the immunizing peptide (26C4+P) or with the isotype-matched control CD3 antibody. Immunoprecipitated material was recovered on protein G-Sepharose, subjected to SDS-PAGE and transferred to nitrocellulose. Exposure time for autoradiography was 2 days. (C) Purified recombinant GST-RhoA bound to glutathione-beads was geranylgeranylated and carboxymethylated in the presence of reticulocyte lysate, supplemented with 20 μM mevalonolactone and pancreatic microsomes and loaded with either GTP γS or GDP. Recombinant GDI (0.5 μg) or BSA was then added for 60 min at 4°C. Following extensive washes, PKA and [^{32}P]ATP were added in phosphorylation buffer as in Figure 2. Proteins were then separated by SDS-PAGE and transferred to nitrocellulose. RhoA phosphorylation was evidenced by autoradiography (top panel), and the filters were then analysed successively by Western blot with anti-GDI antibodies or anti-RhoA.

et al., 1989; Knaus *et al.*, 1992). Phosphorylation of RhoA did not induce any detectable difference in the rate of GTP hydrolysis as measured by conversion to GDP of [α - ^{32}P]GTP pre-loaded onto untreated or phosphorylated GST-RhoA (Figure 3B).

Treatment of human NK cells with Bt_2cAMP induces phosphorylation of membrane-associated RhoA and its translocation to the cytosol

Initial experiments unveiled some difficulties in stimulating the phosphorylation of RhoA in intact, ^{32}P -loaded cells. One such experiment is represented in Figure 4A, where YT cells were labelled with [^{32}P]orthophosphate, and stimulated with 500 μM Bt_2cAMP or 50 μM forskolin (FK) in the presence of 100 μM of the phosphodiesterase inhibitor IBMX. Cells were then lysed and immunoprecipitated with either anti-RhoA or control CD3 antibodies. Under these conditions, the Rho antibody, but not the control antibody, immunoprecipitated, in addition to several background proteins, a faint phosphorylated band which increased upon stimulation, and co-migrated with RhoA as identified in Western blot. The most striking finding in this series of experiments was the low level of RhoA phosphorylation despite the presence of okadaic acid (OKD) during all steps. This could indeed reflect a very fast turnover of this phosphate, but it also suggested that only a small fraction of RhoA might be available for PKA-mediated phosphorylation.

Most of the cellular RhoA is present in the cytosol and complexed with the regulatory protein GDI, which interacts with the geranylgeranylated C-terminus of Rho (Bourmeyster *et al.*, 1992). Thus, GDI could mask the phosphorylation site and only a limited, although functionally essential, portion of RhoA might be accessible to phosphorylation by PKA. This portion of RhoA could be represented by membrane-bound RhoA, that is presumably

in a GTP state, and free from GDI. This was investigated using fractionated membrane and cytosol preparations from the NK-like YT cell line. As shown in Figure 4B, when membrane fractions and cytosol preparations were phosphorylated *in vitro* with PKA and [γ - ^{32}P]ATP, radio-labelled RhoA could only be precipitated by the 26C4 antibody from membranes, whereas cytosolic RhoA did not incorporate any detectable amount of ^{32}P . These data thus demonstrate that membrane-bound RhoA is the only target for PKA and that cytosolic RhoA might be protected from PKA phosphorylation by its binding to GDI. This hypothesis was investigated directly *in vitro* in the following experiments. GST-RhoA proteins bound to glutathione beads were geranylgeranylated and carboxymethylated in the presence of reticulocyte lysate and pancreatic microsomes using previously established conditions (Hancock *et al.*, 1991), and bound to either GDP or GTP γS . Beads were then incubated with recombinant GDI, or bovine serum albumin (BSA) as a control, washed and phosphorylated in the presence of PKA and [^{32}P]ATP. Results presented in Figure 4C clearly show that binding of GDI to the GDP-bound form of RhoA strongly reduced its phosphorylation by PKA. Consistent with previous reports, GDI does not bind with a high affinity to GTP-bound RhoA, as seen in the control Western blot, and has a lesser inhibitory effect upon phosphorylation. Since membrane-bound RhoA represents 2–3% of total RhoA in a cell (Lang *et al.*, 1993), these results provided a likely explanation for the low level of RhoA phosphorylation in intact cells, and point to the functional importance of membrane-bound RhoA.

The subcellular localization and, noticeably, the membrane anchoring of several small GTP binding proteins, including Rap1A and Rab4, is influenced by phosphorylation (Bailly *et al.*, 1991; Lerosey *et al.*, 1991; Quilliam *et al.*, 1991; Van der Sluijs *et al.*, 1992). To investigate

Fig. 5. Stimulation of YT cells with Bt₂cAMP reversibly translocates RhoA out of the cell membrane. (A) YT cells were cultured in the presence of 500 μM Bt₂cAMP and, at the indicated times, membrane fractions were prepared by ultracentrifugation. Fifty μg of proteins from each fraction were analysed by Western blot with the 26C4 antibody. (B) YT cells that had been incubated with 500 μM Bt₂cAMP for 15 min, were washed and further cultured for 30 min in plain medium. Afterwards, membrane fractions were prepared and analysed as above.

whether this was also true for RhoA, YT cells were stimulated with Bt₂cAMP for increasing periods of time, and cell membranes, prepared by ultracentrifugation from freeze-thaw lysates, were analysed by SDS-PAGE and Western blot with the 26C4 antibody to assess the presence of RhoA protein. Following Bt₂cAMP stimulation, the amount of RhoA present in the membranes gradually decreases, becoming almost undetectable after 60 min (Figure 5). In control experiments (not shown), the amount of RhoA detectable in the corresponding cytosolic fractions remained constant: no decrease was observed and, not unexpectedly given the membrane to cytosol ratio of RhoA (2:98), a reciprocal increase in cytosolic RhoA could not be documented. In keeping with the transient morphological effects of cAMP (see above), we showed that, following washes and incubation of treated cells in Bt₂cAMP-free medium, RhoA returns to the membranes of YT cells over a 15–60 min time period (Figure 5B).

