Cdc42 and Rac Stimulate Exocytosis of Secretory Granules by Activating the IP$_3$/Calcium Pathway in RBL-2H3 Mast Cells

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Abstract. We have expressed dominant-active and dominant-negative forms of the Rho GTPases, Cdc42 and Rac, using vaccinia virus to evaluate the effects of these mutants on the signaling pathway leading to the degranulation of secretory granules in RBL-2H3 cells. Dominant-active Cdc42 and Rac enhance antigen-stimulated secretion by about twofold, whereas the dominant-negative mutants significantly inhibit secretion. Interestingly, treatment with the calcium ionophore, A23187, and the PKC activator, PMA, rescues the inhibited levels of secretion in cells expressing the dominant-negative mutants, implying that Cdc42 and Rac act upstream of the calcium influx pathway. Furthermore, cells expressing the dominant-active mutants exhibit elevated levels of antigen-stimulated IP$_3$ production, an amplified antigen-stimulated calcium response consisting of both calcium release from internal stores and influx from the extracellular medium, and an increase in aggregate formation of the IP$_3$ receptor. In contrast, cells expressing the dominant-negative mutants display the opposite phenotypes. Finally, we are able to detect an in vitro interaction between Cdc42 and PLC$_{g1}$, the enzyme immediately upstream of IP$_3$ formation. Taken together, these findings implicate Cdc42 and Rac in regulating the exocytosis of secretory granules by stimulation of IP$_3$ formation and calcium mobilization upon antigen stimulation.

Key words: Cdc42p • Rac • calcium signaling • degranulation • signal transduction

Introduction

A n tigen stimulation of the IgE receptor, Fc$\varepsilon$RI, triggers a signal transduction cascade leading to the degranulation of preformed secretory granules containing a variety of potent inflammatory mediators in mast cells and basophils during the allergic response. The initial stimulus, aggregation of IgE bound to Fc$\varepsilon$RI in response to multivalent ligand, elicits the phosphorylation of Fc$\varepsilon$RI by the tyrosine kinase, Lyn, recruitment of the kinase, Syk, to the plasma membrane, and subsequent phosphorylation and activation of many signaling proteins, including phosphatidylinositol 3-kinase (PI3-K)$_1$, the guanine nucleotide exchange factor Vav, focal adhesion kinase (FAK), and phospholipase C-γ (PLC$_{g}$; for review, see Beaven and Baumgartner, 1996). A activated PLC$_{g}$ cleaves the membrane-bound lipid phosphatidylinositol-4,5-bisphosphate (PIP$_{2}$-4,5) into diacylglycerol, a stimulator of protein kinase C (PKC), and inositol 1,4,5-trisphosphate (IP$_3$), the ligand for the IP$_3$ receptor calcium channel in the ER membrane (for review, see Berridge, 1993). Binding of IP$_3$ to its receptor releases the intraluminal calcium from the ER into the cytoplasm, which in turn causes the influx of extracellular calcium into the cell through the $I_{\text{CRAC}}$ plasma membrane calcium channel (Hoth and Penner, 1992). The combination of both PKC activation and the increase in intracellular calcium is sufficient for promoting the targeting and fusion of secretory granules with the cell surface (Katakami et al., 1984; Sagi-Eisenberg et al., 1985). A role for GTP-binding proteins in the signaling cascade leading to degranulation was originally put forth based on the observation that treatment of permeabilized mast cells with the nonhydrolyzable analogue, GTP$\gamma$S, activated secretion, implying the involvement of either heterotrimeric or small G proteins, or both, in the secretary response (Howell et al., 1987). Introduction of inhibitory or stimulatory peptides into mast cells have implicated both the heterotrimeric G protein, G$_{i3}$ (Aridor et al., 1993), and the small G protein, Rac (Oberhauser et al., 1992), in the later stages of membrane fusion during exocytosis.

