The Small GTP-binding Proteins, Rac and Rho, Regulate Cytoskeletal Organization and Exocytosis in Mast Cells by Parallel Pathways

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In mast cells, activation of GTP-binding proteins induces centripetal reorganization of actin filaments. This effect is due to disassembly, relocalization, and polymerization of F-actin and is dependent on two small GTPases, Rac and Rho. Activities of Rac and Rho are also essential for the secretory function of mast cells. In response to GTP- γ -S and/or calcium, only a proportion of permeabilized mast cells is capable of secretory response. Here, we have compared actin organization of secreting and nonsecreting cell populations. We show that the cytoskeletal and secretory responses are strongly correlated, indicating a common upstream regulator of the two functions. The secreting cell population preferentially displays both relocalization and polymerization of actin. However, when actin relocalization or polymerization is inhibited by phalloidin or cytochalasin, respectively, secretion is unaffected. Moreover, the ability of the constitutively active mutants of Rac and Rho to enhance secretion is also unaffected in the presence of cytochalasin. Therefore, Rac and Rho control these two functions by divergent, parallel signaling pathways. Cortical actin disassembly occurs in both secreting and nonsecreting populations and does not, by itself, induce exocytosis. A model for the control of exocytosis is proposed that includes at least four GTP-binding proteins and suggests the presence of both shared and divergent signaling pathways from Rac and Rho.

INTRODUCTION

Studies with permeabilized mast cells have established a crucial role for GTP-binding proteins in regulation of exocytosis (Cockcroft *et al.*, 1987; Howell *et al.*, 1987). Our studies have shown that, in permeabilized mast cells, activation of GTP-binding proteins by GTP- γ -S results in a centripetal redistribution of filamentous actin. In addition to the involvement of an AlF_4^- -sensitive heterotrimeric GTP-binding protein in this process, the activities of Rac and Rho, two small GTPases related to the oncoprotein Ras, are required (Norman *et al.*, 1994). Moreover, constitutively active mutants of these two proteins, V14RhoA and V12Rac1, enhance regulated secretion from permeab-

ilized mast cells by increasing the proportion of cells that is competent to respond to stimulation. On the other hand, inhibition of the endogenous Rac and Rho activities reduces the secretory response. Thus, in mast cells, Rac and Rho are components of the signaling pathways that lead both to cytoskeleton reorganization and to secretion.

Although the best-established function of Rho-related GTPases is that of mediating signals from receptors to the cytoskeleton (Hall, 1992), recent findings indicate that these proteins may also regulate many other functions independently of their cytoskeletal effects. In this paper, we have addressed the question of the relevance of the cytoskeletal reorganization to the exocytotic process. To what extent is secretion controlled by Rac and Rho via their effects on actin? Rearrangement of the cytoskeleton has long been postulated as a requirement for secretion. In particular, disassembly of the cortical actin network is assumed

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to enable the access of the secretory vesicles to the plasma membrane (Burgoyne and Cheek, 1985; Linstedt and Kelly, 1987; Sontag *et al.*, 1988). Other cytoskeletal functions, such as contractility, gel-sol transformations, transport, compartmentalization, maintenance of tension, and provision of a "scaffold" are relevant to various cellular activities (Stossel, 1989; Ingber *et al.*, 1994).

In permeabilized mast cells, the cytoskeletal responses to calcium and/or GTP- γ -S consist of three separate effects: cortical actin disassembly, de novo actin polymerization, and a relocation of the cortical filaments (Norman *et al.*, 1994; Borovikov *et al.*, 1995). Here, we have studied these three types of cytoskeletal rearrangements in individual permeabilized mast cells after their activation and correlated their extent with that of the exocytotic response. To do so, a method for distinguishing the secreting and non-secreting cell populations was developed. Previous studies have relied on detection of the exposed secreted proteins such as α -amylase (Perrin *et al.*, 1992) or dopamine- β -hydroxylase (Perrin and Aunis, 1985; Vitale *et al.*, 1991; Elferink *et al.*, 1993) to monitor exocytosis from individual parotid acinar or adrenal chromaffin cells, respectively. We have used either fluorescent Concanavalin-A staining and confocal microscopy or determination of light scattering by flow cytometry. The fluorescent lectin (Bodipy-Con-A or FITC-Succinyl-Con-A) binds to granular glycoproteins and proteoglycans, exposed to the cell exterior after mast cell degranulation, and thus provides a useful tool for identification of secreting cells. The analysis of cells by flow cytometry is based on the dramatic morphological changes exhibited by secreting cells and reflected in their light-scattering characteristics. It is important to point out that, whereas glass-attached cells were used for confocal microscopy, flow cytometry analysis and hexosaminidase assays were performed with cells in suspension. The two different approaches allowed examination of the behavior of individual cells as well as that of large cell populations. We have found that the two types of cells exhibit differences in the extent of their responses and in their sensitivities to various inhibitors.

MATERIALS AND METHODS

Materials

All nucleotides (GTP- γ -S, GDP- β -S, GTP, and ATP) were obtained from Boehringer Mannheim (Mannheim, Germany). Streptolysin-O (SL-O) was obtained from Murex Diagnostics (Dartford, United Kingdom) (MR16, Lot K 809810). V14RhoA, V12Rac1, and N17Rac1 proteins were purified as GST-fusion proteins, subsequently cleaved, and characterized as described (Ridley *et al.*, 1992). Glass "Multitest" slides were obtained from ICN-Flow (ICN Biomedicals,

Aurora, OH). Bodipy-Concanavalin-A (Bodipy-Con-A, [BCA]¹) was obtained from Molecular Probes (Eugene, OR). C3-transferase purified from *Clostridium botulinum* was a kind gift from Professor A. Hall (University College, London, United Kingdom). All other reagents were obtained from Sigma Chemical, Poole, Dorset, UK.

The Cells

Rat peritoneal mast cells were prepared as described previously (Cockcroft *et al.*, 1987). The cells were resuspended in a solution containing (in mM) 137 NaCl, 2.7 KCl, 1.0 CaCl₂, 2 MgCl₂, 5.6 glucose, plus 1 mg/ml bovine serum albumin (BSA) and 20 mM NaPIPES (piperazine-*N,N'*-bis (2-ethanesulfonic acid), pH 6.8 (chloride buffer). For confocal microscopy, cells were allowed to attach to glass "Multitest" slides (6-mm-diameter wells) for 1 hr at room temperature (see Figures 1, 3, and 6–8). Suspended cells were used for flow cytometry and for hexosaminidase assays (see Figures 2, 4, and 5).