To confirm that the observed translocation of RhoA was indeed a result of [cAMP]_i increase, other drugs working at different levels of the cAMP pathway were used. For technical reasons, these experiments were performed on a subclone of YT cells (YT2C2) which does not respond as dramatically to cAMP as the parental cell line. However, the experiment shown in Figure 6 clearly demonstrates that FK at 50 μM, prostaglandin E₂ (PGE₂) at 100 nM or isoproterenol at 10 nM induced a reduction of membrane-associated RhoA similar to that induced by cAMP.

Rho-GDI extracts phosphorylated RhoA from cell membranes and binds to phosphorylated RhoA-GTP

Membrane insertion of small G-proteins is mediated through isoprenyl groups that are post-translationally added onto Cys residues present in their C-terminus (Newman and Magee, 1993; Philips *et al.*, 1993). In contrast to most of the Rab proteins, Rho proteins have only one C20 geranylgeranyl prenyl covalently linked to Cys190, and it is possible that membrane attachment is stabilized through a polybasic domain (R-R-G-K-K-K-S) located just upstream of the C-terminus (Adamson *et al.*,

Fig. 6. Effect of various cAMP inducers on membrane-associated RhoA. Cells from the YT2C2 subclone of the YT line were cultured with either 500 μM Bt₂cAMP, forskolin (50 μM), PGE₂ (100 nM) or isoproterenol (10 nM) in the presence of 100 mM IBMX. After 60 min stimulation, membrane fractions were prepared and analysed for RhoA content by Western blot (insert). Scanning densitometry of the film was performed using a computing densitometer and its corresponding Image Quant software (Molecular Dynamics). Results are expressed in arbitrary densitometry units. Numbers in parentheses represent percentages of the control unstimulated value.

Fig. 7. Recombinant GDI extracts phosphorylated RhoA out of the cell membrane. Membranes were prepared from YT cells by ultracentrifugation and separated into two batches: one was left untreated (–); the other was phosphorylated (+) with PKA and ATP and the reaction stopped by addition of PKI. Recombinant GDI (as indicated) was added to aliquots (5×10^6 cell equivalent) of membrane for 60 min at 4°C, and high speed membrane pellets and supernatants were obtained by ultracentrifugation. Each fraction was then analysed by Western blot with the 26C4 antibody. Filters were then stripped and reprobed with anti-GDI antibodies.

1992). It is of interest that this polybasic domain also constitutes the consensus environment for Ser188 to be phosphorylated by PKA. One hypothesis to explain the membrane to cytosol translocation of RhoA following cAMP stimulation would be that the negative charge introduced next to the polybasic domain by a PO_3^{2-} group might be sufficient to repel RhoA from the membrane. However, preliminary experiments indicated that this was not the case, and suggested that a cytosolic factor was required for this to occur. Based on current knowledge of Rho regulatory proteins, a putative candidate to mediate this translocation was GDI (Isomura *et al.*, 1991; Abo *et al.*, 1994). To investigate this further, YT cell membranes that had been left untreated or phosphorylated *in vitro* with PKA, were incubated with various amounts of recombinant GDI, ultracentrifuged, and the high speed supernatants and pellets analysed in parallel Western blots for their contents in RhoA. As shown in Figure 7, phosphorylation alone had no effect on RhoA, which did

not leak out of the membrane spontaneously and was recovered entirely in the pelleted membrane fractions. In the presence of 0.15 μg of GDI, all of RhoA was recovered in the supernatant when the membranes had been previously phosphorylated, whereas it remained in the non-phosphorylated membrane pellet. When GDI concentrations were increased, a proportion of RhoA was eluted from the membrane in the absence of phosphorylation. However, at the highest GDI concentration used in these experiments (1.5 μg), that were estimated to represent an equimolar ratio to RhoA, the proportion of RhoA that was eluted from non-phosphorylated membranes never exceeded 20–25%, as measured by densitometry scanning of the film (not shown). Reprobing of the same filters with anti-GDI antibodies demonstrated that GDI was recovered quantitatively in the supernatant fractions (Figure 7). Furthermore, no added recombinant GDI or endogenous GDI was ever detected in membrane fractions (not shown).

Rho-GDI has been shown to interact with the GDP-bound form of Rac and Rho proteins, but the existence of a stable complex with the GTP-bound form is still controversial. Since at least some (and probably most) of the membrane-bound RhoA should be in a GTP-bound state, these experiments suggested that RhoA phosphorylation may favour interaction of Rho-GDI with RhoA-GTP. That this was indeed the case was demonstrated *in vitro* using purified recombinant GST-RhoA that had been, in sequence (i) geranylgeranylated and carboxymethylated, (ii) phosphorylated in the presence of [^{32}P]ATP and (iii) loaded with either GTP γS or GDP. Beads were then incubated with recombinant GDI (0.5 μg) for 1 h, washed, and the *in vitro* binding of GDI was detected in Western blot analysis with anti-GDI antibodies. These experiments indicated that phosphorylation strongly increased the quantity of GDI that was able to bind to the GTP form of RhoA (Figure 8A, lanes 1 and 3). To approach the affinity of GDI interactions with the various forms of RhoA, additional experiments were performed in which 0.5% NP-40 was added in the washing buffer. Under these more stringent conditions, the only form of RhoA that appeared to bind GDI was phosphorylated RhoA-GTP, suggesting that this interaction was stronger than with any of the other forms of RhoA (Figure 8B).