Several lines of evidence have suggested that members of the Rho family of small GTP-binding proteins, Cdc42,
Rac, and Rhô, also participate in regulating degranulation in mast cells and RBL-2H3 cells, a well-characterized rat mucosal mast cell line. First, secretion efficiency in permeabilized mast cells was enhanced upon incubation with recombinant activated forms of Rhô and Rac and reduced under conditions that inhibit Rhô and Rac activity (Price et al., 1995; Norman et al., 1996). In a similar system, recombinant Rac and Cdc42 were shown to delay the onset of the progressive decrease in secretion efficiency normally observed after streptolysin-O treatment (Brown et al., 1998). The ability to retard the gradual loss of responsiveness to stimulation indicated that Rac and Cdc42 were able to functionally replace proteins that had diffused out of the permeabilized cell, either as regulators or direct components of degranulation. A Rac/RhôGD1 complex purified from bovine brain cytosol was also found to retard the loss of the secretory response (O’Sullivan et al., 1996). However, when RhôGD1, a guanine nucleotide dissociation inhibitor that negatively regulates the Rhô family of GTP-binding proteins, was introduced separately, exocytosis was inhibited (M Ariot et al., 1996).

Second, stable expression of the dominant-negative forms of Cdc42 and Rac in RBL-2H3 cells inhibited antigen-induced degranulation and displayed different effects on cell morphology (G Guillemany et al., 1997). The Rhô family has been implicated in playing critical regulatory roles in cell motility, morphology, and cytoskeletal reorganization (for review, see H all, 1998). Studies examining the redistribution of actin filaments from the cortical region to the cell interior upon antigen stimulation suggested that Rhô and Rac mediate the concomitant changes in cell shape and actin reorganization during degranulation in mast cells (Norman et al., 1996).

Third, treatment of RBL-2H3 cells with Clostridium difficile toxin B, which modifies RhôA and Cdc42 by mono-glucosylation, completely blocked degranulation. In contrast, degranulation was unaffected in cells treated with Clostridium botulinum C3 transference, which selectively impairs RhôA, thus implying that toxin B inhibited exocytosis by inactivating Cdc42 (Prepens et al., 1996).

To further define at which step in the signaling cascade or the exocytosis process the Rhô proteins may be acting, we have constructed a vaccinia virus expression system to produce dominant-active and dominant-negative mutants of Cdc42 and Rac. Using a vaccinia virus infection scheme that exhibited a high level of expression were further amplified in HeLa cells and stored in small aliquots at −80°C. Titters of amplified viral stocks generally ranged from 10^8 to 10^10 pfu/ml.

RBL-2H3 cells were infected with recombinant vaccinia virus at 20 pfu/cell in MEM media supplemented with 5% fetal bovine serum, 2% glutamine, 50 μg/ml gentamicin sulfate, and 1 μg/ml 1249 (Santa Cruz Biotechnology), and rabbit anti-IQGAP (a gift from M. Hart, O’nyx Pharmaceuticals). Blots were developed using either sheep anti-rabbit or sheep anti-mouse coupled to hors eradish peroxidase at a dilution of 1:10,000 and the ECL detection system (Amersham Corp.).
GST alone, GST fused to the limit Cdc42/Rac (p21)-binding domain from PA K3 (GST-PBD), and GST fusions to the wild-type and mutant forms of Cdc42 were expressed in E. coli and purified by affinity binding to agarose beads cross-linked to glutathione (Hart et al., 1994). Immobilized GST-PBD (−50 μg) was incubated with RBL-2H3 cells lysates for 1 h at 4°C, and precipitates were then washed three times with buffer A. Bound proteins were eluted by boiling in SDS sample buffer and subjected to Western blot analysis with the anti-myc antibody. Immobilized GST alone, GST-Cdc42, GST-Cdc42 N17, and GST-Cdc42(N17) (−50 μg) were incubated in 10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 10 mM EDTA, and 1 mM PM SF for 15 min at room temperature to release prebound nucleotide, loaded with 0.5 mM GTPγS for 30 min at room temperature, and supplemented with 10 mM MgCl₂. The GST proteins were then incubated with lysates generated from either nonstimulated RBL-2H3 cells or cells stimulated for 5 min with 100 ng/ml DNP/BSA for 1 h at 37°C in buffer B (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.5% Triton X-100, 1 mM PM SF, and 5 mM MgCl₂). Protein precipitates were washed twice in buffer B supplemented with an additional 100 mM NaCl and once in buffer B minus the Triton X-100. Bound proteins were eluted by boiling in SDS sample buffer and subjected to Western blot analysis with the anti-PLCβ1 or anti-1QGAP antibody. Binding experiments between the GST fusion proteins and purified PLCβ1 from insect cell lysates (a gift from Fuyuo Sekiya and Sue Goo Rhee, NIH) were performed in a similar manner.