Permeabilization. Suspended cells (usually $1\text{--}2 \times 10^6$ cells/ml) were permeabilized for 2 min at 30°C in a buffer containing 137 mM Na glutamate, 2 mM MgCl₂, 1 mg/ml BSA, and 20 mM NaPIPES, pH 6.8 (glutamate buffer, GB) in the presence of 3 mM EGTA and SL-O (0.4 U/ml). To permeabilize attached cells, the cells were washed with GB and then exposed for 8 min at room temperature to SL-O at 0.4 U/ml in GB containing 3 mM EGTA (GB-EGTA). After permeabilization, both suspended and attached cells were washed free of soluble components and excess SL-O with GB-EGTA. In both cases, the time between the permeabilization and stimulation was 10 min. Ca²⁺ was buffered by 3 mM Ca²⁺ EGTA buffer system, pH 6.8, with appropriate dissociation constants as given by Martell and Smith (1974).

Preparation of Cells for Confocal Microscopy

At 10 min after SL-O addition, attached permeabilized cells were treated for 15 min at 30°C with 30–50 μ M GTP- γ -S or 2 μ g/ml V14RhoA or 2 μ g/ml V12Rac1, both in the GTP-bound form, all in GB-EGTA. Where indicated, 0.1 μ g/ml C3-transferase was added at the time of permeabilization in the presence of 0.5 mM NAD⁺, maintained throughout the wash, and removed just before the addition of GTP- γ -S (i.e., 10-min treatment). N17Rac1 was also included with the SL-O and wash at a concentration of 8 μ g/ml but was additionally maintained throughout the incubation with GTP- γ -S. Cytochalasin E (10 μ M) was added 3 min after permeabilization and maintained throughout treatment with GTP- γ -S or N17Rac1. After these treatments, the cells were stained with BCA at 32 μ g/ml in GB-EGTA for 1 min at room temperature. Unbound BCA was removed by two washes in GB-EGTA, and the cells were fixed for 20 min with 3.8% formaldehyde in phosphate-buffered saline (PBS) containing 2 mM MgCl₂ and 3 mM EGTA (PBSME). Rhodamine phalloidin (RP) staining was performed as described below for postlabeling protocol. Each treatment was repeated, and the results were reproduced at least three times.

F-Actin Staining Protocols for Confocal Microscopy

Postlabeling. Total cellular F-actin was visualized by staining fixed cells for 15 min with 2 μ M RP in PBSME containing 80 μ g/ml lysophosphatidylcholine (L-4129; Sigma).

Prelabeling. To follow the movement of existing F-actin filaments, we labeled the cells before the addition of GTP- γ -S; 4 min after permeabilization, 0.18 μ M RP in GB-EGTA was added. The stain was thoroughly washed off 2 min before the addition of GTP- γ -S (i.e., 4-min exposure to RP). After the usual 15-min exposure to

¹ Abbreviations used: BCA, Bodipy-Concanavalin-A; GB, glutamate buffer; GB-EGTA, 3 mM EGTA in GB; PBSME, 2 mM MgCl₂ and 3 mM EGTA in PBS; RP, rhodamine phalloidin; RFI, relative fluorescent intensity; SL-O, streptolysin-O.

GTP- γ -S or other stimuli, the cells were stained with BCA and fixed as described above. The intensity of stain obtained with 0.18 μ M RP was less than that obtained with 2 μ M RP. To obviate fluorescence cross-over problems, the concentration of BCA was lowered to 8 μ g/ml for the 0.18 μ M RP prelabeling experiments.

Pre- and Postlabeling. This protocol was performed to stabilize the F-actin cortex, before the addition of GTP- γ -S, and then to visualize any additional F-actin formed after the treatment with the nucleotide. The cells were prelabeled with 2 μ M RP, and the stain was washed off. GTP- γ -S was added for 15 min, and the cells were stained with 32 ng/ml BCA, fixed, and restained with 2 μ M RP as for the post-labeling protocol.

Confocal Microscopy

After staining with RP and BCA, the cells were mounted in PBSME, and fluorescent images were obtained with a Leica confocal laser scanning microscope attached to a Leitz Fluovert-FU microscope. For the detection of BCA fluorescence, excitation was at 488 nm, and emission was collected with a 540-nm bandpass filter (transmitting between 525 and 555 nm). For the detection of RP fluorescence, excitation was at 514 nm, and emission was collected with a >600 nm barrier filter. Digital images (equatorial optical slices) were displayed with the Leica "bw" look-up table. The equatorial plane of focus was defined as the midpoint between the top and bottom of the cell. The nucleus usually, but not always, lies on this plane. To investigate the possibility of the BCA fluorescence "crossing-over" to the rhodamine channel and vice versa, experiments were performed in which one or another of the stains was omitted. No detectable cross-over was observed with the concentrations of BCA and RP and wavelength filter combinations stated above.

Image Analysis

Correlating F-Actin Content with Secretion. The total pixel intensity of RP and BCA staining found in the equatorial slices of at least 40 cells was determined by integration of the appropriate image areas. The RP staining (RP relative fluorescence intensity, RP RFI) of this equatorial slice was deemed to represent the F-actin content of the whole cell (Norman *et al.*, 1994). The BCA staining (BCA RFI) was considered to be an "all or nothing" index of the occurrence of secretion. The designation of cells into secreting and nonsecreting populations is described in RESULTS.

Correlating Actin Distribution Changes with Secretion. A quantitative estimate of F-actin distribution changes after various treatments was obtained by radial line scan analysis of the images of the equatorial slices. A radial line was described projecting from the cell exterior to a depth of 30 pixels within the cell. This represents a penetration of 7.35 μ m into the cytoplasmic space. The nucleus was normally >8 μ m from the cell edge and therefore not included in this analysis. Three such lines were obtained per cell for a total of at least 40 cells. To obtain the distribution plots, as displayed in Figures 3 and 8, the lines obtained for each set of experimental conditions were averaged to form a mean profile $\pm$ SEM (the statistic is appropriate for the cell number and not the number of lines). The profiles obtained from secreting and nonsecreting cell populations (sorted according to equatorial slice BCA fluorescence, as described in RESULTS) were plotted independently, where appropriate.

Flow Cytometry

Correlating BCA Staining with Light-scattering Characteristics. Intact cells, suspended in chloride buffer, were treated with unconjugated Succinyl-Con-A (100 μ g/ml, 10 min at room temperature) to reduce the background staining. Cells were then permeabilized as above and exposed for 20 min to 3 mM EGTA (control) or to 30 μ M GTP- γ -S/pCa6 (stimulated). After this treatment, cells were quenched with cold GB, spun, resuspended in GB-EGTA containing

FITC-Succinyl-Con-A (64 μ g/ml), and stained for 20 min on ice. The stain was washed off (2 times with GB-EGTA), and the cells were resuspended ($\sim 5 \times 10^5$ ml⁻¹) in GB-EGTA containing 0.5% formaldehyde (without any BSA) and analyzed on an EPICS Elite flow cytometer (Coulter Electronics, Hialeah, FL) equipped with an argon ion laser. Excitation was at 488 nm, and emission was at 525 nm. Forward angle (1.5–19°) and 90° light-scattering and fluorescence readings were collected. Freehand "gates" were drawn around subpopulations on two-parameter light-scattering distributions using the Elite software, and the fluorescence of cells falling within these gates was plotted on one-parameter histograms. Low-level signals from noise and cell debris were removed by this gating procedure. **Correlating F-Actin Content with Secretion.** Suspended mast cells were permeabilized as above and then prelabeled with 0.18 μ M RP in GB-EGTA (2 min at room temperature). Cells were then exposed to various triggering solutions for 20 min, quenched with cold GB, spun, resuspended in GB-EGTA containing 0.5% formaldehyde, and analyzed on an EPICS Elite flow cytometer as above. Excitation was at 488 nm, and emission was at 575 nm. Forward angle and 90° light-scattering and fluorescence readings were collected and analyzed as above. Hexosaminidase release was assayed as previously described (Cockcroft *et al.*, 1987).

