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Purification and Identification of FOAD-II, a Cytosolic Protein that Regulates Secretion in Streptolysin-O Permeabilized Mast Cells, as a Rac/RhoGDI Complex

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Mast cells permeabilized by treatment with streptolysin-O in the presence of Ca^{2+} and GTP-γ-S can secrete almost 100% of their contained *N*-acetyl-β-D-glucosaminidase. If these stimuli are provided to the permeabilized cells after a delay, the response is diminished and the ability of the cells to undergo secretion runs down progressively over a period of about 30 min. This is thought to be due to the loss of key proteins involved in the exocytotic mechanism. Using this effect as the basis of a biological assay, we have isolated a protein from bovine brain cytosol that retards the loss of responsiveness to stimulation by Ca^{2+} and GTP-γ-S. Purification of this protein and peptide sequencing have enabled us to identify it as the small GTP-binding protein rac complexed to the guanine nucleotide exchange inhibitor rhoGDI. Both proteins are required to retard the loss of the secretory response, while purified rhoGDI applied alone accelerates the rundown.

INTRODUCTION

Although it is widely accepted that the mechanisms underlying the membrane fusion events of the secretory pathway are likely to be highly conserved, the control processes that determine if and when two membranes are to undergo fusion appear to be widely divergent. The process of regulated exocytosis, in which the membranes of secretory granules undergo fusion with the cytosolic face of the plasma membrane, provides some excellent opportunities to investigate these mechanisms and their control. Development of techniques of selective plasma membrane permeabilization that permit the direct manipulation of the composition of the cytosol in secretion-competent cells has allowed the identification of low molecular weight solutes that regulate exocytosis (Lindau and Gomperts, 1991) and these have been found to vary among different classes of secretory cells. For exocytosis of catecholamines from adrenal chromaffin cells, calcium alone is believed to be the sufficient trigger (Knight and Baker, 1982), whereas in other systems, such as the acinar cells of the parotid, secretion is thought to be dependent on a rise in the concentration of cyclic AMP (Dormer and Ashcroft, 1974; McMillian *et al.*, 1988). For non-neuroendocrine cells such as the granulocytic mast cells (Howell *et al.*, 1987; Lillie and Gomperts, 1992a), eosinophils (Nüsse *et al.*, 1990; Cromwell *et al.*, 1991), and neutrophils (Barrowman *et al.*, 1986; Cockcroft, 1991) it appears that the final stimulus to exocytosis is provided by GTP and mediated through GTP-binding proteins.

After permeabilization all secretory cells appear to undergo a decline in responsiveness with time to stimuli (such as Ca^{2+} and guanosine 5′-*O*-(3-thiotriphosphate) [GTP-γ-S]).[1] This is understood to be due, at

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[1] Abbreviations used: hexosaminidase, *N*-acetyl-β-D-glucosaminidase; GDI, GDP-dissociation inhibitor protein; GTP-γ-S, guanosine 5′-*O*-(3-thiotriphosphate); SL-O, streptolysin O; FOAD, factor of activation of degranulation; PLD, phospholipase-D; PI-TP, phosphatidylinositol transfer protein.

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least in part, to the loss of cytosolic proteins (Howell and Gomperts, 1987; Sarafian *et al.*, 1987; Koffer and Gomperts, 1989). Using brain cytosols as a source, a number of proteins have been identified that can retard this loss of responsiveness. In particular, this approach has been applied to neuroendocrine cells for which an elevation of cytosol Ca^{2+} is the primary stimulus to secretion. Several regulatory proteins have been identified that can impede the onset of a refractory state. These include the lipid-dependent Ca^{2+}-binding protein annexin-II (Ali and Burgoyne, 1990), members of the 14–3-3 family (Morgan and Burgoyne, 1992; Roth *et al.*, 1993), the catalytic subunit of cAMP-dependent protein kinase (Morgan *et al.*, 1993), 140-kDa calcium-activated protein in secretion (Nishizaki *et al.*, 1992), phosphatidylinositol transfer protein (PI-TP) (Hay and Martin, 1993), phosphatidylinositol-5-kinase (Hay *et al.*, 1995), and calmodulin (Matsuda *et al.*, 1994). We have now extended this approach to an investigation of mast cells in the hope that we could identify specific regulatory components of an exocytotic mechanism in which the stimulus is provided by activation of GTP-binding proteins.

MATERIALS AND METHODS

Deep-frozen bovine brains were obtained from Advanced Protein Products (Brierley Hill, West Midlands, UK). GTP-γ-S was purchased as a 100 mM stock solution from Boehringer Mannheim (Mannheim, Germany) and all other chemicals used were of the highest quality from standard commercial sources. All chromatographic columns used were obtained from Pharmacia (Uppsala, Sweden). The BCA assay for protein was carried out using a kit purchased from Pierce (Chester, UK). Streptolysin-O (SL-O) was obtained from Murex Diagnostics (Dartford, Kent, UK). Rabbit polyclonal antibodies to rac1 and 2 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Antibody to rhoGDI was a gift from Alan Hall.

Rat peritoneal mast cells were prepared by peritoneal lavage of male Sprague Dawley rats (300–400 g) and purified to greater than 99% purity by a Percoll step gradient as previously described (Tatham and Gomperts, 1990) and treated with metabolic inhibitors (2-deoxyglucose, 3mM, and entimycin A, 10M).

Secretion Experiments

The cells were permeabilized by SL-O, a bacterial cytolysin. This was applied to mast cells maintained at ice temperature at a concentration of 1.6 IU ml^{-1}. After 5 min the cells were diluted into buffer A (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.02% NaN_3, 20 mM piperazine-*N*,*N*′-bis(2-ethane sulfonic acid) [Pipes], pH 6.8) supplemented with 0.1 mM EGTA, and the cells were washed free of unbound SL-O by centrifugation and resuspension at 4°. To open the plasma membrane lesions and initiate rundown, 20-μl samples were added to 20 μl of run-down buffer at 37° (buffer A plus 0.6 mM EGTA (Ca^{2+} to < pCa8), 200 μM ATP, 3 mM creatine phosphate, creatine kinase 0.6 mg ml^{-1}), and 20 μl of protein under test made up in buffer B (buffer A plus 100 μM ATP, 0.3 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg ml^{-1} pepstatin, 1 mg ml^{-1} leupeptin). Experience indicates that secretion in response to a maximal stimulus declines after 10–20 min to approximately 10% of its normal level (these cells are capable of releasing 100% of their contained secretory materials). The cells were then stimulated to secrete by addition of 20 μl stimulation (or control) buffer (buffer A containing GTP-γ-S [zero or 200 μM] plus 3 mM EGTA with $CaCl_2$ to regulate the level of free Ca^{2+} at pCa7 or pCa5) (estimated using the program Ligandy; Gomperts *et al.*, 1992). After a further 20-min incubation, the release reaction was quenched by the addition of 100 μl ice cold buffer A. The cells were sedimented and soluble hexosaminidase was assayed as previously described (Gomperts *et al.*, 1992). Secretion is expressed as the % of total cellular *N*-acetyl-β-D-glucosaminidase (hexosaminidase) released by 100 μM GTP-γ-S and 10 μM Ca^{2+} (pCa5), above that released by 100 nM Ca^{2+} (pCa7). All determinations were carried out in quadruplicate unless otherwise stated.

For analysis of activity in the reconstitution of secretion, column fractions were exchanged into buffer B before assay by the use of NAP-5 buffer exchange columns.

Protein Purification

Deep-frozen bovine brains were stored at −80°C. Generally we have used two brains for each preparation. These were defrosted at 4°C for 8 h and then homogenized in buffer B (1 liter per 500 g) using a Waring blender. The homogenate was centrifuged at 14,400 × *g* overnight at 4°C and the supernatant was then subjected to fractionation by the procedures described below.

The supernatant was initially fractionated by ammonium sulphate precipitation. Material capable of reconstituting the activity of cells rendered partially refractory by prior permeabilization was found to be present in proteins precipitating at 85% but remaining soluble at 60% of saturation. The cytosol was stirred for 1 h at 4°C with 60% saturated $(NH_4)_2SO_4$ and then centrifuged at 14,400 × *g* for 30 min. The pellet was discarded and the precipitation was repeated at 85% saturated $(NH_4)_2SO_4$. After centrifugation, the resultant pellet was resuspended in 50 ml buffer B and applied to an octyl sepharose column (120 ml, XK50 column) equilibrated in 2 M $(NH_4)_2SO_4$, 20 mM Pipes, pH 6.5.