β-Hexosaminidase Secretion Assay
RBL-2H3 cells were plated in 48-well plates at a density of 2.5 × 10⁶ cells/well overnight at 37°C and infected the following day. Infected cells were washed twice with Tyrode’s buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 1 mg/ml BSA, and 20 mM HEPES, pH 7.4), and stimulated with 100 ng/ml DNP/BSA for 1 h at 37°C. The β-hexosaminidase content of samples was determined as previously described (Pierini et al., 1996). Typically, 50 μl of sample supernatant were incubated with 200 μl of 1 mM N-(p-nitrophenyl)-N-acetyl-β-D-glucosaminide (Sigma) in 0.05 M citrate buffer (pH 4.5) for 1 h at 37°C. A fter quenching of the reaction with the addition of 500 μl of 0.05 M sodium carbonate and 0.05 M sodium bicarbonate, the β-hexosaminidase release was determined by comparing the OD₄₀₅ measurements of the samples to the total amount of enzyme in cells lysed by 0.5% Triton X-100. For assays in which antigen stimulation was bypassed, cells were incubated in 20 mM PMA for 5 min, and then stimulated with either 1 μM A 23187 or 500 nM thapsigargin (Calbiochem) for 1 h at 37°C.

Calcium Fluorescence
RBL-2H3 cells were plated in 100-mm plates at a density of 4 × 10⁶ cells/plate overnight at 37°C and infected the following day. Infected cells were harvested, washed twice, and resuspended in Tyrode’s buffer supplemented with 0.25 mM sulfipyrazone at 1 × 10⁶ cells/ml, and then loaded with 0.5 μM Indo-1/AM (Calbiochem) for 1 h with constant agitation at 37°C. Cells were then washed twice and resuspended in Tyrode’s buffer and 0.25 mM sulfipyrazone at 1 × 10⁶ cells/ml. 3-mL aliquots of cell suspension were added to acrylic cuvettes maintained at 37°C and constantly stirred during the course of the experiment. DNP/BSA was added at 100 ng/ml to initiate calcium signaling. For experiments that specifically evaluate release of calcium from ER stores, 4 mM EGTA was added to cells before DNP/BSA stimulation. Indo-1 was excited at 330 nm and fluorescence emission was monitored at 400 nm with a Perkin-Elmer LS-5 fluorescence spectrophotometer.

IP₃ Determination
The amount of IP₃ in cells was determined using the [⁹²⁷]IP₃ radioassay kit (DuPont-NEN). RBL-2H3 cells were plated in 70-mm plates at a density of 3 × 10⁶ cells/plate overnight and infected the following day. Infected cells were washed twice with Tyrode’s buffer, stimulated for 5 min with 100 ng/ml DNP/BSA, and lysed according to the manufacturer’s instructions. The aqueous phases of the IP₃ samples were ultrafiltered through a Centricon concentrator (Amicon) with a membrane cut-off of 10,000 mol wt to exclude proteoglycans that interfere with the assay (Choi et al., 1993).

Immunofluorescence
RBL-2H3 cells were plated on glass coverslips in 6-well plates at a density of 2 × 10⁶ cells/well overnight and infected the following day. Infected cells were stimulated with 100 ng/ml DNP/BSA for 30 min, fixed in PBS/3.7% formaldehyde for 8 min, and permeabilized in PBS/0.1% Triton X-100 for 5 min. Incubations with primary and secondary antibody were performed for one hour each in a dark, humid chamber at 25°C. IP₃ receptors were detected using a 1:100 dilution of rabbit anti-IP₃ receptor antibody (Calbiochem) followed by a 1:200 dilution of anti-rabbit IgG conjugated to the Bodipy fluorophore (Molecular Probes). Cells were mounted in non-fade medium (Kirkegaard and Perry) and photographed on a Zeiss axiophot microscope with a Zeiss filter set (Carl Zeiss, Inc.) with T-M ax 400 film (Eastman Kodak Co., Inc.).