Discussion

Recent publications have started to unveil the multiple relationships that may exist between serine/threonine kinases and small G-proteins. Ras-GTP binds to, and regulates, the Raf-1 kinase, and recently the Rho family proteins Cdc42 and Rac1 have been shown to complex with, and activate, p65^{PAK}, a brain serine/threonine kinase related to the yeast protein STE20 (Smeal *et al.*, 1991; Dickson *et al.*, 1992; Vojtek *et al.*, 1993; Manser *et al.*, 1994). On the other hand, several Ras-related proteins are known kinase substrates. Cyclic AMP-elevating agents induce PKA-mediated phosphorylation of Rap1A in neutrophils, and of Rap1B in platelets (Kawata *et al.*, 1989; Lapetina *et al.*, 1989; Quilliam *et al.*, 1991). Rad, a new member of the Ras family, is also a substrate for PKA (Zhu *et al.*, 1995). In contrast, Rab3B, Rab6 and Rab8 are phosphorylated in response to thrombin, but not

Fig. 8. *In vitro* binding of Rho-GDI to phosphorylated RhoA-GTP. (A) Purified recombinant GST-RhoA bound to glutathione-beads was successively (i) geranylgeranylated as in Figure 4C, (ii) phosphorylated with PKA and [^{32}P]ATP and (iii) loaded with either GTP γS or GDP. Recombinant GDI (0.5 μg) was then added for 60 min at 4°C and, following extensive washes in detergent-free buffer, proteins bound to glutathione-beads were eluted by boiling in sample buffer, separated by SDS-PAGE and transferred to nitrocellulose. RhoA phosphorylation was evidenced by autoradiography (top), and the filters were then analysed successively by Western blot with anti-GDI antibodies or anti-RhoA antibodies. (B) The same experiment as above except that final washes were performed with 0.5% NP-40-containing buffer. GG: geranylgeranylation; PP: phosphorylation; GNP: guanine nucleotide phosphate; T: GTP; D: GDP.

in response to PGE1 in platelets (Karniguian *et al.*, 1993). Behaving still differently, Rab1 and Rab4 are phosphorylated by p34^{cdc2} in a cell cycle-dependent manner (Baillly *et al.*, 1991; Van der Sluijs *et al.*, 1992). In most cases, phosphorylation occurs close to the C-terminus and affects the protein's subcellular localization.

The experiments reported here add, for the first time, a member of the Rho subfamily to the list of Ras-related small G-proteins that are kinase substrates. We demonstrate that recombinant RhoA is phosphorylated *in vitro* by PKA on Ser188 and that phosphorylation of RhoA does not appear to affect its ability to bind and hydrolyse GTP.

Experiments performed on cytosol and membrane extracts *in vitro* indicated that only membrane RhoA, which accounts for <2% of total cellular RhoA, could be phosphorylated by PKA, presumably because it is free from GDI. Indeed, we showed that GDI, which binds to the C-terminus of RhoA, prevents its phosphorylation *in vitro* by PKA. This observation is likely to account for the low level of RhoA phosphorylation in ^{32}P -loaded and B $_2$ cAMP-stimulated lymphocytes. However, stimulation of intact cells with inducers of cAMP translocates RhoA from the membrane to the cytosol in a process that was shown, using membrane preparations *in vitro*, to involve GDI. GDI are regulatory proteins, so far only described for the Rho and Rab subfamilies, whose primary function is to inhibit the GDP/GTP exchange (Fukumuto *et al.*, 1990; Hancock and Hall, 1993; Novick and Brennwald, 1993). Consistent with this, GDI interacts with a much higher affinity with the GDP-bound than with the GTP-bound form of the protein (Garrett *et al.*, 1993). Weak interactions of Rho-GDI with the GTP-bound form of Rho and Rac have, however, been described, that resulted in blocking GTP hydrolysis, presumably by preventing recognition by GAP (Sasaki *et al.*, 1993). Besides guanine nucleotide exchange regulation, or because of it, another

Fig. 9. A model depicting the membrane to cytosol translocation of phosphorylated RhoA independent of its cycling from GTP to GDP. RhoA in its GTP-bound form is shown interacting with a putative, membrane-associated effector molecule. From this point on, RhoA signalling proceeds together with hydrolysis of GTP, and inactive RhoA-GDP is shuttled by GDI back to the cytosol, where, following GDI dissociation and GTP exchange, a new cycle resumes. Alternatively, RhoA-GTP is phosphorylated by PKA, and extracted from the membrane in its GTP-bound form by GDI, thus terminating RhoA-GTP signalling prematurely. The fate of phosphorylated RhoA-GTP has not been established.

function of GDI appears to be the control of the membrane association of small G-proteins (Hori *et al.*, 1991; Ullrich *et al.*, 1993). Activation of the NADPH-oxidase is one of the best studied models, where Rac-GTP translocates to the cell membrane together with the multimolecular oxidase complex (Quinn *et al.*, 1993; Abo *et al.*, 1994). GTP hydrolysis then occurs and Rac-GDP is shuttled by GDI back to the cytosol where GTP exchange takes place, initiating a new cycle (Bokoch and Knaus, 1994). A similar role has been assigned to Rab-GDI in regulating intracellular membrane traffic (Araki *et al.*, 1990; Ullrich *et al.*, 1994). At the present time, no anti-RhoA-specific antibody is available that can immunoprecipitate RhoA in its native form, thus allowing an analysis of the GTP/GDP state of the protein. Yet, most membrane-bound RhoA is likely to be in the active GTP-bound conformation and free from GDI, in contrast to cytosolic RhoA that is thought to be essentially GDP-bound, and complexed to GDI (Ueda *et al.*, 1990). Consistent with this hypothesis, we showed that GDI could only extract a small, presumably GDP-bound proportion of RhoA from untreated membranes. In contrast, as a result of membrane phosphorylation, all of RhoA was eluted from the membrane by GDI, and we showed that GDI interacts *in vitro* with RhoA-GTP when it is phosphorylated. With regard to RhoA function, phosphorylation may thus provide an alternate way of terminating RhoA signalling, independently from its cycling from GTP to GDP, simply by removing RhoA-GTP from the proximity of its putative membrane-associated effector molecules (Figure 9). The fate of phosphorylated RhoA-GTP has not been investigated. However, GDI has been shown to inhibit both GAP-stimulated and spontaneous GTP hydrolysis of Rho and Rac (Sasaki *et al.*, 1993). Thus, in a phosphorylated RhoA-GTP/GDI complex, RhoA would remain in a GTP state. RhoA could then be dephosphorylated, which would

reduce GDI affinity dramatically and free RhoA-GTP from the complex.