All buffers used for chromatography contained 0.02% NaN_3 to prevent microbial growth and all chromatography steps were carried out on a fast performance liquid chromatography at 4°C to reduce proteolysis. Proteins were eluted from the octyl sepharose column using a linear gradient down to 20 mM Pipes, pH 6.5, with a flow rate of 5 ml min^{-1}. The active fractions were pooled and dialyzed overnight against 20 mM Tris-HCl, pH 8.5. This was then applied to a Q-sepharose-FF column, (60 ml, XK26 column) pre-equilibrated with 20 mM Tris-HCl, pH 8.5, and proteins were eluted with an increasing gradient of NaCl with a flow rate of 5 ml min^{-1}. The active fractions were combined and concentrated to 3 ml on a YM10 membrane in an Amicon pressure filtration cell. The samples were exchanged into 20 mM Pipes, pH 6.9, using 3 NAP-10 columns and then applied to a 5 ml HI-Trap SP column and eluted with a rising gradient of NaCl at a flow rate of 2 ml min^{-1}. At this stage two activities were recovered. The active fractions from the second peak were pooled and concentrated to 1 ml on a Centricon (Amicon, Beverly, MA) spin filter having a 10 kDa cut-off. This was injected on to a G75 preparation grade gel filtration column (Hi Load 16/60 Superdex 75pg) pre-equilibrated in buffer A (flow rate 0.12 ml min^{-1}), and the activity eluted as a single peak with an estimated molecular weight of 43 kDa. The active fractions were again pooled and exchanged into 20 mM Tris-HCl, pH 8.5, and then applied to a monoQ column (1 ml) from which a stimulatory activity and an inhibitory activity were recovered. The stimulatory activity was then rechromatographed on the G75 column.

Protein concentration was assayed by the BCA method. The peptide composition of column fractions were analyzed on 12% SDS polyacrylamide gels using the method of Laemmli (1970) and visualized by silver staining (Morrissey, 1981).

Protein sequencing samples were pyridylethylated before analysis according to a slight modification of a published method (Thomsen and Bayne, 1988). Briefly, the sample was concentrated to dryness on an Amicon 3 (3-kDa cut-off; Beverly, MA) spin filter and resuspended in 70 μl guanidine buffer (6 M guanidine HCl, 0.25 M

TrisCl, 2 mM EDTA, pH 7.5) before incubation with 2 μl 10% 2-mercaptoethanol in guanidine buffer for 15 min under nitrogen in the dark at room temperature. This was followed by the addition of 2 μl 4-vinylpyridine in ethanol (1 + 5 vol) and further incubation for 15 min under similar conditions. Sample components were separated by reversed phase high performance liquid chromatography (Hewlett Packard 1090) using an Aquapore RP-300 column (10 cm × 4.6 mm id) and a linear gradient of 0.1% trifluoroacetic acid/acetonitrile at 1 ml min^{-1}. Peak fractions were characterized by gel electrophoresis and portions were directly sequenced on an ABI 476A protein sequencer or dried and incubated with 50 μl of cyanogen bromide solution (7 mg ml^{-1}; 70% trifluoroacetic acid) for 18 h under nitrogen in the dark. The reactions were terminated by addition of 500 μl water. The samples were dried and resuspended in tricine sample buffer (Novex, San Diego, CA) and the protein fragments separated on a 16% tricine gel (Novex) (Schagger and von Jagow, 1987). These were transferred to polyvinylidene difluoride (ABI ProBlott, Columbia, MD) by electroblotting in 10% methanol/10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 11 (Matsudaira, 1987). Bands were visualized using sulforhodamine B stain and excised for sequencing.

Leakage of RhoGDI

Cells were purified and treated with 100 μM diisopropyl phosphofluoridate for 10 min and then with metabolic inhibitors as described above. They were then permeabilized as usual and samples (0.3×10^3) were taken at various times of rundown. Cells were immediately sedimented by centrifugation and the proteins present in the supernatant were aggregated by the addition of ice-cold acetone and precipitated by centrifugation. Precipitated proteins were then dissolved in sample buffer and run on a 12% polyacrylamide gel. Proteins were transferred to nitrocellulose which was probed with an antibody (anti-rhoGDI from Transduction Laboratories, Lexington, KY) according to the manufacturer's instructions. Antibody binding was detected using the Amersham ECL kit (Buckinghamshire, UK) according to instructions.

All data reported are representative of experiments that were repeated on at least three separate occasions. The purification shown in Figures 1–6 derives from a single preparation that was repeated on at least 25 occasions.

RESULTS

This paper describes the isolation of a factor that modulates secretion (degranulation) from permeabilized mast cells by altering the rate of onset of a refractory state understood to be due to leakage of cytosol proteins. Figure 1 illustrates the extent of secretion elicited by application of a stimulus (pCa5 with 100 μM GTP-γ-S) to mast cells either at the time of permeabilization (i.e. the time of elevation of the temperature to 37°, which allows the pre-bound SL-O to generate plasma membrane lesions), or at various times thereafter. Typically these cells can respond to the stimulus by releasing close to 100% of their contained hexosaminidase, but after a period of about 10 min this declines to about 50% and then to zero at about 30 min (nb the actual timecourse of rundown varies widely between experiments). As shown in Figure 1, the factor we have isolated retards the rate of decline; we have called this activity FOAD-II, a factor of activation of degranulation.

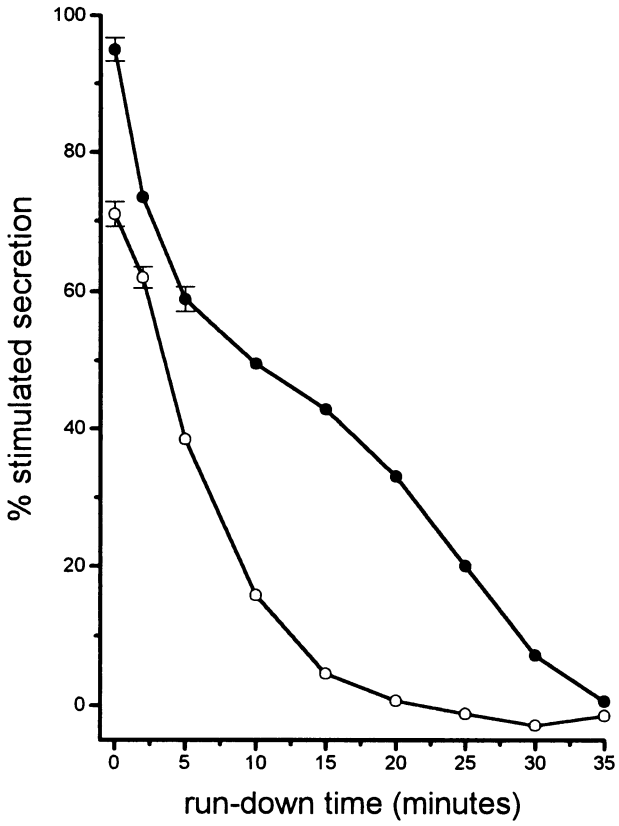

Figure 1. Loss of sensitivity (rundown) to stimulation for secretion by mast cells following permeabilization and its rescue by FOAD-II. Mast cells were permeabilized by treatment with streptolysin-O at 0° for 5 min, washed, and then brought to 37°. A stimulus to secretion (pCa5 with GTP-γ-S, 100 μM) was applied at the indicated times and incubation was continued for a further 20 min, at which time the cells were sedimented by centrifugation and the supernatant was assayed for the presence of secreted hexosaminidase. In this experiment (the rate of rundown is very variable between experiments) the half time for rundown was about 7.5 min (open symbols), declining to zero at about 25 min. For cells permeabilized in the presence of FOAD 5 μg ml^{-1} (solid symbols), the initial rate of rundown was similar to the control cells, but from 5 min onward, the rate was retarded so that the half time was about 12.5 min, and secretion declined to zero at about 35 min. Error bars represent SEM (n = 3).

Preliminary work was carried out with extracts of fresh rat brain. When rat brain cytosol proteins at 2 mg ml^{-1} are provided to permeabilized mast cells during the run-down period before stimulation, the rate of decline of the secretory responsiveness to stimulation by 10 μM Ca^{2+} with 100 μM GTP-γ-S is reduced. This activity was found to be sensitive to heat and to treatment with trypsin. Initial fractionation of the rat brain cytosol by $(NH_4)_2SO_4$ precipitation indicated the activity to be present in the fraction sedimenting at 60–85% of saturation. Although crude bovine brain cytosol was found to be ineffective, the fraction sedimented by $(NH_4)_2SO_4$ at 60–85% saturation was again

found to contain FOAD activity. This material comprised the starting point for further fractionation. We have no reason to believe that other activities might not be present in proteins sedimenting at lower concentrations of $(NH_4)_2SO_4$ but the advantage of the high salt fraction for our work at this stage was that it has low levels of contaminating hexosaminidase activity. Because of this, the bioassay procedure, involving the measurement of hexosaminidase secretion, was much easier.