RESULTS

Fluorescent Con-A as a Tool for Detection of Exocytosis

As a result of exocytotic fusion, proteins within secretory vesicles become exposed to the cell exterior. We have used a fluorescent lectin, BCA, to visualize granular glycoproteins and proteoglycans accessible to this stain after degranulation of mast cells. These include proteins in incompletely extruded secretory granule matrices as well as lumen-facing granule membrane glycoproteins.

The left-hand panels of Figure 1 show confocal micrographs of permeabilized mast cells treated with BCA before fixation. Figure 1A is an image of control cells (exposed to EGTA). The plasma membrane was the only structure visible. After stimulation with GTP- γ -S in the absence of calcium (Figure 1, B and C, left-hand panels), an increased BCA staining was observed. However, not all of the permeabilized cells respond to GTP- γ -S in the same way; a subpopulation of cells ($\sim 30\%$) remained unresponsive and did not exhibit the increase in BCA fluorescence.

Because of dramatic changes in their morphology and refractive properties, degranulating mast cells can also be easily recognized by using phase-contrast microscopy: they are more transparent and have less well-defined edges. In all cases, we have found that cells that stained brightly with BCA corresponded to those observed to degranulate by phase-contrast microscopy. To confirm that Con-A-stained cells are those undergoing degranulation, populations of suspended cells stained with FITC-Succinyl-Con-A were analyzed by flow cytometry. Morphological changes of secreting cells, reflected by pronounced changes in light-scattering characteristics, were compared with the fluorescent Con-A binding properties of these

Figure 1. Confocal micrographs of Bodipy-Con-A and rhodamine phalloidin-stained permeabilized mast cells. After stimulation, cells were stained with BCA to visualize degranulating cells (left-hand panels) and then fixed and stained with 2 μ M RP to visualize F-actin (right-hand panels). (A) SL-O permeabilized cells treated for 15 min at 30°C with 3 mM EGTA or (B) with 50 μ M GTP- γ -S/3 mM EGTA. (C) Permeabilized cells stimulated with GTP- γ -S, displayed at higher magnification. The bars in A and C represent 20 and 10 μ m, respectively.

cells. Figure 2, A and B, shows histograms of permeabilized cells exposed to EGTA (control) and GTP- γ -S/pCa6 (stimulated), respectively. The inclusion of calcium at this point was necessary because suspended cells, used for flow cytometric studies, are less responsive to GTP- γ -S than their attached counter-

parts that were used for the confocal studies. Most of the control cells were contained within the population X (95% of total cell number; Figure 2A). After stimulation 53% of cells exhibited lower side scatter (population Z; Figure 2B). The fluorescence intensity of the population Z (Figure 2D) was found to be much higher than that of the control cells (Figure 2C) or of the cells not responding to the stimulus (i.e., those remaining within population Y; Figure 2D). Thus, fluorescent Con-A is an effective tool for assessment and identification of the degranulating population of mast cells. To be precise, Con-A-stained cells are those in which at least some of the granules have undergone fusion with the plasma membrane. However, because some granules are completely extruded, the intensity of the fluorescence cannot necessarily be considered as proportional to the extent of secretion.

Correlation of Cytoskeletal Changes with Secretion

The right-hand panels of Figure 1 show confocal micrographs of permeabilized mast cells stained (after fixation) with RP to visualize actin filaments. As reported previously, F-actin is present primarily at the cortex of quiescent permeabilized cells (Figure 1A). After stimulation by GTP- γ -S (Figure 1, B and C, right-hand panels), the RP staining of the cell cortex becomes less intense and that of the cell interior stronger. GTP- γ -induced secretion is accompanied by an overall increase in the total F-actin content (20–30%; Norman *et al.*, 1994). Comparison of BCA and RP-stained cells revealed that those cells that have a high BCA fluorescence also have a higher F-actin content. This is shown clearly by the high magnification image in Figure 1C. Of the four GTP- γ -treated cells, three are degranulating. These three cells also exhibit a network of actin filaments within the cytoplasmic space. The nonresponding cell (bottom left-hand corner) is primarily devoid of these structures.

BCA and RP fluorescence intensities were quantified by evaluation of the total pixel intensity per cell in images similar to those shown in Figure 1. For the purpose of classification, the value of BCA relative fluorescence intensity (BCA RFI), below which the control group of nonsecreting cells was contained, was taken as a borderline. Figure 3, A and B, represents such quantitative evaluation of the content and the distribution of F-actin in secreting and nonsecreting cell populations. The bar diagram (Figure 3A) shows that cells unresponsive to GTP- γ -S (NS; cells with BCA RFI < 150) had F-actin content similar to that of the control cells. In contrast, secreting cells (S; cells with BCA RFI > 150) exhibited a relative increase in F-actin (RP RFI is higher relative to control cells). The radial line analysis in Figure 3B shows that, although the increase in the content of F-actin in the interior region was much greater in secreting cells, the reduction in

Figure 2. Flow cytometry of permeabilized, FITC-Succinyl-Con-A-stained mast cells. Cells were treated with EGTA (control) or 30 μ M GTP- γ -S/pCa6 (stimulated) and stained, as described in MATERIALS AND METHODS. (A) Light-scattering characteristics of control cells (population X, 95% of total). (B) Light-scattering characteristics of stimulated cells (Y, nonsecreting, 43%; Z, secreting, 53%). (C and D) Corresponding fluorescence of these cell populations.

the cortical region was the same for both populations. Cortical disassembly in response to GTP- γ -S occurred irrespective of whether a cell secreted or not.

Effect of Actin Filament Stabilization on Secretion

Pretreatment of permeabilized cells with 2 μ M RP stabilizes actin filaments and prevents their disassembly and Rac-dependent relocalization (Norman *et al.*, 1994). Surprisingly, the phalloidin pretreatment did not significantly reduce the secretory response. We have obtained this result with both suspended and attached permeabilized cells. Thus, when GTP- γ -S-induced secretion from phalloidin pretreated, suspended cells was assayed (either as percentage of released hexosaminidase or by flow cytometry of FITC-Succinyl-Con-A-stained cells), phalloidin had no effect (our unpublished results). The response of glass-attached cells is shown in Figure 3, C and D. In this experiment, 2 μ M RP pretreated cells were stained with BCA after secretion to identify the secreting cell population, fixed, and restained with RP to visualize any additional F-actin that might be newly polymerized after GTP- γ -S addition. The bar diagram (Figure 3C) shows that GTP- γ -S induced a similar proportion of cells (85%) to stain with BCA (i.e., to degranulate) re-

gardless of the RP pretreatment (compare with Figure 3A, in which 73% of cells degranulated). Thus, two cytoskeletal effects induced by GTP- γ -S (cortical filament disassembly and relocalization) could be inhibited by phalloidin without affecting the secretory response.