We have used human cytotoxic lymphocytes to investigate the morphological and functional effects of *C.botulinum* C3 transferase and of cAMP. Both compounds modify cell shape, alter motility and inhibit cytotoxicity, presumably through disassembly of the actin cytoskeleton that occurs as a result of RhoA functional inhibition. In the case of C3, RhoA function may be inhibited due to a lack of interaction of regulatory proteins with the ADP-ribosylated effector domain, whereas cAMP-induced phosphorylation decreases membrane association of RhoA-GTP. The question then is to understand whether phosphorylation occurs under physiological conditions and what are its consequences with regard to cytotoxic function. It is well known that cytolytic T cells, or NK cells, recycle from one target to another, in a process that requires the effector to detach from the target cell and spare cytolytic granules. This implies the existence of a mechanism which reduces the affinity of adhesion molecules and blocks granule release. A role for transient elevation of intracellular cAMP in this recycling has been discussed elegantly by Valitutti *et al.* (1993), and points to cytoskeleton function as being in control of this recycling. Our findings that RhoA phosphorylation results in its disappearance from the cell membrane, and that this is a reversible phenomenon, indeed fits the killer cell recycling model. Furthermore, the ability of GDI to remove RhoA from the membrane in its GTP-bound form provides a mechanism whereby signalling can be interrupted at an early step, before cytotoxic cells, in this case, have exhausted all of their lytic potential.

Finally, it is of interest that, thus far, no functional differences have been reported between the highly homologous RhoA and RhoC proteins (178/193 amino acid identity). Out of the 15 amino acid substitutions, 10 are completely conservative and, in the light of the data reported here, the most striking change is the absence of Ser188 in RhoC. We would like to suggest the possibility that RhoC mediates housekeeping functions, e.g. regulating cytoskeleton organization during the normal cell cycle, whereas RhoA may be involved in stimulus-induced responses, that in some instances could be regulated through phosphorylation. From this point of view, it is striking that in several cell types that have been studied for cAMP response and for C3 transferase-mediated effects, the functional consequences of both agents are superimposable: (i) in platelets, C3 and cAMP inhibit aggregation and mediator release (Ashby *et al.*, 1990; Morii *et al.*, 1992); (ii) the rat PC12 pheochromocytoma cell line responds to either forskolin or C3 transferase by proliferation arrest and neurite outgrowth (Chijiwa *et al.*, 1990; Nishiki *et al.*, 1990; Jalink *et al.*, 1994); (iii) dramatic changes in the actin microfilament network are observed in fibroblasts in response to both agents (Lamb *et al.*, 1988; Rubin *et al.*, 1988) and (iv) cAMP and C3 transferase inhibit microfilament assembly in neutrophils, which affects cell motility (Downey *et al.*, 1991; Stasia *et al.*, 1991). Thus, it is likely that the mechanisms we describe here in cytotoxic lymphocytes also apply to other cell types. Notwithstanding other possible mechanisms, such as direct phosphorylation of actin, our observations are consistent with a central role for PKA-mediated

phosphorylation of RhoA in mediating the cellular effects of cAMP.

Materials and methods

Cell cultures and microcinematography

All cell cultures were carried in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine and antibiotics. The YT cell line is a transformed, NK-like cell line established from a leukaemic patient (Yodoi *et al.*, 1985). IL-2-dependent normal human NK cell lines were established as previously described and were used after 3–6 weeks of growth in IL-2 (Vitté-Mony *et al.*, 1990). For time-lapse recording, NK cells (10^5 /ml) were introduced into Rose chambers (Rose *et al.*, 1958) and mounted on an inverted Zeiss ICM 505 microscope equipped with Nomarsky differential interference contrast. The microscope was kept at a controlled constant temperature (37°C) and connected to an Arriflex 16 mm camera. A time-lapse device gave an impulse every 5 s, at which time the field under observation was illuminated and a microphotograph was taken. For analysis of cell motility, the position of individual cells was located manually by projecting 5 min spaced pictures on graph paper, and the total distance covered during a 60 min time period was measured and averaged for each group of cells.

Electroporation

Cells were electroporated using a Cellject apparatus (Eurogentec, Belgium) generating a decaying exponential waveform double electric pulse (800 V, 40 μ F for 1.7 ms then 100 V, 1500 μ F for 66 ms) with 4 μ g/ml of highly purified recombinant *C. botulinum* C3 exoenzyme, kindly provided by P. Boquet, Pasteur Institute, Paris (Rubin *et al.*, 1988). Cells were allowed to recover membrane integrity for 1 h at 37°C in culture medium, and viability was assessed by trypan blue exclusion, before time-lapse observation.

Recombinant proteins

Escherichia coli expression of RhoA and Rac1 was achieved by the use of PGEX vectors, kindly provided by A. Hall (MRC, London), encoding GST fusion proteins. The procedures used for expression and purification of RhoA and Rac1 were essentially as described by Hall *et al.* (Adamson *et al.*, 1992). Briefly, PGEX-transformed *E. coli* (strain HB101) were grown to mid-exponential phase, induced for 3 h with 1 mM isopropylthiogalactoside and lysed by freeze–thawing in hypotonic buffer as described. The GST fusion proteins were purified from the bacterial lysate by incubation with glutathione-coupled Sepharose beads (Pharmacia). Separation of the small GTP binding protein from the GST moiety was achieved by cleavage with thrombin (Sigma, 500 U/ml) for 30 min at 25°C.

For RhoA Ser188→Ala mutation, a two-step PCR procedure was used, whereby the C-terminal end of RhoA was first amplified from a PBS-SK-RhoA plasmid using a mutated oligonucleotide (GA CGT GGG AAG AAG AAA GCT GGT TGC) and a T7 primer. The purified PCR I product was then used, in conjunction with a T3 primer, to further amplify the full-length sequence. The PCR II DNA product was cut with *Bam*HI and *Eco*RI and inserted into PGEX-2T to yield GST-mutated RhoA fusion proteins.