Results
Expression of Functional Wild-Type and Mutant Forms of Cdc42 and Rac Using a Vaccinia Virus Expression System
We have employed the vaccinia virus expression system to introduce dominant-active and dominant-negative mutants of Cdc42 and Rac, tagged with the myc epitope, into RBL-2H3 cells. To investigate whether the virus-expressed GTPases would exhibit the expected properties—dictated by the different mutations, in vitro binding experiments were performed between lysates from infected RBL-2H3 cells expressing the various mutants and GST fused to the GTPase (p21)-binding domain (PBD) of the p21-activated kinase, PA K3 (Bagrodia et al., 1995). The PBD, or CR1B motif, for Cdc42/Rac interaction binding, is conserved in a number of different effector proteins and mediates their interactions with Cdc42 and Rac in a GTP-dependent manner.

Administration of the virus-expressed mutant protein bound to GST-PBD by Western blotting using the anti-myc antibody demonstrated that both of the dominant-active mutants, Cdc42(N17) and Rac(N17), interacted with GST-PBD (Fig. 1a, lanes 4 and 9). In contrast, the two dominant-negative mutants, Cdc42(N17) and Rac(N17), did not exhibit any detectable binding to the fusion protein (Fig. 1b, lanes 5 and 10), as was the case with lysates from uninfected cells (Fig. 1b, lanes 1 and 6) and cells infected with empty vector (Fig. 1b, lanes 2 and 7). The wild-type forms of Cdc42 and Rac displayed relatively weaker binding, presumably reflecting a subpopulation of the wild-type GTPases that had acquired an activated state (Fig. 1b, lanes 3 and 8). Protein expression levels from infections between the three forms of Cdc42 (Fig. 1a, lanes 3–5) and Rac (Fig. 1a, lanes 8–10) were comparable, indicating that uniform expression with different vaccinia viruses can be achieved. These results indicate that the virus-expressed forms of Cdc42 and Rac displayed the behavior predicted by their respective mutations and can be used to functionally interfere with the endogenous GTPases in RBL-2H3 cells.

Antigen-stimulated Degranulation Is Enhanced by Dominant-Active Cdc42 and Rac and Inhibited by Dominant-Negative Cdc42 and Rac
The effects of expressing dominant-active or dominant-negative mutants of Cdc42 and Rac on degranulation were examined by measuring the secretion efficiency of infected RBL-2H3 cells after antigen stimulation. Since any perturbation in the degranulation process will require sufficient expression of the recombinant protein in order to compete

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with endogenous wild-type protein for target binding, the relative levels of the virus-expressed and endogenous proteins in infected RBL-2H3 cells were determined. The recombinant proteins migrated with a slower mobility on Western blots probed with anti-Cdc42 and anti-Rac antibodies, compared with endogenous protein, due to the presence of the myc epitope (Fig. 2a, lanes 3-5 and 7-9) and were absent in uninfected cells (Fig. 2a, lane 1) and cells infected with empty vector (Fig. 2a, lanes 2 and 6). Comparison of the protein levels between the two different forms revealed that approximately \( \approx 50\% \) of the total Cdc42 and Rac in the cell was derived from the virus-expressed source.

Secretion assays were performed in infected RBL-2H3 cells by measuring the relative amounts of \( \beta \)-hexosaminidase, a secretory granule enzyme that is released into the extracellular media upon antigen stimulation with DNP/BSA. The infection process itself produced a modest inhibitory effect on degranulation, as evidenced by cells infected with empty vector exhibiting a twofold increase compared with release extracellular media upon antigen stimulation with DNP/BSA. The infection process itself produced a modest inhibitory effect on degranulation, as evidenced by cells in infected RBL-2H3 whole cell lysates were incubated with immobilized GST-PBD for 1 h at 4°C. Bound proteins were washed and subjected to Western analysis using the anti-myc antibody.

dominant-negative mutants, Cdc42(N17) and Rac(N17), strongly implicate Cdc42 and Rac in the signal transduction pathway leading to exocytosis.