Preferential De Novo Actin Polymerization in the Secreting Cell Population

Figure 3C also shows that those RP-pretreated cells with BCA RFI values above control level exhibit an increased F-actin content (higher RP RFI) similar to the cells shown in Figure 3A. As shown by radial line scans (Figure 3D), the RP pretreatment inhibited the cortical actin disassembly in both secreting and nonsecreting cell populations. Thus, also in this case, the F-actin increase was exclusively within the cell interior and was much greater in secreting (BCA-positive) cells. Because no relocalization of the existing cortical filaments occurred, the increase in the cell interior resulted from de novo actin polymerization only. This actin polymerization is dependent on the activity of a small GTPase, Rho (Norman *et al.*, 1994); therefore, the results of Figure 3D indicate that Rho is more active in degranulating cells.

Figure 3. Response of permeabilized mast cells to GTP-γS. Top panels show RP relative fluorescence intensities (RFI) of SL-O permeabilized cells after their treatment with 3 mM EGTA (control) or 50 μM GTP-γS/EGTA (plus GTP-γS). The data were obtained from evaluation of the total pixel intensity per cell in images similar to those shown in Figure 1. Radial line scan analyses (bottom panels) show distribution of F-actin in basal (●, circles) and GTP-γS-stimulated cells (triangles). Cells with RFI BCA above and below control levels are represented by Δ and ▲ triangles, respectively. (A and B) Postlabeling with 2 μM RP. Cells were stained as described for Figure 1. The upper limit RFI BCA of the control cells was 150; 73% of cells secreted in response to GTP-γS (with RFI BCA >150). Mean relative F-actin content of GTP-γS-treated cells was $127 \pm 2.8\%$ ($n > 50$). Both secreting and nonsecreting cells exhibit cortical actin disassembly, but the level of F-actin in the cell interior is much greater in the secreting cell population. (C and D) Pre- and postlabeling with 2 μM RP. To stabilize F-actin, permeabilized cells were treated with 2 μM RP before exposure to the triggering solutions. After 15 min at 30°C, cells were stained with BCA, fixed, and then restained with 2 μM RP to visualize any additional F-actin polymerized after GTP-γS treatment. The upper limit RFI BCA of the control cells was 180; 85% of cells secreted in response to GTP-γS. Mean relative F-actin content of GTP-γS-treated cells was $122 \pm 2.6\%$ ($n > 50$). De novo actin polymerization occurred preferentially in secreting cells. (E and F) Prelabeling with 0.18 μM RP. To follow actin filament relocation, permeabilized cells were treated with 0.18 μM RP before exposure to the triggering solutions. After 15 min at 30°C, cells were stained with BCA and fixed. The upper limit RFI BCA of the control cells was 130; 68% of cells secreted in response to GTP-γS. Mean relative F-actin content of GTP-γS-treated cells was $87 \pm 4.0\%$ ($n > 50$). Relocation of cortical filaments to the cell interior occurred preferentially in secreting cells.

Increased Relocalization of Cortical Actin Filaments in the Secreting Cell Population

In addition to the newly polymerized actin, the increase in F-actin in the cell interior is also due to relocalization of cortical actin filaments. The entrapment of the released cortical filaments is dependent on the activity of another small GTPase, Rac (Norman *et al.*, 1994). It is possible to follow the fate of the cortical filaments by pretreatment of permeabilized cells with a low (0.18 μM) concentration of RP (insufficient to stabilize the cortex, but sufficient to tag label the F-actin). The movement of these labeled filaments to the

cell interior or their loss from the cell may then be monitored.

Figure 3E shows that under the above protocol, the secreting cell population retained its F-actin, whereas the nonsecreting cells lost a substantial proportion of their prelabeled filaments. The radial line scans in Figure 3F show redistribution of cortical actin to the cytoplasmic region of the secreting cells. Thus, the entrapment of released cortical filaments also occurred preferentially in secreting cells. On the other hand, the disassembly of the cortical filaments occurred in both secreting and nonsecret-

ing cell populations and was, therefore, on its own, insufficient to induce exocytosis.

Preferential Retention of F-Actin in Secreting Cells Confirmed by Flow Cytometry

Secretion from washed permeabilized mast cells can be induced in the absence of GTP- γ -S by calcium (pCa 5) and ATP (>1 mM; Koffer, 1993). In this case, F-actin disassembles, but no relocalization or polymerization of actin occurs (Figure 4; Borovikov *et al.*, 1995). Using the 0.18 μ M RP prelabeled protocol (as described for Figure 3, E and F) and measuring light-scattering characteristics of suspended cells (as described for Figure 2B), we analyzed cell populations after their exposure to 10 μ M calcium/3 mM ATP (Figure 4, C and E). Relative to the control EGTA-exposed cells (Figure

4A), both secreting (S) and nonsecreting (NS) cells exhibited lower RP fluorescence (Figure 4C). Both populations lost ~30% of their F-actin (Figure 4E). However, when GTP- γ -S was also present in the triggering solution, the content of F-actin was significantly higher in the secreting cell population (Figure 4D). Although nonsecreting cells lost ~20% of F-actin, the F-actin content of secreting cells remained close to that of the control cells (Figure 4F). Thus, the results obtained by both confocal microscopy and flow cytometry have shown that the preferential retention of actin in secreting cells is dependent on activation of a GTP-binding protein(s).

Enhancement of Secretion by V14RhoA and V12Rac1 Is Not Inhibited by Cytochalasin

The above results indicate that the effects of Rac and Rho proteins on both the actin cytoskeleton and on secretion are closely correlated. To test how important for secretion are the cytoskeletal effects of Rho (i.e., de novo actin polymerization), we have tested the effects of cytochalasin. This actin-destabilizing drug, which blocks barbed ends of actin filaments (Cooper, 1987), inhibits Rho-dependent de novo polymerization of actin (Norman *et al.*, 1994). Addition of a constitutively active mutant of Rho (V14RhoA) to permeabilized mast cells enhances calcium/ATP-induced secretion (Price *et al.*, 1995). As shown in Figure 5, the presence of cytochalasin E had no effect on the calcium/ATP-induced secretory response of control or V14RhoA pretreated, suspended cells. This result was obtained by assaying the released hexosaminidase (Figure 5A) as well as by measuring the proportion of secreting cells by flow cytometry (Figure 5B). The enhancement induced by V12Rac1 was also unaffected by cytochalasin (Figure 5). Thus despite the close correlation, the effects of Rac and Rho on the cytoskeleton and on secretion are independent. A common (upstream) factor must exist that activates both processes within a particular cell.