The 60–85% pellet was resuspended in buffer B, made up to 2 M $(NH_4)_2SO_4$ by addition of an appropriate volume of 3.4 M concentrated stock and applied to an octyl sepharose column (Figure 2), which was used to fractionate the proteins according to their hydrophobicity. Unadsorbed proteins (i.e., the most polar proteins) were first washed through with 170 ml of 2 M $(NH_4)_2SO_4$ (fractions 1–17) and the column was then eluted with a declining salt gradient down to 0.5 M $(NH_4)_2SO_4$ (fractions 18–79). Fractions 45–63, eluting at 1.25–0.83 M $(NH_4)_2SO_4$ were found to contain FOAD activity. These were pooled and dialyzed overnight at 4° against 20 mM Tris, pH 8.5, and then applied to a Q-sepharose-(anion exchange)-fast flow column (Figure 3) that was eluted with a linear gradient of NaCl. FOAD activity was found to elute at 0.096–0.128 M NaCl. An inhibitory activity that emerged when the salt concentration was taken above 0.2 M was discarded. The active material (fractions 22–27) were pooled and concentrated under pressure to 3 ml using an Amicon YM-10 filter. The sample was then applied to three NAP-10 columns to exchange the buffers into 20 mM Pipes, pH 6.9, and applied to a Hi-Trap SP (cation exchange) column that was eluted with a gradient of NaCl.

The SP column resolves the FOAD activity into two components (Figure 4). The first of these to emerge, FOAD-I, does not bind to the column and elutes in the flow-through volume, before the NaCl gradient is applied. FOAD-II elutes at 80–220 mM NaCl (fractions 17–26). We have concentrated on the FOAD-II component and the FOAD-I has not so far been further purified.

The fractions containing FOAD-II were concentrated to 1 ml using a Filtron Microsep centrifugal concentrator (Brooklyn, Australia) having a 10-kDa cut-off filter and the material was then injected onto a G75 (gel filtration) column that was eluted with 20 mM PIPES, pH 6.8. Activity was found to elute at 58.4 ml (approximately 43 kDa) (Figure 5A). The proteins from the fractions containing active material were subjected to analysis by SDS-gel electrophoresis and were found to contain two peptides of 22 and 28 kDa (see Figure 5B). The ratio of staining of the two peptides is dependent on the technique; although on silver stain the intensity of the 22-kDa band is the greater, when visualized by colloidal Coomassie blue (Neuhoff *et al.*, 1988), the 28-kDa band is more intense (our unpublished observations). Analysis by ^{32}P-GTP overlay of the peptides following electrophoretic transfer on to Immobilon-P revealed that the 22-kDa component is a GTP-binding protein (our unpublished observations).

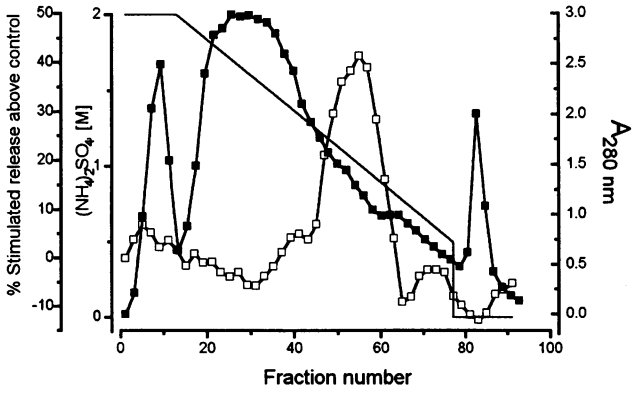

Figure 2. Chromatography of FOAD on octyl Sepharose. Brain cytosol proteins precipitated by 60–85% saturated $(NH_4)_2SO_4$ were dissolved in buffer B and then made up to 2 M $(NH_4)_2SO_4$ and loaded onto octyl Sepharose. This was eluted with a gradient (2–0.5 M $(NH_4)_2SO_4$) and 10-ml fractions were collected. Small samples (0.5 ml) of the fractions were passed through NAP-5 columns for exchange into buffer B, suitable for bioassay. Fractions (45–63) eluting in the range 1.2–0.8 M $(NH_4)_2SO_4$, which contained FOAD activity, were pooled and dialyzed overnight against 20 mM Tris-HCl. pH 8.5, for loading onto a Q-Sepharose (anion exchange) column. Solid symbols indicate protein concentration (A_{280}), open symbols indicate FOAD activity, and continuous line indicates concentration of salt gradient.

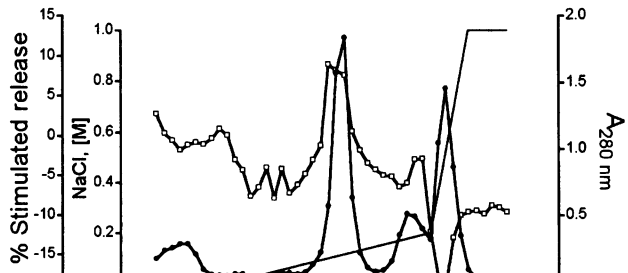

Figure 3. Chromatography of FOAD on Q-Sepharose. The active material eluted from chromatography on octyl Sepharose was loaded onto a Q-Sepharose column at pH 8.5 and eluted with an increasing gradient of NaCl (0–0.2 M). Ten-milliliter fractions were collected. Samples (0.5 ml) of the fractions were passed through NAP-5 columns for buffer exchange into buffer B, suitable for bioassay. Fractions (22–27, eluting in the range 0.08–0.12 M NaCl) that contained FOAD activity were combined and concentrated to 3 ml on a YM10 membrane and then exchanged into 20 mM Pipes, pH 6.9, using 3 NAP-10 columns for application to a HI-Trap SP column. Symbols are as for Figure 2.

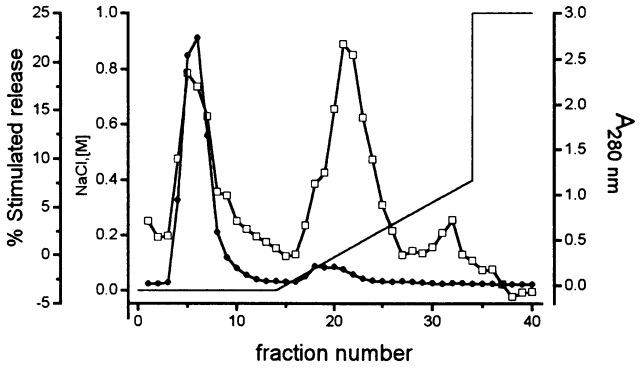

Figure 4. Chromatography of FOAD on Hi-Trap SP. The active material eluted by chromatography on Q-Sepharose was loaded onto a 5-ml Hi-Trap SP (cation exchange) column at pH 6.9 and eluted with an increasing gradient of NaCl (0–0.4 M) and 1-ml fractions were collected. Samples were removed, prepared, and assayed for FOAD activity. Active material was found to elute in the flow-through buffer (zero salt, nonadherent) and again in fractions 17–26 (0.06–0.24 M NaCl). The nonadherent activity was discarded and the active fractions from the second peak were pooled and concentrated to 1 ml on a Centricon spin filter having a 10-kDa cut off, in preparation for gel filtration chromatography. Symbols are as for Figure 2.