Infection with wild-type Cdc42 and Rac resulted in levels of secretion comparable to those obtained with an empty vector control, albeit at slightly inhibited levels of \( \approx 18\% \) and \( \approx 23\% \), respectively. The wild-type GTPases might have been predicted to either slightly stimulate secretion, since a fraction of each GTPase is present in an activated state (Fig. 1b, lanes 4 and 9), or not affect secretion levels at all compared with the empty vector control. Instead, we have consistently observed that the wild-type Cdc42 or Rac has a slight inhibitory effect, which suggests that the wild-type GTPase enter into nonproductive binding interactions with other proteins that function downstream in this signaling pathway leading to degranulation.

**Figure 1.** Wild-type and mutant forms of Cdc42 and Rac expressed using vaccinia virus are functionally active. (a) RBL-2H3 cells were infected with vaccinia virus containing empty vector (lanes 2 and 7), or a vector expressing Cdc42 (lane 3), Cdc42(V12) (lane 4), Cdc42(N17) (lane 5), Rac (lane 8), Rac(V12) (lane 9), or Rac(N17) (lane 10) for 6 h at 37°C, or were uninfected (lanes 1 and 6). Proteins were solubilized, resolved by SDS-PAGE, and the recombinant proteins were detected by Western blotting using anti-myc antibody. The additional protein bands present in the lanes containing lysates of the different forms of Rac are nonspecific (lanes 8-10), since they also appear in the uninfected and empty vector lanes (lanes 6 and 7). (b) Uninfected and infected RBL-2H3 cells were incubated with immobilized GST-PBD for 1 h at 4°C. Bound proteins were washed and subjected to Western analysis using the anti-myc antibody.

**Figure 2.** Dominant-active Cdc42 and Rac enhance secretion, whereas dominant-negative Cdc42 and Rac inhibit secretion. (a) RBL-2H3 cells were infected with vaccinia virus containing empty vector (lanes 2 and 6), or a vector expressing Cdc42 (lane 3), Cdc42(V12) (lane 4), Cdc42(N17) (lane 5), Rac (lane 7), Rac(V12) (lane 8), or Rac(N17) (lane 9) for 6 h at 37°C, or were uninfected (lane 1). Proteins were solubilized and resolved by SDS-PAGE. Cdc42 and Rac were detected by Western blotting using either anti-Cdc42 or anti-Rac antibody, respectively. Virus-expressed proteins migrate at a slightly higher molecular mass compared with the endogenous protein due to the presence of the myc epitope. (b) \( \beta \)-Hexosaminidase secretion was measured in RBL-2H3 cells infected with the different forms of Cdc42 and Rac upon stimulation with 100 ng/ml DNP/BSA for 1 h at 37°C and under nonstimulated conditions. Degranulation is given as the percent of total cellular \( \beta \)-hexosaminidase released, as measured by treatment of RBL-2H3 cells in parallel with 0.5% Triton X-100. Error bars represent the standard deviation of four independent experiments. The paired t test was used to evaluate the statistical significance between cells infected with empty vector and Cdc42 (0.0264), empty vector and Cdc42(V12) (0.0023), empty vector and Rac(N17) (0.0191), Cdc42 and Cdc42(V12) (0.0437), and Rac and Rac(N17) (0.0424). A score of \( \approx 0.05 \) denotes statistical significance. The comparison between cells infected with empty vector and Rac was not statistically significant.
One possibility is that the binding of GDP-bound Cdc42 and/or GDP-bound Rac to a target effector occurs with a measurable affinity, perhaps not significantly different from that for the GTP-bound forms of these proteins, but that the GTP-bound Cdc42/Rac interaction does not activate the target effector. In such a case, the over-expression of wild-type Cdc42 or Rac would competitively inhibit the binding of the activated GTP-binding protein to the target and yield an inhibitory effect.

Cells containing the empty vector or expressing the wild-type and mutant forms of Cdc42 and Rac exhibited similar levels of basal secretion (≤5%) in the absence of antigen treatment (Fig. 2b, −DNP/BSA). It is significant to note that RBL-2H3 cells expressing the dominant-negative mutants, Cdc42V12 and RacV12, do not bypass the requirement for DNP/BSA to effect degranulation. Thus, antigen binding may generate multiple signals in order to trigger degranulation, at least one of which involves a pathway that is not dependent on the activation of Cdc42 or Rac.