It should be noted here that permeabilized cells do not secrete in response to the constitutively active forms of Rho and Rac (V14RhoA and V12Rac1) when these are applied alone under basal conditions (3 mM EGTA). Introduction of V14RhoA into permeabilized cells is, however, sufficient to induce the de novo actin polymerization in the cell interior in a cytochalasin-sensitive manner (Norman *et al.*, 1994). No changes in the cytoskeleton are observed upon addition of V12Rac1, and this lack of response is probably due to the absence of cortical actin disassembly, without which no relocalization of filaments can occur.

Rac and Rho Communicate

C3-transferase is an exoenzyme from *Clostridium botulinum*, which inactivates Rho by ADP-ribosylation

Figure 4. Degranulated mast cells retain their F-actin in the presence of GTP- γ -S. Flow cytometry of permeabilized mast cells prelabeled with a low concentration of RP and then stimulated with calcium (pCa 5) and 3 mM MgATP without (C and E) or with (D and F) 50 μ M GTP- γ -S. The appropriate control populations (3 mM EGTA) are shown in A and B. At minimum, 5000 cells were analyzed, and the results are representative of at least five such experiments. Relative F-actin content shown in E and F is the mean $\pm$ SE, calculated as mean fluorescence channel number; the control levels are taken as 100%.

Figure 5. Enhancement of secretion by V14RhoA and V12Rac1 is not inhibited by cytochalasin. Permeabilized cells were stimulated with calcium (pCa 5) and 3 mM MgATP in the presence or absence of V12Rac1 (16 μ g/ml), V14RhoA (8 μ g/ml), and cytochalasin E (cyto; 1 μ M). Hexosaminidase secretion (A) was assayed, cells were then fixed, and the proportion of secreting cells was determined by flow cytometry (B). The error bars represent SD of triplicate determinations; the results are representative of three separate experiments.

(Aktories *et al.*, 1989). N17Rac1 is a dominant inhibitory mutant of Rac (Ridley *et al.*, 1992). GTP- γ -S-induced secretion from suspended permeabilized mast cells is strongly (40–60%) inhibited by both of these agents (Price *et al.*, 1995), indicating participation of endogenous Rac and Rho in the secretory response. The presence of both of these proteins in mast cells has been confirmed by immunoblotting (Price *et al.*, 1995). Similar to the cytochalasin effects, C3 inhibits GTP- γ -S-induced actin polymerization in mast cells, whereas N17rac1 prevents the retention of the released cortical filaments (Norman *et al.*, 1994).

The secretory response of glass-attached cells to GTP- γ -S is much stronger than that of the suspended cells, indicating that adhesion-dependent signaling may participate in the secretory response (shown by BCA staining; our unpublished results). We have therefore examined the sensitivity of glass-attached mast cells to various combinations of C3-transferase, cytochalasin E, and N17Rac1. Exocytosis was visualized either by BCA staining (Figure 6A) or by phase microscopy (Figure 6B). In contrast to their effects on

suspended cells, C3 had only a small effect on secretion. Evaluation of the response by phase microscopy (counting the more transparent degranulating cells) has shown that the addition of C3 reduced the proportion of cells that secrete in response to GTP- γ -S from 77 to 58%. N17Rac1 was not inhibitory at all. However, simultaneous addition of both C3 and N17Rac1 caused a profound reduction in the proportion of secreting cells (from 77 to 15%), indicating that Rac and Rho can counteract each other. On the other hand, simultaneous addition of N17Rac1 and cytochalasin E had no effect on exocytosis. This indicates that inhibition of secretion by C3 is not due to its inhibition of actin polymerization.

Differential Effects of GDP- β -S on Secretion and Actin Organization

Secretory response of washed, permeabilized mast cells to GTP- γ -S is very sensitive to GDP- β -S (Koffer, 1993). Here, we have examined how GDP- β -S affects the cytoskeletal rearrangements induced by GTP- γ -S. Figure 7 shows confocal micrographs of permeabilized cells triggered with 50 μ M GTP- γ -S in the absence (Figure 7A) or presence of 1 mM (Figure 7B) or 10 mM (Figure 7C) GDP- β -S. The left- and right-hand panels show the same cells stained with BCA (for exocytosis) and RP (for F-actin), respectively. Exocytosis was blocked by 1 mM GDP- β -S but the changes in F-actin distribution were unaffected. This was confirmed by the radial line scan analyses shown in Figure 8. Only on increasing the concentration of GDP- β -S to 10 mM was a complete blockade of both exocytosis and F-actin redistribution observed (Figures 7 and 8). This indicates that a GTP-binding protein required for exocytosis is more sensitive to GDP- β -S than Rho, Rac, and the AlF_4^- -sensitive heterotrimeric G-protein required for redistribution of the actin cytoskeleton.

DISCUSSION

We have shown previously that Rac and Rho are involved in regulating both secretion and the actin reorganization that accompanies activation of mast cells (Norman *et al.*, 1994; Price *et al.*, 1995). How then does the cytoskeletal rearrangement relate to exocytosis? Here we show that cytoskeletal reorganization occurs preferentially in the secreting cell population. The two responses are strongly correlated; therefore, a common upstream regulatory element must exist. However, the relationship is not a simple one, because the two activities can be divorced from one another. This indicates that the signaling pathways to secretion and the cytoskeleton may diverge and act in parallel rather than in series.

Figure 6. Effects of various agents on GTP- γ S-induced exocytosis. Permeabilized cells were treated with C3-transferase (C3; 0.1 μ g/ml), cytochalasin E (Cy; 10 μ M), N17Rac1 (N17; 8 μ g/ml), C3-transferase with N17Rac1, or cytochalasin E with N17Rac1, as described in MATERIALS AND METHODS. The cells were then stimulated to secrete with GTP- γ S/EGTA (G; 50 μ M). (A) Exocytosis visualized by BCA. (B) The proportions of secreting cells as calculated from phase micrographs. The values represent data from at least three experiments and cells counted from at least five different fields ($n > 5$).

Cytoskeleton-independent Functions of Rac and Rho

In fibroblasts, Rac, Rho, and Cdc42 control the assembly of actin into membrane ruffles, stress fibers, and filopodia, respectively (Ridley and Hall, 1992; Ridley *et al.*, 1992; Nobes and Hall, 1995). However, these GTPases can, independently of actin polymer-

ization, induce the assembly of specific multimolecular focal complexes that link actin networks to the plasma membrane (Nobes and Hall, 1995). The Rho-dependent formation of focal adhesions requires activities of protein tyrosine kinases (Ridley and Hall, 1994), indicating that kinases may be an-

other target for Rho-related GTPases. Recent findings that a serine/threonine kinase p65PAK (p21-activated kinase) binds to and is activated by the GTP-bound forms of Rac and Cdc42 is relevant; p65PAK kinase may be an effector of Rac (Manser *et al.*, 1994).