Highly purified recombinant GDI (Chuang *et al.*, 1993) was kindly provided by G. Bockoch (Scripps Res. Institute, La Jolla, CA).

Antibodies and protein analysis

The production and characterization of the RhoA-specific 26C4 mAb have been reported previously (Lang *et al.*, 1993). Rabbit antiserum generated against purified bovine neutrophil GDI was kindly contributed by N. Bourmeyster (CENG, Grenoble). Other antibodies used in this study include commercially available anti-GST goat antibodies (Pharmacia) and goat anti-mouse (GAM, Amersham) or swine anti-rabbit (SAR, Dako) antibodies coupled to peroxidase.

Separation of proteins on 8–20% gradient SDS–PAGE, and electrotransfer to nitrocellulose were performed using conventional techniques. Immunoblots were revealed by enhanced chemiluminescence (ECL, Amersham).

In vitro phosphorylation

Phosphorylation of purified GST proteins, bound to glutathione beads, by PKA (10 U, catalytic subunit, Sigma) was carried out in a 20 μ l final volume of 50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 20 μ M ATP, and 10 μ Ci [γ -³²P]ATP (3000 Ci/mmol, Amersham)

for 30 min at 30°C. The reaction was stopped by addition of 1 ml of cold phosphorylation buffer. Following three washes in the same buffer, proteins were eluted from the beads by boiling in SDS–PAGE sample buffer, and processed for Western blot and autoradiography.

Translocation of RhoA

YT cells (30×10^6 /ml) were pre-incubated in aliquots of 500 μ l with OKD (100 nM) for 15 min at 37°C before adding 500 μ M Bt₂cAMP for increasing periods of time at 37°C. Incubation was terminated by addition of 500 μ l of 2 $\times$ cold stop buffer (10 mM Tris pH 7.5, 50 mM NaCl and 50 mM NaF). After a quick spin, cells were lysed by freeze–thawing in hypotonic buffer [10 mM Tris pH 7.5, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM aprotinin, 100 nM OKD]. The post-nuclear fraction was then harvested following centrifugation at 15 000 r.p.m. for 5 min at 4°C, and ultracentrifuged (100 000 g for 60 min) to separate membrane and soluble cytosol fractions. The resulting pellet (membrane fraction) was washed twice in hypotonic lysis buffer, and analysed in parallel with the corresponding cytosol preparation for RhoA content.

In order to observe the transient effect of translocation of RhoA from membrane to cytosol, cells were incubated for 30 min at 37°C with Bt₂cAMP. They were then washed three times, and further incubated for 30 min in Bt₂cAMP-free culture medium. Cells were then lysed at various times and treated as above. Bt₂cAMP, FK, IBMX, OKD, PGE₂ and isoproterenol were purchased from Sigma.

Effects of Rho-GDI on the translocation of RhoA

YT cells were lysed in hypotonic buffer and the resulting post-nuclear supernatant centrifuged at 100 000 g for 60 min. Membrane fractions were washed twice with phosphorylation buffer minus OKD, and aliquoted. Each fraction (5×10^6 cell equivalent) was resuspended in 20 μ l of phosphorylation buffer supplemented with 0.1% Triton X-100 and 6 mM ATP. Samples were treated with or without the catalytic subunit of PKA (10 μ g/ml) for 30 min at 30°C. The reaction was stopped by addition of 5 μ g of protein kinase inhibitor (Sigma). Where indicated, recombinant GDI, at increasing concentrations, was added to the mixture for 60 min at 4°C. The reaction was terminated by ultracentrifugation (100 000 g) for 30 min. The corresponding pellets and supernatants were treated for Western blot as above to analyse their respective RhoA contents.

GTP loading and hydrolysis

GST–RhoA proteins (200 ng) bound to glutathione–beads were incubated for 30 min at room temperature with 6 nM [α -³²P]GTP in 50 mM Tris pH 7.5, 50 mM NaCl, 0.1 mM DTT supplemented with either 0.5 μ M or 5 mM MgCl₂. After six washes in high MgCl₂ buffer, radioactivity bound to the beads was measured in a Beckmann 6000LS scintillation counter. Non-specific binding was measured in the presence of a 100-fold excess of unlabelled GTP or GDP.

To compare the rate of GTP hydrolysis by RhoA or phosphorylated RhoA, proteins adsorbed to glutathione–beads were loaded with [α -³²P]GTP as above, washed and transferred to 37°C. At selected times, the reaction was stopped by washing in cold high MgCl₂ buffer. Guanine nucleotides were then recovered by adding 20 μ l of elution buffer (2 mM EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM GTP and GDP) to the beads pellet and further incubating at 68°C for 20 min. Thin layer chromatography was then performed on polyethyleneimine cellulose plates in 0.8 M HCl, 1.2 M ammonium formate. Following autoradiography, GTP and GDP spots were cut out and counted.

In vitro interaction of GDI with recombinant RhoA

Aliquots of GST–RhoA fusion proteins were processed *in vitro* as follows. First geranylation and carboxymethylation were performed by incubation with a mixture of reticulocyte lysate and canine pancreatic microsomes supplemented with 20 μ M mevalonolactone, for 1 h at 37°C according to Hancock *et al.* (1991). Beads were then washed six times with phosphorylation buffer and aliquots processed for [³²P]ATP phosphorylation as above. Separate aliquots were loaded with GTP γ S or GDP as described previously. Recombinant GDI (0.2 μ g in 10 mM Tris pH 7.5, 5 mM MgCl₂, 1 mM PMSF, 1 mM aprotinin) was then added to aliquots (0.2 μ g) of processed or unprocessed RhoA in their GTP- or GDP-bound state. Incubation was for 60 min at 4°C with agitation, and beads were washed five times with 0.15 M NaCl, 20 mM Tris pH 7.5 and 5 mM EDTA. Proteins bound to the beads were eluted in sample buffer and prepared for autoradiography and Western blot analysis.