**Cdc42 and Rac Function Upstream of the Calcium Influx Step and PKC Activation in Signaling Degranulation**

To locate the step that Cdc42 and Rac may be modulating in the antigen stimulation pathway, infected cells were induced to degranulate by treatment with a combination of the calcium ionophore A23187 and phorbol 12-myristate 13-acetate (PMA). These agents synergistically act to bypass the requirement for antigen by directly initiating calcium influx into the cytoplasm, and by activating PKC, respectively (Katakami et al., 1984; Sagi-Eisenberg et al., 1985). Under these conditions, the continued maintenance of a secretion defect in cells expressing the dominant-negative mutants would indicate that the inhibition imparted by Cdc42V12 and RacV12 occurs downstream of calcium influx and PKC activation, whereas the ability to restore secretion would indicate that dominant-negative Cdc42 or Rac intervene at an upstream step.

Treatment of control cells with A23187 and PMA activated degranulation more strongly than DNP/BSA, increasing the relative amount of β-hexosaminidase released from ~39% to ~59% in uninfected cells (Fig. 3). In cells infected with empty vector, degranulation was elevated from ~28% in antigen-stimulated cells to ~68% in A23187/PMA-treated cells, thus demonstrating that the slight decrease in secretion caused by vaccinia virus infection could be bypassed by treatment with A23187 and PMA. Secretion was substantially less inhibited in cells infected with either Cdc42V12 or RacV12 upon stimulation with A23187 and PMA, with percent degranulation increased from ~6% and ~16% in antigen-stimulated cells to ~45% and ~46% in A23187/PMA-treated cells, respectively. These levels of secretion were similar to those observed in cells expressing the dominant-active Cdc42V12 and RacV12 mutants upon A23187 and PMA treatment. The more modest inhibition that had been observed upon expression of wild-type Cdc42 or Rac was also relieved, with percent degranulation increased from ~10% and ~23% in antigen-stimulated cells to ~48% and ~45% in A23187/PMA-treated cells, respectively. Both A23187 and PMA were required to achieve substantial rescue from the inhibition. Stimulation with the ionophore alone restored secretion to only half that observed with both treatments in cells infected with the dominant-negative mutants, whereas PMA by itself did not activate degranulation above basal levels (data not shown).

It should be noted that we have consistently found that treatment of cells expressing any form of Cdc42 or Rac with ionophore plus PMA yields a final level of secretion slightly below the levels measured in control cells (vector alone or uninfected). At the present time, we do not know the explanation for the slight dampening of the secretory response that is caused by expressing either of these GTPases. However, the fact that it is independent of their state of activation suggests that the dampening effect may reflect a perturbation of the GTP-binding/GTPase cycle of Cdc42 or Rac that occurs as an outcome of the general overexpression of any of these GTPases.

An alternative strategy to activating calcium influx with an ionophore is to treat cells with thapsigargin, a sesquiterpene lactone inhibitor of the ER Ca2⁺-ATPase pump, which normally maintains an intracellular calcium gradient by transporting free calcium from the cytoplasm back into the ER lumen (Lytton et al., 1991). Thus, blocking the function of the pump with thapsigargin effectively increases the cytoplasmic levels of free calcium. Using thapsigargin and PMA to stimulate degranulation, cells infected with Cdc42V12 and RacV12 also exhibited elevated levels of secretion, such that the percent degranulation was comparable to cells infected with empty vector (data not shown). The considerable stimulation of secretion in cells expressing the dominant-negative and wild-type Cdc42/Rac proteins implies that the combined effects of calcium mobilization and PKC activation are able to bypass the defect, thereby placing Cdc42 and/or Rac downstream of the calcium influx step. This observation strongly suggests that Cdc42 and Rac may modulate the calcium influx step and PKC activation in the signaling pathway that leads to degranulation in RBL-2H3 cells.
upstream of these two events in the antigen signaling pathway.