Figure 7. Confocal micrographs showing differential effects of GDP- β -S on secretion and F-actin distribution. Cells were permeabilized in the presence of (A) none, (B) 1 mM, or (C) 10 mM GDP- β -S and then treated (15 min at 30°C) with 50 μ M GTP- γ -S/EGTA, maintaining the GDP- β -S concentrations throughout this stimulation. Cells were then stained with both BCA (left-hand panels) and RP (right-hand panels). GDP- β -S did not affect control (3 mM EGTA-treated) cells, which resembled the basal cells shown in Figure 1A.

Figure 8. Radial line scan analyses of confocal images of GDP- β -S-treated cells. Cells were permeabilized in the presence of GDP- β -S as for Figure 8 and then exposed to 3 mM EGTA ($\bullet$) or to 50 μ M GTP- γ -S/EGTA in the presence of 1 mM GDP- β -S ($\blacktriangle$) or 10 mM GDP- β -S ($\triangle$). The distribution of F-actin in basal cells was unaffected by 1 or 10 mM GDP- β -S; only one scan is shown for clarity ($n > 50$).

In phagocytic leukocytes, Rac has been found to regulate the production of superoxide by NADPH oxidase without any involvement of the cytoskeleton (Abo *et al.*, 1992; Rotrosen *et al.*, 1992). Rho may also play a role in thrombin-induced aggregation of platelets (Morii *et al.*, 1992) and PMA-induced aggregation of lymphocytes (Tominaga *et al.*, 1993), as shown by experiments with C3 transferase. In both platelets and lymphocytes, cytochalasin inhibited the motile responses but not cell aggregation, and the authors suggested that the latter is regulated by Rho independently of its effects on actin polymerization. Rac, in addition to its effect on actin, controls the release of arachidonic acid from membrane phospholipids, probably by activating cPLA₂ (Peppelenbosch *et al.*, 1995). The subsequent generation of leukotrienes was shown to activate Rho and thus to induce formation of stress fibers. Recent evidence also points to Rac and Cdc42 involvement in the control of the MEKK-JNK pathway and therefore of gene expression (reviewed, Vojtek and Cooper, 1995). The serine/threonine kinase p65PAK has been suggested as a possible link between Cdc42/Rac1 and MEKK.

Other "noncytoskeletal" functions of Rho-related GTPases include control of enzymes that regulate the turnover of phosphoinositides, such as PI3-K, PIP5-K, and PLD (Bowman *et al.*, 1993; Chong *et al.*, 1994; Malcolm *et al.*, 1994; Hawkins *et al.*, 1995). Because activities of many actin-binding proteins are regulated by phosphoinositides (Lassing and Lindberg, 1988), at least some effects that these GTPases exert on the cytoskeleton may well be a consequence of their effects

on phospholipid metabolism. In permeabilized platelets, polyphosphoinositides were shown to induce F-actin uncapping and thus to provide nucleation sites for actin assembly (Hartwig *et al.*, 1995). This effect was mimicked by activation of thrombin receptor, by GTP- γ -S, and, most significantly, by V12Rac1. In parallel, V12Rac1 also induced PIP₂ synthesis. Using immunofluorescence confocal microscopy, we have previously observed a reduction of cortical gelsolin after GTP- γ -S/EGTA treatment of permeabilized mast cells (Borovikov *et al.*, 1995). This could be relevant to the cortical F-actin disassembly. Gelsolin caps actin filaments, and polyphosphoinositide-induced uncapping would cause their destabilization.

F-Actin Disassembly in Mast Cells

Cortical disassembly accompanies secretion induced by all stimuli examined so far, which is consistent with the postulate that cortical actin forms a barrier to exocytosis. However, as shown in Figure 3C, high concentrations of phalloidin that prevent GTP- γ -S-induced cortical F-actin disassembly and filament relocalization have no significant effect on secretion or on the proportion of cells that has undergone exocytotic fusion. It is possible that the response to GTP- γ -S/EGTA involves only those peripheral granules that are already docked and therefore unaffected by cortex stabilization. On the other hand, evidence that microfilament disassembly may control the proportion of granules recruited for exocytosis was obtained by introducing exogenous gelsolin into permeabilized cells (Borovikov *et al.*, 1995). Severing of actin filaments by gelsolin may facilitate recruitment of the internal granules. Another signal may also be directed at filament cross-linking proteins rather than at actin itself (Perrin and Aunis, 1985). This would result in loosening of the actin network but would not be detected by our methodology. As reported previously, phalloidin does not prevent cortical disassembly induced by Ca²⁺ and does not have any effect on calcium-induced secretion (Koffer *et al.*, 1990). Thus, the mechanisms of calcium- and GTP- γ -S-induced cortical disassembly are different and may synergize.

Cortical disassembly occurs in both secreting and nonsecreting cell populations (Figures 3, B and F, and 4E). In addition, GTP- γ -S in the presence of 1 mM GDP- β -S (Figure 7) causes disassembly without inducing secretion. Thus, the disassembly by itself is insufficient to induce secretion, and an additional Rac and/or Rho-regulated step is required. This contrasts with permeabilized pancreatic acinar cells, which were reported to secrete in response to thymosin β -induced actin depolymerization without any additional cofactors (Muallem *et al.*, 1995).

The Appearance of New Actin Filaments

A very strong correlation was detected between the appearance of internal actin filaments (in the cytoplasmic space of mast cells) and secretion. The two events, however, can occur independently of each other. For example, actin polymerization occurs independently of cortical disassembly or secretion when V14Rho is introduced into permeabilized cells (Norman *et al.*, 1994). GDP- β -S (1 mM) inhibits the secretory but not the cytoskeletal response (Figure 7). Conversely, secretion occurs without the appearance of internal actin filaments when cells are stimulated by calcium and ATP in the absence of GTP- γ -S (Figure 4). Cytochalasin inhibits de novo actin polymerization without any significant effects on secretion (Figures 5 and 6). The ability of Rho and Rac to promote secretion in the presence of cytochalasin clearly demonstrates that this signaling is not via the cytoskeleton (Figure 5).

When both relocalization and actin polymerization are prevented by N17Rac1 and C3, respectively, secretion is strongly inhibited. The same effect on the cytoskeleton is obtained by a combination of N17Rac1 with cytochalasin E but without altering exocytosis (Figure 6). This again indicates that the cytoskeletal rearrangements do not necessarily precede degranulation but that the activities of either Rho or Rac must be intact. The collapsed appearance of cells, secreting under conditions in which both relocalization and de novo actin polymerization have been inhibited by N17Rac1 and cytochalasin, may give a clue to the function of this internal actin cytoskeleton. It could provide structural compensation for the disassembled cortex of degranulating cells as well as restrict granules from any uncontrolled secretory response. Another possible function for the internal actin filaments is an involvement in the endocytotic process that follows degranulation (Lindau *et al.*, 1993; Thomas *et al.*, 1994).