In our attempts to resolve the 43-kDa active material further, we found that any procedure that separated the 22- and 28-kDa components completely had the effect of destroying the ability of FOAD-II to retard rundown of the permeabilized cells. This indicated that both peptides are required for the activity. However, using a monoQ (anion exchange) column we found that the activity could be resolved into an activating and an inhibitory component. The column was eluted with a gradient of NaCl buffered with Tris at pH 8.5. Active material eluted at 160–200 mM NaCl and the inhibitory component emerged at 220–240 mM NaCl (Figure 6A). The resolution of these peptides is sensitive to the flow rate of the eluting solvent. At flow rates greater than 0.5 ml min^{-1} the fine resolution of the stimulatory peak is lost and there is a concomitant increase in the inhibitory fraction. Analysis of the fractions by SDS-gel electrophoresis (Figure 6B) showed that FOAD activity again correlates with the presence of the 22- and 28-kDa peptides, although the ratio 22 kDa:28 kDa is now higher than that of the active G75 fractions, while the inhibitory component appears to consist of the 28-kDa protein alone. The active material from the monoQ anion exchange column was concentrated to 1 ml using a Filtron centrifugal concentrator and applied to a G75 column, and after analysis of the active fractions by SDS-gel electrophoresis (Figure 6C) was found to consist only of the 22- 28-kDa complex. The monoQ column appears to allow partial separation of the complex of 22- and 28-kDa peptides, providing two components, one of which is the original active complex, the other, 28 kDa, causing inhibition. The monomeric 22-kDa component, which appears to be inactive, co-elutes with the complex and can be removed by gel filtration.

The purified proteins emerging from the second G75 column were subjected to sequence analysis. From the 22-kDa protein, four peptides, representing 47% of the total, were sequenced (see Table 1) and these were found to match the human ras-like protein TC25 (human rac1) with 97.8% identity (by comparison there is 92.0% identity with human rac2, with seven specific differences being consistent with rac1 rather than rac2). We have been unable to obtain any sequence for the C-terminus, and antibodies raised against C-terminal peptides from human rac1 and rac2 showed no cross-reactivity (Western blotting) with the bovine protein that we isolated, nor did they react with pro-

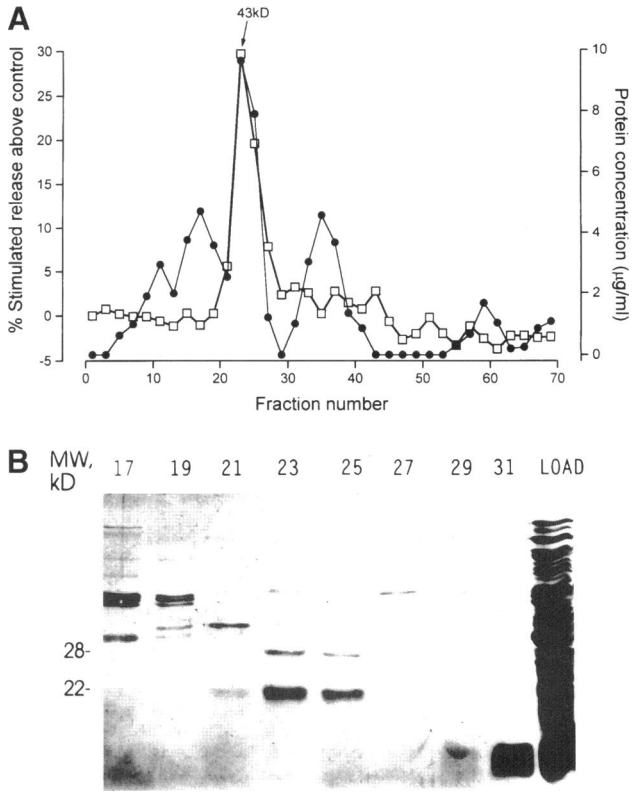

Figure 5. Chromatography of FOAD-II by gel filtration on G75. (A) The active material eluted by chromatography on HiTrap-SP was loaded onto a G75 Sephadex (gel filtration) column, eluted with buffer A, and 800-μl fractions were collected. Samples from the fractions were assayed for FOAD activity without further preparation and the active material was found to elute as a 43-kDa protein. The active fractions were pooled, concentrated, and exchanged using NAP-10 columns into 20 mM Tris-HCl, pH 8.5, for application to a monoQ column. Symbols are as for Figure 2. (B) Samples from fractions 17–31 were extracted into standard Laemmli sample buffer (Laemmli, 1970) under denaturing conditions and the proteins were separated on 12% SDS gels. Visualization was by silver staining and the gels were photographed using a deep blue filter to enhance contrast.

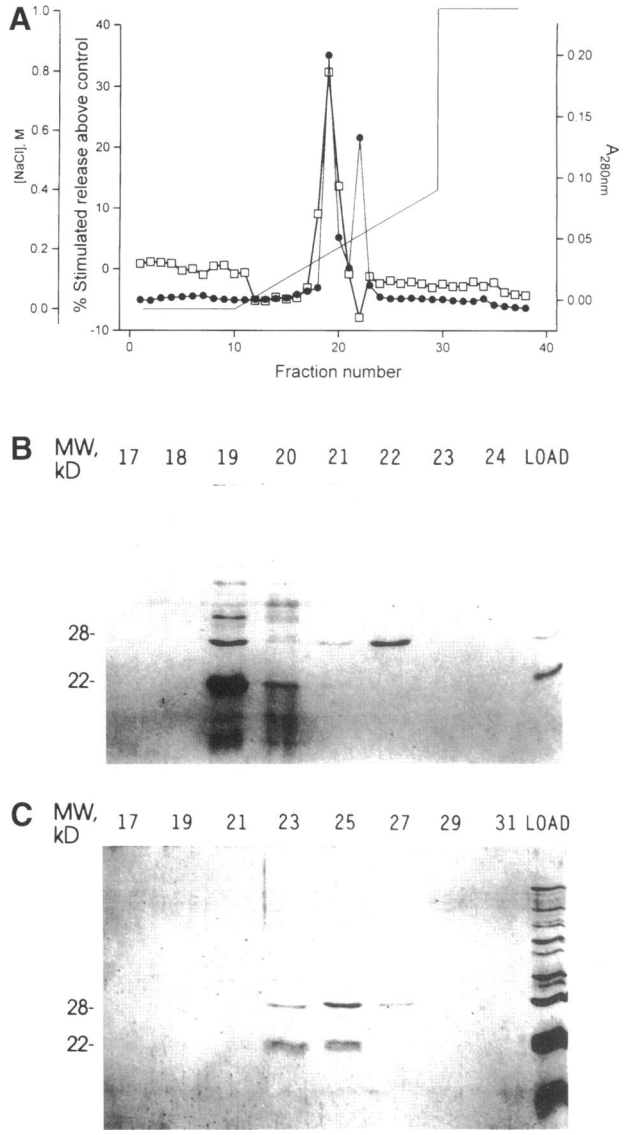

Figure 6. Chromatography of FOAD-II on monoQ Sepharose. (A) The 43-kDa protein eluted from a gel filtration column was loaded onto a monoQ column. This was eluted with a gradient of NaCl (0–0.4 M) and 0.5-ml fractions were collected. Samples were removed and exchanged using NAP-5 columns into buffer B for bioassay. Two activities, coinciding with the emergence of protein (A_{280} trace) were detected, the first of which (FOAD-II) retarded the rundown in the secretion assay, the second being inhibitory. The pooled material from the stimulatory peak was concentrated and reapplied to a G75 Sephadex column. Symbols are as for Figure 2. (B) Samples from fractions 21–28 were extracted into standard Laemmli sample buffer (Laemmli, 1970) under denaturing conditions and the proteins were separated on 12% SDS gels. Visualization was by silver staining. (C) SDS gel analysis of FOAD-II–containing fractions after re-chromatography by gel filtration on Superdex G75pg.

teins present in bovine brain cytosol. We are therefore unable to determine conclusively whether the GTP-binding protein that we have isolated is rac1 or rac2, however, as there is apparently no mRNA coding rac2 in (human) brain (Didsbury *et al.*, 1989) the protein is likely to be rac1. Two peptides, representing 28% of the total protein, were sequenced from the 28-kDa protein (see Table 2) and these were found to match the sequence of bovine rhoGDI. An antibody against RhoGDI reacted (Western blotting) against the 28-kDa protein (our unpublished observations).

RhoGDI leaks rapidly from the permeabilized cells and this is effectively complete within 5 min as shown by analysis of the cell-conditioned salts solution (Figure 7). Due to the nonavailability of suitable antibodies we have been unable to carry out a similar analysis for the leakage of rac.

Figure 8 illustrates the concentration-activity relationship of the rac/rhoGDI complex in the reconstitution of secretory activity in partially run-down mast cells. In this experiment the permeabilized mast cells were incubated in the presence of various concentrations of the complex under nonstimulating conditions (pCa8) and allowed to run down to the point that no more than 20% secretion could be elicited on stimulation (pCa5 with 100 μM GTP-γ-S) in the absence of added protein. The extent of induced secretion increased with increasing concentrations of FOAD. The optimal concentration was found to be in the range of 5 to 15 $\mu g\ ml^{-1}$ (four different preparations) with EC_{50} of 2.6 $\pm$ 0.4 $\mu g\ ml^{-1}$ (i.e., 50 $\pm$ 8 nM) (mean $\pm$ SEM, n = 4 experiments, each determination carried out in triplicate). At no concentration in the range tested did the FOAD have any effect at pCa7. As shown in the experiment illustrated in Figure 1 (see above) illustrating the effect of the complex on the rate of rundown of the secretory response, it is only capable of enhancing secretion in cells that still maintain some residual response to stimulation.