Acknowledgements

We are indebted to A.Hall for kindly providing PGEX-RhoA and PGEX-Rac1 vectors, P.Boquet for C3 transferase and G.Bokoch and T.-H.Chuang for recombinant GDI. We thank N.Bourmeyster and P.Vignais for anti-RhoGDI antibodies and F.Troalen and J.-P.Levillain for peptide sequencing. We thank J.Brard, A.Vazquez and J.Pierre for reviewing the manuscript, and members of our laboratory for many useful discussions. This work was supported by INSERM, Association pour la Recherche contre le Cancer (grant ARC 6310), and by a fellowship from Ligue Nationale contre le Cancer to P.L.

References

- Abo, A., Webb, M.R., Grogan, A. and Segal, A.W. (1994) Activation of NADPH oxidase involves the dissociation of p21 rac from its inhibitory GDP/GTP exchange protein (rho GDI) followed by its translocation to the plasma membrane. *Biochem. J.*, **298**, 585–591.
- Adamson, P., Marshall, C.J., Hall, A. and Tilbrook, P.A. (1992) Post-translational modifications of p21 rho proteins. *J. Biol. Chem.*, **267**, 20033–20038.
- Aktories, K., Rsener, S., Blaschke, U. and Chatwal, G.S. (1988) Botulinum ADP-ribosyltransferase C3: purification of the enzyme and characterization of the ADP-ribosylation reaction in platelet membranes. *Eur. J. Biochem.*, **172**, 445–450.
- Araki, S., Kikuchi, A., Hata, Y., Isomura, M. and Takai, Y. (1990) Regulation of reversible binding of smg p25A, a ras p21-like GTP-binding protein, to synaptic plasma membranes and vesicles by its specific regulatory protein, GDP dissociation inhibitor. *J. Biol. Chem.*, **265**, 13007–13015.
- Ashby, B., Daniel, J.D. and Smith, J.B. (1990) Mechanisms of platelets activation and inhibition. *Hematol./Oncol. Clin. North Am.*, **4**, 1–26.
- Bailly, E., Mac Caffrey, M., Touchot, N., Zahraoui, A., Goud, B. and Bornens, M. (1991) Phosphorylation of two small GTP binding proteins of the rab family by p34 cdc2. *Nature*, **350**, 715–718.
- Bokoch, G.M. and Knaus, U.G. (1994) Ras-related GTP-binding proteins and leukocyte signal transduction. *Curr. Opin. Hematol.*, **1**, 53–60.
- Bourmeyster, N., Stasia, M., Garin, J., Gagnon, J., Boquet, P. and Vignais, P. (1992) Copurification of rho protein and the rho-GDP dissociation inhibitor from bovine neutrophil cytosol. Effect of phosphoinositides on rho ADP-ribosylation by the C3 exoenzyme of *Clostridium botulinum*. *Biochemistry*, **31**, 12863–12869.
- Chardin, P., Boquet, P., Madaule, P., Popoff, M.R. and Gill, D.M. (1989) The mammalian G protein rhoC is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilaments in Vero cells. *EMBO J.*, **8**, 1087–1092.
- Chavrier, P., Parton, R.G., Hauri, H.P., Simons, K. and Zerial, M. (1990) Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell*, **62**, 317–329.
- Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Toshioka, T. and Hidaka, H. (1990) Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized inhibitor of cyclic AMP-dependent protein kinase, *N*-(2-(*p*-bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J. Biol. Chem.*, **265**, 5267–5272.
- Chuang, T.H., Xu, X., Knaus, U.G., Hart, M.J. and Bokoch, G.M. (1993) GDP dissociation inhibitor prevents intrinsic and GTPase activating protein-stimulated GTP hydrolysis by the rac GTP-binding protein. *J. Biol. Chem.*, **268**, 775–778.
- Dickson, B., Sprenger, F., Morisson, D. and Hafen, E. (1992) Raf functions downstream of ras1 in the sevenless signal transduction pathway. *Nature*, **360**, 600–603.
- Downey, G.P., Elson, E.L., Schwab, B.III, Erzurum, S.C., Young, S.K. and Worthen, G.S. (1991) Biophysical properties and microfilament assembly in neutrophils: modulation by cyclic AMP. *J. Cell Biol.*, **114**, 1179–1190.
- Downward, J. (1992) Rac and Rho in tune. *Nature*, **359**, 273–274.
- Fukumoto, Y., Kaibuchi, Y., Hori, Y., Fujioka, H., Araki, S., Ueda, T., Kikuchi, A. and Takai, Y. (1990) Molecular cloning and characterization of a novel type of regulatory protein (GDI) for the rho proteins, ras p21-like small GTP-binding proteins. *Oncogene*, **5**, 1321–1328.
- Garrett, M.D., Self, A.J., Van Oers, C. and Hall, A. (1989) Identification of distinct cytoplasmic target for ras/R-ras and rho regulatory proteins. *J. Biol. Chem.*, **264**, 10–13.
- Garrett, M.D., Kacene, A.K., Zahner, J.E., Kaibuchi, K., Sasaki, T., Takai, Y., Cheney, C.M. and Novick, P.J. (1993) Interaction of sec4 with GDI proteins from bovine brain, *Drosophila melanogaster* and *Saccharomyces cerevisiae*: conservation of GDI membrane dissociation activity. *FEBS Lett.*, **331**, 233–238.