A Model for the Involvement of GTPases in Mast Cell Activation

Figure 9 depicts a schematic summary of proposed pathways regulating mast cell actin and secretion, showing four GTPases that are either common to both processes or limited to one. 1) An AIF₄⁻-sensitive G-protein regulates disassembly of the actin cortex and is independent of the other three GTPases. A possible candidate for this protein is G_{i3}, a pertussis toxin-sensitive heterotrimeric G-protein that can be activated directly by compound 48/80 (Aridor *et al.*, 1990). This is our working hypothesis at present. The disassembly facilitates secretion by allowing an increased number of granules to be recruited for exocytosis, but it is not necessary for the fusion of (presumably docked) granules with the plasma membrane. In intact cells, the G-protein (activated by compound

Figure 9. Model showing interactions between GTP-binding proteins that regulate the cytoskeleton and exocytosis. An AlF_4^- -sensitive G-protein leads to disassembly of cortical actin filaments. The disassembly facilitates secretion by allowing an increased number of granules to be recruited for exocytosis but is not necessary for the fusion of (presumably docked) granules with plasma membrane. Activation of Rac and Rho cause entrapment of released cortical filaments and de novo actin polymerization, respectively. The activity of either Rac or Rho is also necessary for exocytotic fusion. Exocytosis requires the activity of another GTP-binding protein that is more sensitive to GDP- β -S than Rho, Rac, and the AlF_4^- -sensitive G-protein. A cytosolic factor mediates communication between the G-protein and Rac and Rho. Calcium, although not included in the model, may affect each step of the pathway.

48/80) would communicate with Rac and Rho proteins via a cytosolic factor that leaks from permeabilized cells (Norman *et al.*, 1994). 2) Rac activity is required for entrapment of released cortical filaments, and 3) Rho regulates actin polymerization from a membrane-bound monomeric pool. The activity of either Rho or Rac is also required for exocytosis. The two proteins apparently communicate, because major inhibition of secretion from glass-attached cells is seen only when both proteins are inhibited. However, the two cytoskeletal responses elicited by Rac and Rho proteins can be evoked independently of each other: de novo actin polymerization can occur in the absence of the relocation (Figure 3D); conversely, relocation occurs in the presence of C3 or cytochalasin, when actin polymerization is inhibited (Norman *et al.*, 1994). The pathways to exocytosis and to the cytoskeleton are divergent but are controlled by a common

upstream regulator, an activator of Rac and/or Rho. 4) Exocytosis requires activation of another GTP-binding protein that is more sensitive to inhibition by GDP- β -S than Rho, Rac, or the AlF_4^- -sensitive G-protein. Its activity is not involved in the cytoskeletal response. In Figure 9, the GDP- β -S-sensitive protein is located downstream of Rac/Rho proteins although it could act independently. Rab or Arf, small GTPases implicated in the control of exocytosis (Oberhauser *et al.*, 1992; Fensome *et al.*, 1996), are possible candidates for this additional GTP-binding protein.

The shared pathway from Rac and Rho to a common downstream fusogenic effector could be due to a common target protein for Rho and Rac, possibly one of the GAPs (GTPase-activating proteins) for Rho-related proteins (Boguski and McCormick, 1993; Lancaster *et al.*, 1994). On the other hand, the divergent pathways must be mediated by specific targets and/or accessory proteins, such as bcr and n-chimaerin for Rac and p190 for Rho (Diekmann *et al.*, 1991; Ridley *et al.*, 1993). These more specific proteins may constitute part of the filament entrapment or actin polymerization responses. Calcium has not been included in this model, because this work is mainly concerned with the contribution of GTP-binding proteins. However, it has an important role in mast cell secretion and may well affect all the steps shown in the model (Howell *et al.*, 1987).

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REFERENCES

- Abo, A., Boyhan, A., West, I., Thrasher, A., and Segal, A. (1992). Reconstitution of neutrophil NADPH oxidase activity in the cell-free system by four components: p67-phox, p47-phox, p21-rac1, and cytochrome b245. *J. Biol. Chem.* 267, 16767–16770.
- Aktories, K., Braun, S., Rosener, S., Just, I., and Hall, A. (1989). The Rho gene product expressed in *E. coli* is a substrate for botulinum ADP-ribosyltransferase C3. *Biochem. Biophys. Res. Commun.* 158, 209–213.
- Aridor, M., Traub, L.M., and Sagi-Eisenberg, R. (1990). Exocytosis in mast cells by basic secretagogues: evidence for direct activation of GTP-binding proteins. *J. Cell Biol.* 111, 909–917.
- Boguski, M.S., and McCormick, F. (1993). Proteins regulating Ras and its relatives. *Nature* 366, 643–654.
- Borovikov, Y.S., Norman, J.C., Price, L.S., Weeds, A., and Koffer, A. (1995). Secretion from permeabilised mast cells is enhanced by addition of gelsolin: contrasting effects of endogenous gelsolin. *J. Cell Sci.* 108, 657–666.
- Bowman, E.P., Uhlinger, D.J., and Lambeth, J.D. (1993). Neutrophil phospholipase D is activated by a membrane-associated Rho family