Under the normal operating conditions of these experiments, secretion from mast cells requires the presence of both Ca^{2+} and an activating guanine nucleotide. The dependence on these two effectors is best presented in the form of a matrix in which the concentrations of both are treated as variables. Such an experiment is illustrated in Figure 9 in which permeabilized mast cells were first allowed to run down in the presence (or absence) of an optimum concentration of FOAD-II and then stimulated with various concentrations of GTP-γ-S and Ca^{2+}. When the cells run down, both the extent of maximal secretion and the sensitivity to the two effectors declines. In the experiment illustrated, the cells were allowed to run down to the extent that the stimulus (pCa5 and 100 μM GTP-γ-S) induced about 5% secretion. Provision of FOAD enhanced this secretion in a manner that was dependent on the concentrations of both effectors. Even so, there was very little secretion unless the maximal concentrations were provided. (This result can be compared with the concentrations required to

Table 1. Sequence analysis and matching of FOAD-II peptide with human TC25

P15153 Human rac2

P15154 Human ras-like protein TC25 (human Rac1)

Rac2	MQAIKCVVVG	DGAVGKTCLL	ISYTTNAFPG	EYIPTVFDNY	SANVMVDSKP
Rac1	MQAIKCVVVG	DGAVGKTCLL	ISYTTNAFPG	EYIPTVFDNY	SANVMVDGKP
	QAIKCVVV				**VDGKP**
				*	*
Rac2	VNLGLWDTAG	QEDYDRLRPL	SYPQTDVFLI	CFSLVSPASY	ENVRAKWFPE
Rac1	VNLGLWDTAG	QEDYDRLRPL	SYPQTDVFLI	CFSLVSPASF	ENVRAKWYPE
	VNLGqtDTA-	**QED**		**SLVSPASF**	**ENVRAK-YPE**
				*	* *
Rac2	VRHHCPSTPI	ILVGTKLDLR	DDKDTIEKLK	EKKLAPITYP	QGLALAKEID
Rac1	VRHHCPNTPI	ILVGTKLDLR	DDKDTIEKLK	EKKLTPITYP	QGLAMAKEIG
	VRHHC			**TPITYP**	**QGLAMAKEIG**
	*				
Rac2	SVKYLECSAL	TQRGLKTVFD	EAIRAVLCPQ	PTRQQKRACS	LL
Rac1	AVKYLECSAL	TQRGLKTVFD	EAIRAVLCPP	PVKKRKRKCL	LL
	AVKYLECSAL	**TQRGLKTVFD**	**EAIRA**		

Bold type indicates amino acids sequenced, uppercase indicates matches, and lowercase indicates mismatch with published sequence rac1 obtained from the Swiss-Prot Database. * indicates mismatch of sequence with rac2.

stimulate secretion from cells triggered at the time of permeabilization: in the presence of ATP and 100 μM GTP-γ-S, secretion can be induced at any concentration of Ca^{2+} above pCa6.5; conversely, for cells permeabilized in the presence of Ca^{2+} at pCa5, secretion commences at any concentration of GTP-γ-S above 10^{-8} M [Howell *et al.*, 1987; Gomperts and Tatham, 1988]). Surprisingly, for FOAD-II–supported cells, Ca^{2+} alone (i.e., in the absence of GTP-γ-S) at the highest concentration (pCa5) is able to elicit a small (4%), but significant extent of secretion. It is unlikely that this secretion is due to in situ generation of GTP by transphosphorylation from ATP since deoxyUDP (1 mM), a competitive inhibitor of nucleotide diphosphate kinase, was without effect. The dependence on the concentration of FOAD-II for such Ca^{2+}-induced secretion is the same as when GTP-γ-S is also provided.

The separation of the FOAD-II complex into two components by chromatography on monoQ anion exchange resin (Figure 6) revealed that rhoGDI is inhibitory to exocytosis in run-down cells. The experiment illustrated in Figure 10 shows that the purified rhoGDI accelerates the rate of rundown of the permeabilized cells. It is possible that this reflects the detachment and leakage of membrane-bound components of the rho family of low molecular weight GTP-binding proteins, including rac. The extended lag, about 5 min, before the onset of inhibition is probably due to the time required for diffusion of the exogenous rhoGDI, the solubilization of the rho and rac proteins, and their diffusion to the exterior. (One would expect that soluble complexes of the GTP-binding proteins with the rhoGDI should be active when present inside the cells).

Table 2. Sequence analysis and matching of FOAD-II peptide with bovine rhoGDI

Bovine RhoGDI (Swiss prot P19803)

RhoGDI	MAEQEPTAEQ	LAQIAAENEE	DEHSVNYKPP	AQKSIQEIQE	LDKDDESLRK
RhoGDI	YKEALLGRVA	VSADPNVPNV	VVTRLTLVCS	TAPGPLELDL	TGDLESFKKQ
		PNV	**VVTRLTLVCS**	**TAPGPLE**	
RhoGDI	SFVLKEGVEY	RIKISFRVNR	EIVSGMKYIQ	HTYRKGVKID	KTDYMVGSYG
			KYIQ	**HTYRKGVKID**	**KTDYMVGSYG**
RhoGDI	PRAEEYEFLT	PMEEAPKGML	ARGSYNIKSR	FTDDDRTDHL	SWEWNLTIKK
	PRAEEYEFLT	**PM-EA**			
RhoGDI	EWKD				

Bold type indicates amino acids sequenced, uppercase indicates matches, and lowercase indicates mismatch with published sequence of bovine rhoGDI obtained from the Swiss-Prot Database.

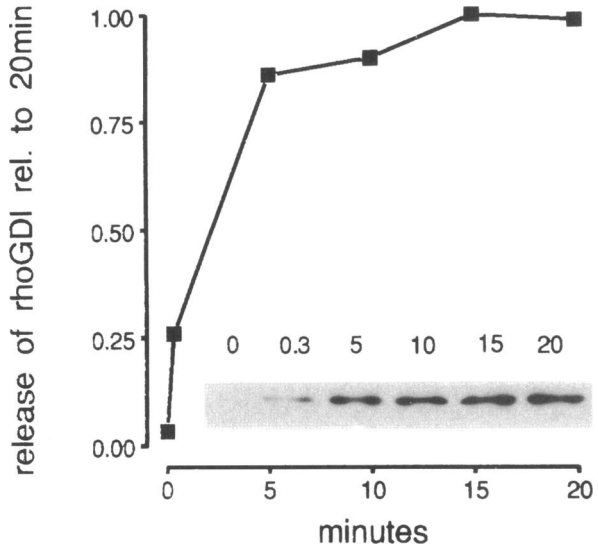

Figure 7. Leakage of rhoGDI from mast cells permeabilized by treatment with streptolysin-O. Cells pretreated with diisopropylphosphofluoridate to prevent proteinolysis, were treated as detailed in the legend to Figure 1 and timed samples were removed as indicated. The cells were immediately sedimented and the proteins present in the external salts solution were separated by SDS-gel electrophoresis. After electrophoretic transfer to nitrocellulose, the proteins were probed with an antibody to rhoGDI and detected by the ECL technique. The figure illustrates relative density of each band by densitometry and the inset shows the appearance of the ECL film.