- Goto, T., Herberman, R.B., Maluish, A. and Strong, D. (1983) Cyclic AMP as a mediator of prostaglandin E-induced suppression of human natural killer cell activity. *J. Immunol.*, **130**, 1350–1355.
- Hall, A. (1990) The cellular functions of small GTP-binding proteins. *Science*, **249**, 635–640.
- Hancock, J.F. and Hall, A. (1993) A novel role for RhoGDI as an inhibitor of GAP proteins. *EMBO J.*, **12**, 1915–1921.
- Hancock, J.F., Cadwallader, K. and Marshall, C.J. (1991) Methylation and proteolysis are essential for efficient membrane binding of prenylated p21 K-ras(B). *EMBO J.*, **10**, 641–646.
- Hori, Y., Kikuchi, A., Isomura, M., Katayama, M., Miura, Y., Fujioka, H., Kaibuchi, K. and Takai, Y. (1991) Post translational modifications of the C-terminal region of the rho protein are important for its interaction with the membranes and the stimulatory and inhibitory GDP/GTP exchange proteins. *Oncogene*, **6**, 515–522.
- Isomura, M., Kikuchi, A., Ohga, N. and Takai, Y. (1991) Regulation of binding of rhoB p20 to membranes by its specific regulatory protein, GDP dissociation inhibitor. *Oncogene*, **6**, 119–124.
- Jalink, K., van Corven, E.J., Hengeveld, T., Morii, N., Narumiya, S. and Moolenaar, W.H. (1994) Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. *J. Cell Biol.*, **126**, 801–810.
- Karnigian, A., Zaharoui, A. and Tavittian, A. (1993) Identification of small GTP-binding rab proteins in human platelets: thrombin induced phosphorylation of rab3b, rab6 and rab8 proteins. *Proc. Natl Acad. Sci. USA*, **90**, 7647–7651.
- Kawata, M., Kikuchi, A., Hoshijima, M., Yamamoto, K., Hashimoto, E., Yamamura, H. and Takai, Y. (1989) Phosphorylation of smg p21, a ras-like GTP-binding protein, by cyclic AMP-dependent protein kinase in a cell-free system and in response to prostaglandin E1 in intact human platelets. *J. Biol. Chem.*, **264**, 15688–15695.
- Knaus, U.G., Heyworth, P.G., Kinsella, B.T., Curnutte, J.T. and Bokoch, G.M. (1992) Purification and characterization of rac2: a cytosolic GTP-binding protein that regulates human neutrophil NADPH oxidase. *J. Biol. Chem.*, **267**, 23575–23582.
- Lamb, N.J., Fernandez, M.A., Conti, A., Adelstein, R., Glass, D., Welch, W.J. and Feramisco, J.R. (1988) Regulation of actin microfilament integrity in living non muscle cells by the cAMP-dependent protein kinase and the myosin light chain kinase. *J. Cell Biol.*, **106**, 1955–1971.
- Lang, P., Guizani, L., Vitt-Mony, I., Stancou, R., Dorseuil, O., Gacon, G. and Bertoglio, J. (1992) ADP-ribosylation of the ras-related GTP-binding protein RhoA inhibits lymphocyte mediated cytotoxicity. *J. Biol. Chem.*, **267**, 11677–11680.
- Lang, P., Gesbert, F., Thiberge, J., Troalen, F., Dutartre, H., Chavrier, P. and Bertoglio, J. (1993) Characterization of a monoclonal antibody specific for the ras-related GTP-binding protein RhoA. *Biochem. Biophys. Res. Commun.*, **196**, 1522–1528.
- Lapetina, E.G., Lacal, J.C., Reep, B.R. and Molina y Vedia, L. (1989) A ras-related protein is phosphorylated and translocated by agonists that increase cAMP levels in human platelets. *Proc. Natl Acad. Sci. USA*, **86**, 3131–3134.
- Lerosey, I., Pizon, V., Tavittian, A. and Gunzburg, J. (1991) The cAMP-dependent protein kinase phosphorylates the rap1 protein *in vitro* as well as in intact fibroblasts, but not the closely related rap2 protein. *Biochem. Biophys. Res. Commun.*, **175**, 430–436.
- Lowy, D.R. and Willumsen, B.M. (1993) Function and regulation of ras. *Annu. Rev. Biochem.*, **62**, 851–891.
- Manser, E., Leung, T., Salihuddin, H., Zhao, Z.S. and Lim, L. (1994) A brain serine/threonine protein kinase activated by CDC42 and Rac1. *Nature*, **367**, 40–46.
- Morii, N., Teru-Uchi, T., Tominaga, T., Kumagai, N., Kozaki, S., Ushikubi, F. and Narumiya, S. (1992) A rho gene product in human blood platelets: effects of the ADP-ribosylation by the C3 ADP-ribosyltransferase on platelet aggregation. *J. Biol. Chem.*, **267**, 20916–20920.
- Nelson, J.W. (1992) Regulation of cell surface polarity from bacteria to mammals. *Science*, **258**, 948–964.
- Nemoto, Y., Namba, T., Teru-Uchi, T.L., Ushikubi, F., Morii, N. and Narumiya, S. (1992) A rho gene product in human blood platelets: identification of the platelet substrate for botulinum C3 ADP-ribosyltransferase as rhoA protein. *J. Biol. Chem.*, **267**, 20916–20920.
- Newman, C.M.H. and Magee, A.I. (1993) Posttranslational processing of