- small molecular weight GTP-binding protein. *J. Biol. Chem.* 268, 21509–21512.
- Burgoyne, R.D., and Cheek, T.R. (1985). Reorganisation of peripheral actin filaments as a prelude to exocytosis. *Biosci. Rep.* 7, 281–288.
- Chong, L.D., Traynor Kaplan, A., Bokoch, G.M., and Schwartz, M.A. (1994). The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell* 79, 507–513.
- Cockcroft, S., Howell, T.W., and Gomperts, B.D. (1987). Two G-proteins act in series to control stimulus-secretion coupling in mast cells: use of neomycin to distinguish between G-proteins controlling polyphosphoinositide phosphodiesterase and exocytosis. *J. Cell Biol.* 105, 2745–2750.
- Cooper, J.A. (1987). Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* 105, 1473–1478.
- Diekmann, D., Brill, S., Garrett, M.D., Totty, N., Hsuan, J., Monfries, C., Hall, C., Lim, L., and Hall, A. (1991). Bcr encodes a GTPase-activating protein for p21-rac. *Nature* 351, 400–402.
- Elferink, L.A., Peterson, M.R., and Scheller, R.H. (1993). A role for synaptotagmin (p65) in regulated exocytosis. *Cell* 72, 153–159.
- Fensome, A., Cunningham, E., Prosser, S., Khoon Tan, S., Swigart, P., Thomas, G., Hsuan, J., and Cockcroft, S. (1996). ARF and PIP₂ restore GTP- γ S-stimulated protein secretion from cytosol-depleted HL60 cells by promoting PIP₂ synthesis. *Curr. Biol.* 6, 730–738.
- Hall, A. (1992). Ras-related GTPases and the cytoskeleton. *Mol. Biol. Cell* 3, 475–479.
- Hartwig, J.H., Bokoch, G.M., Carpenter, C.L., Janmey, P.A., Taylor, L.A., Toker, A., and Stossel, T.P. (1995). Thrombin receptor ligation and activated Rac uncouple actin filament barbed ends through phosphoinositide synthesis in permeabilised human platelets. *Cell* 82, 643–653.
- Hawkins, P.T., Eguinoa, A., Qui, R., Stokoe, D., Cooke, F.T., Walters, R., Wennstrom, S., Claesson-Welsh, L., Evans, T., Symons, M., and Stephens, L. (1995). PDGF stimulates an increase in GTP-Rac via the activation of phosphoinositide 3OH kinase. *Curr. Biol.* 5, 393–400.
- Howell, T.W., Cockcroft, S., and Gomperts, B.D. (1987). Essential synergy between Ca²⁺ and guanine nucleotides in exocytotic secretion from permeabilised mast cells. *J. Cell Biol.* 105, 191–197.
- Ingber, D.E., et al. (1994). Cellular tensegrity: exploring how mechanical changes in the cytoskeleton regulate cell growth, migration, and tissue pattern during morphogenesis. *Int. Rev. Cytol.* 150, 173–224.
- Koffer, A. (1993). Calcium-induced secretion from permeabilised rat mast cells: requirements for guanine nucleotides. *Biochim. Biophys. Acta* 1176, 236–244.
- Koffer, A., Tatham, P.E.R., and Gomperts, B.D. (1990). Changes in the state of actin during the exocytotic reaction of permeabilised rat mast cells. *J. Cell Biol.* 111, 919–927.
- Lancaster, C.A., Taylor-Harris, P.M., Self, A.J., Brill, S., van Erp, H.E., and Hall, A. (1994). Characterization of RhoGAP. A GTPase-activating protein for Rho-related small GTPases. *J. Biol. Chem.* 269, 1137–1142.
- Lassing, I., and Lindberg, U. (1988). Evidence that the phosphatidylinositol cycle is linked to cell motility. *Exp. Cell Res.* 174, 1–15.
- Lindau, M., Nüsse, O., Bennett, J.P., and Cromwell, O. (1993). The membrane fusion events in degranulating guinea pig eosinophils. *J. Cell Sci.* 104, 203–209.
- Linstedt, A.D., and Kelly, R.B. (1987). Overcoming barriers to exocytosis. *Trends Neurosci.* 10, 446–448.
- Malcolm, C.K., Ross, A.H., Qiu, R.G., Symons, M., and Exton, J.H. (1994). Activation of rat liver phospholipase D by the small GTP-binding protein RhoA. *J. Biol. Chem.* 269, 25951–25954.
- Manser, E., Leung, T., Salihuddin, H., Zhao, Z., and Lim, L. (1994). A brain serine/threonine protein kinase activated by cdc42 and Rac1. *Nature* 367, 40–46.
- Martell, A.E., and Smith, R.M. (1982). Critical stability constants. In: *Critical Stability Constants*, New York: Plenum Press.
- 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. II. Effects of the ADP-ribosylation by botulinum C3 ADP-ribosyltransferase on platelet aggregation. *J. Biol. Chem.* 267, 20921–20926.
- Muallem, S., Kwiatkowska, K., Xu, X., and Yin, H.L. (1995). Actin disassembly is a sufficient final trigger for exocytosis in nonexcitable cells. *J. Cell Biol.* 128, 589–598.
- Nobes, C.D., and Hall, A. (1995). Rho, Rac, and Cdc42 regulate the assembly of multi-molecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81, 53–62.
- Norman, J.C., Price, L.S., Ridley, A.J., Hall, A., and Koffer, A. (1994). Actin filament organization in activated mast cells is regulated by heterotrimeric and small GTP-binding proteins. *J. Cell Biol.* 126, 1005–1015.
- Oberhauser, A.F., Monck, J.R., Balch, W.E., and Fernandez, J.M. (1992). Exocytotic fusion is activated by Rab3a peptides. *Nature* 360, 270–273.
- Peppelenbosch, M.P., Qiu, R.G., de Vries Smits, A.M., Tertoolen, L.G., de Laat, S.W., McCormick, F., Hall, A., Symons, M.H., and Bos, J.L. (1995). Rac mediates growth factor-induced arachidonic acid release. *Cell* 81, 849–856.
- Perrin, D., and Aunis, D. (1985). Reorganization of a-fodrin by stimulation in secretory cells. *Nature* 315, 589–592.
- Perrin, D., Möller, K., Hanke, K., and Söling, H. (1992). cAMP and Ca²⁺-mediated secretion in parotid acinar cells is associated with reversible changes in the organization of the cytoskeleton. *J. Cell Biol.* 116, 127–134.
- Price, L.S., Norman, J.C., Ridley, A.J., and Koffer, A. (1995). Small GTPases, Rac and Rho, as regulators of secretion in mast cells. *Curr. Biol.* 5, 68–73.
- Ridley, A.J., and Hall, A. (1992). The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70, 389–399.
- Ridley, A.J., and Hall, A. (1994). Signal transduction pathways regulating Rho-mediated stress fiber formation: requirement for a tyrosine kinase. *EMBO J.* 13, 2600–2610.
- 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.
- Ridley, A.J., Self, A.J., Kasmi, F., Paterson, H.F., Hall, A., Marshall, C.J., and Ellis, C. (1993). Rho family GTPase-activating proteins p190, bcr, and RhoGAP show distinct specificities in vitro and in vivo. *EMBO J.* 12, 5151–5160.
- Rotrosen, D., Yeung, C.L., Leto, T.L., Malech, H.L., and Kwong, C.H. (1992). Cytochrome b558: the flavin-binding component of the phagocyte NADPH oxidase. *Science* 256, 1459–1462.
- Sontag, J., Aunis, D., and Bader, M. (1988). Peripheral actin filaments control calcium-mediated catecholamine release from strep-

- tolysin-O-permeabilized chromaffin cells. *Eur. J. Cell Biol.* 46, 316–326.
- Stossel, T.P. (1989). From signal to pseudopod. *J. Biol. Chem.* 264, 18261–18264.
- Thomas, P., Lee, A.K., Wong, J.G., and Almers, W. (1994). A triggered mechanism retrieves membrane in seconds after Ca^{2+} -stimulated exocytosis in single pituitary cells. *J. Cell Biol.* 124, 667–756.
- 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. *J. Cell Biol.* 120, 1529–1537.
- Vitale, M.L., Rodriguez del Castillo, A., Tchakarov, L., and Trifaro, J. (1991). Cortical filamentous actin disassembly and scinderin redistribution during chromaffin cell stimulation precede exocytosis, a phenomenon not exhibited by gelsolin. *J. Cell Biol.* 113, 1057–1067.
- Vojtek, A.B., and Cooper, J.A. (1995). Rho family members: activators of MAP kinase cascades. *Cell* 82, 527–529.