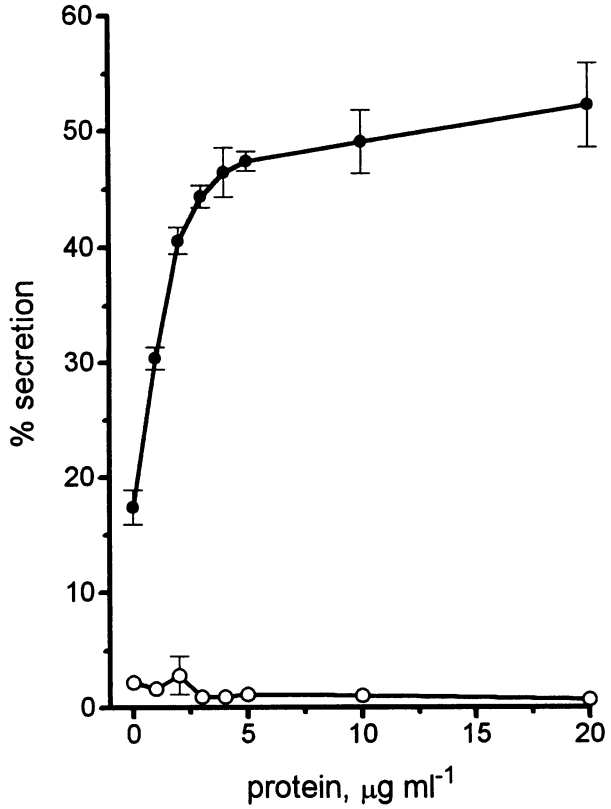

Figure 8. Concentration-activity relationship for purified FOAD-II (rac/rhoGDI complex). Mast cells were permeabilized in buffer A in the presence of various concentrations of FOAD-II and allowed to run down for 17 min before being stimulated to secrete by addition of Ca^{2+} (pCa5) and GTP-γ-S (100 μM) (solid symbols). Open symbols indicate no-stimulus controls (pCa7, zero GTP-γ-S). Error bars indicate SEM (n = 3).

In this assay, we also tested phosphatidylinositol transfer protein (PI-TP), 14–3-3 proteins (Exo1), NSF (NEM sensitive factor), α-SNAP, γ-SNAP (soluble NSF attachment protein), calmodulin, and annexin-II, all known to modulate exocytosis in other secretory systems. These were all tested at concentrations known to support secretion in other cells. None of these proteins had any discernible effect on the rate of rundown of permeabilized mast cells (our unpublished observations). Three of these (α-SNAP, γ-SNAP, and NSF) were subsequently tested on cultured bovine adrenal chromaffin cells and shown to be active in that system (Morgan and Burgoyne, personal communication).

DISCUSSION

Mast cells, permeabilized with streptolysin-O and stimulated simultaneously with Ca^{2+} and GTP-γ-S, can undergo total degranulation and release 100% of their contained hexosaminidase and histamine. If the stimulus is provided some minutes after the cells are permeabilized, then the extent of release is reduced. This phenomenon is referred to as rundown and probably occurs, in part at least, as a consequence of the leakage of proteins through the lesions in the plasma membrane induced by SL-O. For example, leakage of lactate dehydrogenase (Howell and Gomperts, 1987) and phosphoglycerate kinase (Gomperts *et al.*, 1987) is rapid, being essentially complete by 5 min and the competence of cells to undergo stimulated exocytosis extends well beyond this time. Although the rate of rundown varies considerably between experiments, our experience indicates that the sensitivity to stimulation extends well beyond 20 min before diminishing to zero. For this reason, the decline in responsiveness is unlikely to be related to the loss of true soluble proteins of the cytosol, but to proteins that are tethered, either in aggregates or anchored to membranes.

We have isolated a factor, FOAD-II, from the cytosol fraction of bovine brain, which retards the rate of rundown, and sequence information has identified this as a heterodimer consisting of rac and rhoGDI. These have to be present together to have an enhancing effect on secretion: rac provided alone is without effect, while rhoGDI accelerates the rate of rundown. The simplest conclusion to draw from these observa-

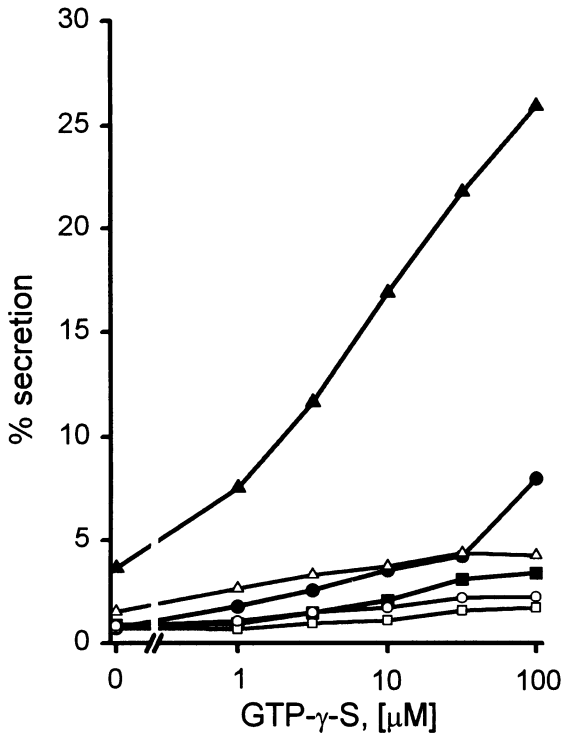

Figure 9. Effect of FOAD-II (rac/rhoGDI complex) on the requirement for Ca^{2+} and GTP-γ-S for secretion from run-down mast cells. The cells were permeabilized and allowed to run down for 17 min in buffer A in the presence and absence of FOAD-II (5 μg ml^{-1}) before stimulation with various combinations of GTP-γ-S (abcissa) and Ca^{2+} (□,■ pCa5.5; ○,● pCa5.25; △,▲ pCa5). Open symbols indicate absence of FOAD and solid symbols indicate presence of FOAD. Points indicate average of three separate determinations.

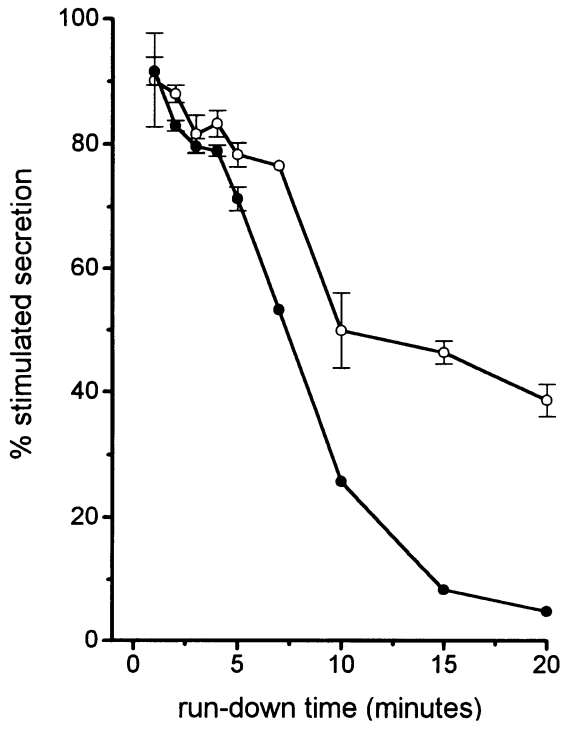

Figure 10. Loss of sensitivity to stimulation for secretion as a result of permeabilization (rundown) and its acceleration by rhoGDI. Cells were treated as detailed in the legend to Figure 1 and incubated with rhoGDI 10 μg ml^{-1}. Open symbols indicate controls (absence of rhoGDI) and solid symbols indicate presence of rhoGDI during the run-down period, and subsequent incubation. Error bars indicate SEM (n = 3).

tions is that the rundown occurs as a consequence of the leakage of rac from the cells and that the complex of exogenous rac/rhoGDI is capable of restoring this; however, we have not shown so far that rac is indeed released from the permeabilized cells and there may be other explanations. Our conclusion is supported by the observation that recombinant rac2, preloaded with GTP-γ-S, is capable of inducing exocytosis in the absence of added guanine nucleotide (our unpublished observation).

Unfortunately we have been unable to demonstrate the leakage of rac from the permeabilized cells. Antibodies raised against rac1 and rac2 C-terminal peptides, although reactive against the recombinant forms and also a peptide running at 30 kDa (Abo *et al.*, 1994; our unpublished observations), failed to detect the wild-type protein (24 kDa) on Western blots. Similarly, an antiprotein antibody (gift of Alan Hall) now has low sensitivity to peptides running at 24 kDa and displays considerable cross-reactivity against other proteins. Leakage of the mobile component of rhoGDI is at least as rapid as other soluble proteins such as lactate dehydrogenase (Howell and Gomperts, 1987) and phosphoglycerate kinase (Gomperts *et al.*, 1987), and is effectively complete by 5 min. This is considerably faster than the leakage of actin that continues to emerge during 15–60 min (Koffer and Gomperts, 1989).