- the ras superfamily of small GTP-binding proteins. *Biochim. Biophys. Acta*, **1155**, 79–96.
- Nishiki.T., Narumiya.S., Morii.N., Yamamoto.M., Fujiwara.M., Kamata.Y., Sakaguchi.G. and Kozaki.S. (1990) ADP-ribosylation of the rho/rac proteins induces growth inhibition, neurite outgrowth and acetylcholine esterase in cultured PC-12 cells. *Biochem. Biophys. Res. Commun.*, **167**, 265–272.
- Novick.P. and Brennwald.P. (1993) Friends and family: the role of the rabGTPases in vesicular traffic. *Cell*, **75**, 597–601.
- Ojcius.D.M., Zheng.L.M., Sphicas.E.C., Zychlinski.A. and Young.J.D.E. (1991) Subcellular localization of perforin and serine esterase in lymphokine-activated cells and cytotoxic T cells by immunogold labeling. *J. Biol. Chem.*, **146**, 4427–4432.
- Philips.M.R., Pillinger.M.H., Staud.R., Volker.C., Rosenfeld.M.G., Weissmann.G. and Stock.J.B. (1993) Carboxyl methylation of ras-related proteins during signal transduction in neutrophils. *Science*, **259**, 977–980.
- Quilliam.I.A., Mueller.H., Bohl.B.P., Prossnitz.V., Sklar.I.A., Der.C.J. and Bokoch.G.M. (1991) rap1A is substrate for cyclic AMP dependent protein kinase in human neutrophils. *J. Biol. Chem.*, **147**, 1628–1635.
- Quinn.M.T., Evans.T., Priscu.L.R., Jesaitis.A.J. and Bokoch.G.M. (1993) Translocation of rac correlates with NADPH oxidase activation. *J. Biol. Chem.*, **268**, 20983–20987.
- Ridley.A.J. and Hall.A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesion and actin stress fibers in response to growth factors. *Cell*, **70**, 389–399.
- Ridley.A.J., Paterson.H.F., Johnston.C.L., Diekmann.D. and Hall.A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*, **70**, 401–410.
- Rose.G.G., Promerat.C.M., Shindler.T.O. and Trunnel.J.B. (1958) A cellophane-strip technique for culturing tissue in multipurpose culture chambers. *J. Biophys. Biochem. Cytol.*, **4**, 761–763.
- Rubin.E.J., Gill.D.M., Boquet.P. and Popoff.M. (1988) Functional modification of a 21-kilodalton G protein when ADP-ribosylated by exoenzyme C3 of *Clostridium botulinum*. *Mol. Cell. Biol.*, **8**, 418–426.
- Sasaki.T., Kato.M. and Takai.Y. (1993) Consequences of weak interaction of Rho GDI with the GTP-bound forms of rho p21 and rac p21. *J. Biol. Chem.*, **268**, 23959–23963.
- Sekine.A., Fujiwara.M. and Narumiya.S. (1989) Asparagine residue in the rho gene product is the modification site for ADP-ribosyltransferase. *J. Biol. Chem.*, **264**, 8602–8609.
- Self.A.J., Paterson.H.F. and Hall.A. (1993) Different structural organization of ras and rho effector domains. *Oncogene*, **8**, 655–661.
- Smeal.T., Binetruy.B., Mercolas.D.A., Birrer.M. and Karin.M. (1991) Oncogenic and transcriptional cooperation with Ha-ras requires phosphorylation of c-jun on serines 63 and 73. *Nature*, **354**, 494–496.
- Springer.T.A. (1989) Adhesion receptors of the immune system. *Nature*, **336**, 425–434.
- Stasia.M.J., Louan.A., Bourmeyster.N., Boquet.P. and Vignais.P.V. (1991) ADP-ribosylation of a small size GTP-binding protein in bovine neutrophils by the C3 exoenzyme of *Clostridium botulinum* and effect on the cell motility. *Biochem. Biophys. Res. Commun.*, **180**, 615–622.
- Taylor.S.S., Buechler.J.A. and Yonemoto.W. (1990) cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. *Annu. Rev. Biochem.*, **59**, 971–1005.
- Tominaga.T., Sugie.K., Hirata.M., Morii.N., Fukata.J., Uchida.A., Imura.H. and Narumiya.S. (1993) Inhibition of PMA-induced, LFA-1 dependent lymphocyte aggregation by ADP ribosylation of the small molecular weight GTP binding protein, rho. *J. Cell. Biol.*, **120**, 1529–1537.
- Ueda.T., Kikuchi.A., Ohga.N., Yamamoto.J. and Takai.Y. (1990) Purification and characterization from bovine brain cytosol of a novel regulatory protein inhibiting the dissociation of GDP from and the subsequent binding of GTP to rhoB p20, a ras p21-like GTP-binding protein. *J. Biol. Chem.*, **265**, 9373–9380.
- Ullrich.O., Stenmark.H., Alexandrov.K., Huber.L.A., Kaibuchi.K., Sasaki.T., Takai.Y. and Zerial.M. (1993) Rab GDP dissociation inhibitor as a general regulator for the membrane association of rab proteins. *J. Biol. Chem.*, **268**, 18143–18150.
- Ullrich.O., Horiuchi.H., Bucci.C. and Zerial.M. (1994) Membrane association of rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. *Nature*, **368**, 157–160.
- Valencia.A., Chardin.P., Wittinghofer.A. and Sander.C. (1991) The ras protein family: evolutionary tree and role of conserved amino acids. *Biochemistry*, **30**, 4637–4648.
- Valitutti.S., Dessing.M. and Lanzavecchia.A. (1993) Role of cAMP in regulating cytotoxic T lymphocyte adhesion and motility. *Eur. J. Immunol.*, **23**, 790–795.
- Van der Sluijs.P., Hull.M., Huber.L.A., Male.P., Goud.B. and Mellman.I. (1992) Reversible phosphorylation–dephosphorylation determines the localization of rab4 during the cell cycle. *EMBO J.*, **11**, 4379–4389.
- Vitté-Mony.I., Stancou.R. and Bertoglio.J. (1990) Functional consequences of cAMP accumulation in human NK cells: implications for IL-2 and IL-4 signal transduction. *J. Immunol.*, **145**, 4272–4278.
- Vojtek.A.B., Hollenberg.S.M. and Cooper.J.A. (1993) Mammalian ras interacts directly with the serine threonine kinase raf. *Cell*, **74**, 205–214.
- Yodoi.T. *et al.* (1985) TCGF (IL2)-receptor inducing factor (s). I: regulation of IL2 receptor on a natural killer-like cell line. *J. Immunol.*, **134**, 1623–1629.
- Zhu.J., Reynet.C., Caldwell.J.S. and Kahn.C.R. (1995) Characterization of Rad, a new member of Ras/GTPase superfamily, and its regulation by a unique GTPase-activating protein (GAP)-like activity. *J. Biol. Chem.*, **270**, 4805–4812.

Received on May 8, 1995; revised on October 26, 1995