One possibility is that the FOAD does not act to increase the level of rac in the depleted cells, but prevents the detachment and loss of a factor, which may not be rac. Alternatively, an important factor other than rac is lost from the cells causing rundown, and provision of rac substitutes for its lost function. The GDI proteins are known to retard GDP dissociation from specific low molecular weight GTPases (Takai *et al.*, 1992) and also to confer aqueous solubility (Isomura *et al.*, 1991) and thus to act as escorts to the wild-type forms, all of which are otherwise insoluble due to the presence of acyl and isoprenyl attachments at the carboxyterminals (Kinsella *et al.*, 1991; Takai *et al.*, 1992). Guanine nucleotide exchange is enhanced only when the GTPases are transferred to their membrane binding sites (Takai *et al.*, 1992; Bokoch *et al.*, 1994). The essential role of the GDI gives an important clue that the first explanation, namely that rac (or a closely related GTP-binding protein) detaches and

leaks from the permeabilized cells, and that provision of rac, in its complex with rhoGDI, is capable of reversing this loss, thus ensuring that the membrane-bound pool of rac is not exhausted. The rac content is reported as approximately 11 ng/10^6 mast cells (Price *et al.*, 1995). For the purposes of this discussion we shall assume that it is indeed the loss of rac that causes the rundown.

The rac/rhoGDI complex, FOAD-II, is only capable of restoring activity to cells that have retained some capacity for stimulated secretion; for cells that have become fully refractory, the presence of FOAD-II is without effect. This indicates that during the initial phases of the rundown, provision of FOAD-II is able to make good whatever has been lost, which determines the limiting step in exocytosis. As the rundown proceeds and the cells become insensitive to stimulation even in the presence of FOAD-II, the loss of other proteins (possibly FOAD-I [Figure 4], and maybe others) may become critical. These may detach from their binding sites and leak through the SL-O–induced lesions at a slower rate. Note, however, that FOAD-II is still capable of causing substantial enhancement even when the unsupported level of secretion has declined to well below 5% (Figures 11 and 9).

Wild-type rac on its own has no ability to retard rundown. In contrast, recombinant, constitutively active forms of rac1 or rhoA are capable of enhancing secretion from permeabilized (and washed) mast cells (Price *et al.*, 1995). This difference is probably due to the lack of post-translational modification of the recombinant proteins. The rhoGDI is necessary to maintain the wild-type protein in solution. Although the major effect of the rac/rhoGDI is to enhance secretion induced by GTP-γ-S, as would be expected for a function mediated by a GTP-binding protein, the complex still appears to induce secretion to a small extent in the absence of an activating guanine nucleotide (Figure 10). Although this behavior is apparently anomalous, there is a precedent for this observation in the report of activation of the NADPH oxidase complex by rac in neutrophils (Abo *et al.*, 1994; Bromberg *et al.*, 1994). In the search for an explanation of this paradox it may be worth recalling that the stimulation of cell surface receptors can also occur in the absence of activating ligands (Bond *et al.*, 1995). This has been rationalized on the basis of a two-state model in which the receptors are in equilibrium between the active and the inactive conformations. The conventional agonist, rather than activating the receptor directly, has the effect of shifting the equilibrium to increase the proportion of the receptor in the active form. Similarly, we offer the idea that the ability of rac to induce a low level activation of exocytosis in the absence of GTP could be due to a two-state equilibrium in which there exists a small proportion of the rac in an active form.

The inhibitory effect of rhoGDI is best explained by a mechanism involving the extraction of rac, together with other rho-related peptides, from their native binding sites within the cells. A similar mechanism has been invoked to explain the inhibition by rhoGDI of phospholipase-D (PLD) activation in permeabilized HL-60 cells (Bowman *et al.*, 1993). For these cells it has been suggested that the activation of PLD is central to the triggering of secretion (Stutchfield and Cockcroft, 1993) and so rhoGDI would be expected to be an inhibitor. A more direct experimental approach is needed to confirm this mechanism.

GTP is required for activation of mast cells but it appears that depending on the nature of the stimulus, separate GTP-binding proteins mediate the terminal stages of the secretory pathway. For activation by receptor-mimetic agents (mastoparan, compound 48/80, etc.), exocytosis can be inhibited by treatment with pertussis toxin, implicating a heterotrimeric G-protein as the G_E, the GTP-binding protein mediating exocytosis (Aridor *et al.*, 1990). Introduction of specific probes into permeabilized mast cells has identified this as G_{i3} (Aridor *et al.*, 1993). Conversely, secretion stimulated by cross-linking the receptors for IgE or with Ca^{2+}-ionophores is quite insensitive to ADP-ribosylating toxins (Saito *et al.*, 1987) and yet still requires the presence of GTP (Wilson *et al.*, 1989; Marquardt *et al.*, 1987). Rac can therefore be considered as a candidate for the G_E-mediating exocytosis in mast cells stimulated by activation of the IgE pathway. Alternatively, it could be the regulator of a common late event, distal to the site of action of the pertussis-sensitive step in the pathway induced by the receptor-mimetic agents, and conferring the requirement for GTP in the pathway initiated by activation of the receptor for IgE. However, the finding that exocytosis from permeabilized cells can be induced by $[AlF_4]^-$ (Lillie and Gomperts, 1992b; Aridor *et al.*, 1993) is indicative of a direct pathway of activation through a heterotrimeric G-protein. For this reason it is more likely that there are discrete pathways of activation for stimulation of IgE receptors or with receptor-mimetic agents.

The mechanism of action of rac in mast cells remains unclear, but there are a number of possible ways in which it may act. As discussed above PLD has been implicated in the regulation of secretion from HL-60 cells and it is possible that the rac acts directly or indirectly on this protein and in this way regulates secretion in mast cells. An alternative possibility is that the action of rac is exerted via regulation of the cytoskeleton: rac, rho, and cdc42 have all been shown to exert profound effects on the cytoskeleton (Nobes and Hall, 1995; Hall, 1994). Rac and rho proteins have been demonstrated to be involved in the disassembly of the cortical actin network in rat peritoneal mast cells stimulated by Ca^{2+} and GTP-γ-S (Norman *et al.*, 1994).

This cortical web of actin has been proposed to regulate exocytosis in a number of secretory cells (for review see Trifaro *et al.*, 1992) by acting as a barrier to fusion of the secretory granules and plasma membrane. However, no direct demonstration of this has yet been achieved and it remains possible that the correlation between actin disassembly and secretion is not mechanistically significant and that the effects of rac on both secretion and the cytoskeleton are due to their having a similar signaling pathway possibly through an effector such as a rac-activated kinase (Manser *et al.*, 1994).

The importance of rac is underlined by the finding that several other cytosolic proteins, including PI-TP, 14–3-3 proteins, NSF, α-SNAP, γ-SNAP, calmodulin, and annexin-II (both the heterotetramer and the monomeric forms), all known to modulate exocytosis in other secretory systems (Ali and Burgoyne, 1990; Morgan and Burgoyne, 1992; Nishizaki *et al.*, 1992; Hay and Martin, 1993; Hay *et al.*, 1995; Morgan *et al.*, 1993; Roth *et al.*, 1993; Bond *et al.*, 1995), are without effect in the mast cell under the conditions of our experiments. It is likely that their leakage from these cells following permeabilization by SL-O is substantial and probably rapid, but although their loss is apparently not rate limiting, this does not mean that they are without a role. In contrast, the function supported by the FOAD-II complex of rac and rhoGDI is clearly of considerable importance.

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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 (rhoGDI) followed by its translocation to the plasma membrane. Biochem. J. *298*, 585–591.

Ali, S.M., and Burgoyne, R.D. (1990). The stimulatory effect of calpactin (annexin II) on calcium-dependent exocytosis in chromaffin cells: requirement for both the N-terminal and core domains of p36 and ATP. Cell. Signalling *2*, 265–276.

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.

Aridor, M., Rajmilevich, G., Beaven, M.A., and Sagi-Eisenberg, R. (1993). The heterotrimeric G-protein G_E(i3) stimulates final steps in regulated exocytosis. Science *262*, 1569–1572.

Barrowman, M.M., Cockcroft, S., and Gomperts, B.D. (1986). Two roles for guanine nucleotides in the stimulus secretion sequence of neutrophils. Nature *319*, 504–507.

Bokoch, G.M., Bohl, B.P., and Chuang, T.H. (1994). Guanine nucleotide exchange regulates membrane translocation of Rac/Rho GTP-binding proteins. J. Biol. Chem. *269*, 31674–31679.

Bond, R.A., *et al.* (1995). Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the β-adrenoceptor. Nature *374*, 272–276.

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.

Bromberg, Y., Shani, E., Joseph, G., Gorzalczany, Y., Sperling, O., and Pick, E. (1994). The GDP-bound form of the small G protein rac1 is a potent activator of the superoxide-forming NADPH oxidase of macrophages. J. Biol. Chem. *269*, 7055–7058.

Cockcroft, S. (1991). Relationship between arachidonate release and exocytosis in permeabilised human neutrophils stimulated with formylmethionyl-leucyl-phenylalanine (fMetLeuPhe), guanosine 5'-[gamma-thio]triphosphate (GTP[S]) and Ca^{2+}. Biochem. J. *275*, 127–131.

Cromwell, O., Bennett, J.P., Kay, A.B., Hide, I., and Gomperts, B.D. (1991). Mechanisms of granule enzyme secretion from permeabilized guinea pig eosinophils: dependence on Ca^{2+} and guanine nucleotides. J. Immunol. *147*, 1905–1911.

Didsbury, J., Weber, R.F., Bokoch, G.M., Evans, T., and Snyderman, R. (1989). rac: a novel ras-related family of proteins that are botulinum toxin substrates. J. Biol. Chem. *264*, 16378–16382.

Dormer, R.L., and Ashcroft, S.J.H. (1974). Studies on the role of calcium ions in the stimulation by adrenaline of amylase release from rat parotid. Biochem. J. *144*, 543–550.

Gomperts, B.D., Cockcroft, S., Howell, T.W., Nüsse, O., and Tatham, P.E.R. (1987). The dual effector system for exocytosis in mast cells: obligatory requirement for both Ca^{2+} and GTP. Biosci. Rep. *7*, 369–381.

Gomperts, B.D., and Tatham, P.E.R. (1988). GTP-binding proteins in the control of exocytosis. Cold Spring Harbor Symp. Quant. Biol. *53*, 983–992.

Gomperts, B.D., and Tatham, P.E.R. (1992). Regulated exocytotic secretion from permeabilized cells. In: Methods in Enzymology, ed. J. Rothman, New York: Academic Press, 178–189.

Hall, A. (1994). Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu. Rev. Cell Biol. *10*, 31–54.

Hay, J.C., Fisette, P.L., Jenkins, G.H., Fukami, K., Takenawa, T., Anderson, R.A., and Martin, T.F.J. (1995). ATP-dependent inositide phosphorylation required for Ca^{2+}-activated secretion. Nature *374*, 173–177.

Hay, J.C., and Martin, T.F.J. (1993). Phosphatidylinositol transfer protein required for ATP-dependent priming of Ca^{2+}-activated secretion. Nature *366*, 572–575.

Howell, T.W., Cockcroft, S., and Gomperts, B.D. (1987). Essential synergy between Ca^{2+} and guanine nucleotides in exocytotic secretion from permeabilized mast cells. J. Cell Biol. *105*, 191–197.

Howell, T.W., and Gomperts, B.D. (1987). Rat mast cells permeabilised with streptolysin-O secrete histamine in response to Ca^{2+} at concentrations buffered in the micromolar range. Biochim. Biophys. Acta *927*, 177–183.

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.

Kinsella, B.T., Erdman, R.A., and Maltese, W.A. (1991). Carboxy-terminal isoprenylation of ras-related GTP-binding proteins encoded by rac1, rac2, and ralA. J. Biol. Chem. *266*, 9786–9794.

Knight, D.E., and Baker, P.F. (1982). Calcium dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. J. Membr. Biol. *68*, 107–140.

Koffer, A., and Gomperts, B.D. (1989). Soluble proteins as modulators of the exocytotic reaction of permeabilized rat mast cells. J. Cell Sci. *94*, 585–591.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Lillie, T.H.W., and Gomperts, B.D. (1992a). Guanine nucleotide is essential, Ca^{2+} is a modulator, in the exocytotic reaction of permeabilised rat mast cells. Biochem. J. *288*, 181–187.

Lillie, T.H.W., and Gomperts, B.D. (1992b). Nucleotides and divalent cations as effectors and modulators of exocytosis in permeabilised rat mast cells. Phil. Trans. Roy. Soc. Lond. B. *336*, 25–34.

Lindau, M., and Gomperts, B.D. (1991). Techniques and concepts in exocytosis: focus on mast cells. Biochim. Biophys. Acta *1071*, 429–471.

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.

Marquardt, D.L., Gruber, H.E., and Walker, L.L. (1987). Ribavirin inhibits mast cell mediator release. J. Pharmacol. Exp. Ther. *240*, 145–149.

Matsuda, M., Okabe, T., Sugimoto, N., Senda, T., and Fujita, H. (1994). Tetanus toxin and *Clostridium perfringens* enterotoxin as tools for the study of exocytosis. Ann. NY Acad. Sci. *710*, 94–106.

Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. *262*, 10035–10038.

McMillian, M.K., Soltoff, S.P., and Talamo, B.R. (1988). Mediation of norepinephrine effects on free cytosolic calcium in rat parotid acinar cells by α_1 adrenergic receptors. Biochem. Pharmacol. *37*, 3790–3793.

Morgan, A., Wilkinson, M., and Burgoyne, R.D. (1993). Identification of Exo2 as the catalytic subunit of protein kinase A reveals a role for cyclic AMP in Ca^{2+}-dependent exocytosis in chromaffin cells. EMBO J. *12*, 3747–3752.

Morgan, A., and Burgoyne, R.D. (1992). Interaction between protein kinase C and Exo1 (14-3-3 protein) and its relevance to exocytosis in permeabilized adrenal chromaffin cells. Biochem. J. *286*, 807–811.

Morrissey, J.H. (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. *117*, 307–310.

Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie brilliant blue G-250 and R-250. Electrophoresis *9*, 255–262.

Nishizaki, T., Walent, J.H., Kowalchyk, J.A., and Martin, T.F.J. (1992). A key role for a 145-kDa cytosolic protein in the stimulation of Ca^{2+}-dependent secretion by protein kinase C. J. Biol. Chem. *33*, 23972–23981.

Nobes, C.D., and Hall, A. (1995). Rho, Rac and Cdc42 GTPases regulate the assembly of multimolecular 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.

Nüsse, O., Lindau, M., Cromwell, O., Kay, A.B., and Gomperts, B.D. (1990). Intracellular application of GTP-5′-*O*-(3-thiotriphosphate) induces exocytotic granule fusion in guinea pig eosinophils. J. Exp. Med. *171*, 775–786.

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.

Roth, D., Morgan, A., and Burgoyne, R.D. (1993). Identification of a key domain in annexin and 14-3-3 proteins that stimulate calcium-dependent exocytosis in permeabilized adrenal chromaffin cells. FEBS Lett. *320*, 207–210.

Saito, H., Okajima, F., Molski, T.F.P., Sha'afi, R.I., Ui, M., and Ishizaka, T. (1987). Effects of ADP-ribosylation of GTP-binding protein by pertussis toxin on immunoglobulin E-dependent and -independent histamine release from mast cells and basophils. J. Immunol. *138*, 3927–3934.

Sarafian, T., Aunis, D., and Bader, M. (1987). Loss of proteins from digitonin-permeabilized adrenal chromaffin cells essential for exocytosis. J. Biol. Chem. *262*, 16671–16676.

Schagger, H., and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. *166*, 368–379.

Stutchfield, J., and Cockcroft, S. (1993). Correlation between secretion and phospholipase D activation in differentiated HL60 cells. Biochem. J. *293*, 649–655.

Takai, Y., Kaibuchi, K., Kikuchi, A., and Kawata, M. (1992). Small GTP-binding proteins. Int. Rev. Cytol. *133*, 187–230.

Tatham, P.E.R., and Gomperts, B.D. (1990). Cell permeabilization. In: Peptide Hormones–A Practical Approach, ed. K. Siddle and J.C. Hutton. Oxford, UK: IRL Press, 257–269.

Thomsen, J., and Bayne, S. (1988). Microscale alkylation with 4-vinylpyridine. J. Prot. Chem. *7*, 295–296.

Trifaro, J.M., Rodriguez-del-Castillo, A., and Vitale, M.L. (1992). Dynamic changes in chromaffin cell cytoskeleton as prelude to exocytosis. Mol. Neurobiol. *6*, 339–358.

Wilson, B.S., Deanin, G.G., Standefer, J.C., Vanderjagt, D., and Oliver, J.M. (1989). Depletion of guanine nucleotides with mycophenolic acid suppresses IgE receptor-mediated degranulation in rat basophilic leukemia cells. J. Immunol. *143*, 259–265.