**Cdc42 and Rac Regulate the Intracellular Calcium Response after Antigen Stimulation**

In light of our finding that direct mobilization of calcium into the cell can synergize with PKC activation to bypass the inhibitory effects on degranulation caused by the dominant-negative mutants, we examined the possibility that Cdc42 and Rac may regulate the calcium influx pathway. This could occur either by modulating the release of calcium from ER stores or by directly activating plasma membrane calcium channels. The calcium responses of RBL-2H3 cells infected with the Cdc42 or Rac mutants were compared with those of cells infected with empty vector, in order to control for variations in cell number and loading efficiency of the fluorescent dye Indo-1. Upon antigen stimulation (Fig. 4, unfilled arrows), cells infected with either Cdc42V12 or RacV12 exhibited an enhancement of both phases of the calcium response, namely the initial, rapid peak contributed by release from ER stores and the influx pathway, and the more sustained plateau derived primarily from influx (Fig. 4, g and i). Expression of either dominant-negative mutants Cdc42N17 or RacN17 produced the opposite effect, dampening the two phases of calcium signaling (Fig. 4, h and j). The calcium response of cells infected with empty vector was slightly inhibited, but did not significantly differ from that of uninfected cells compared with the effects generated by the Cdc42 and Rac mutants (Fig. 4 a). Surprisingly, calcium signaling in cells expressing wild-type Cdc42 and Rac was very similar to that of cells expressing the empty vector (Fig. 4, b and c). We had expected the calcium response in the cells expressing the wild-type GTPases to be less robust compared with that of the vector control, consistent with our observation that expression of wild-type Cdc42 and Rac produced a small inhibition of antigen-stimulated secretion. The absence of an effect on calcium signaling suggested the possibility that the inhibitory effects produced by the wild-type GTPases during antigen-stimulated secretion may be manifested subsequent to calcium signaling. Rho GTPases have previously been suggested to play a role in the latter stages of degranulation (Norman et al., 1996).

To specifically evaluate release of calcium from intracellular stores, fluorescence assays performed in the presence of EGTA, to chelate total extracellular calcium, were carried out in parallel with experiments that contained free calcium in the medium. The release of calcium from ER stores has been implicated in directly activating the calcium influx pathway (Hoth and Penner, 1992). Thus, an alteration in the amount of calcium exiting the ER may provide an explanation for the stimulation in the calcium response observed with Cdc42V12 and RacV12, as well as the inhibition obtained with Cdc42N17 and RacN17. A addition of EGTA decreased calcium fluorescence to a lower resting level, presumably due to a small amount of extracellular Indo-1 releasing its bound calcium (Fig. 4, d–f and k–n, filled arrows). Subsequent antigen stimulation (Fig. 4, d–f and k–n, unfilled arrows) triggered a sharp peak that quickly returned close to the starting baseline fluorescence, confirming that the sustained plateau phase of the calcium response seen in the absence of EGTA represents the influx of extracellular calcium. Expression of the dominant-active mutants, Cdc42V12 and RacV12, elevated the peak of the calcium response compared with infection with empty vector (Fig. 4, k and m). In contrast, infection of cells with either Cdc42N17 or RacN17 led to a reduced amplitude in calcium signaling (Fig. 4, l and n). Although

![Figure 4](https://rupress.org/jcb/article-pdf/148/3/481/1289134/9908070.pdf)
expression of Rac\(^{\text{V17}}\) generally produced a more pronounced decrease in the release of calcium from intracellular stores compared with Cdc42\(^{\text{V12}}\), the calcium responses in the presence of extracellular calcium are very comparable. Since expression of the two dominant-negative GTPases inhibited overall calcium signaling to a very similar extent, it may be that there is a threshold level of calcium release from the ER, below which an inhibited calcium signal is produced. Finally, the release of calcium from ER stores in uninfected cells or cells expressing wild-type Cdc42 and Rac did not significantly differ from the empty vector control (Fig. 4, d–f).

The opposing effects produced by the dominant-active and dominant-negative mutants indicate that Cdc42 and Rac are able to directly modulate calcium signaling in response to antigen stimulation. The finding that these opposing effects are also maintained in the presence of EGTA strongly suggests that Cdc42 and Rac act upstream of the release of calcium from the ER lumen rather than directly on plasma membrane calcium channels. Altering the ability of calcium to be released from stores would subsequently influence the extent of calcium influx from the extracellular medium.

**Cellular IP\(_3\) Levels Are Enhanced in Cells Expressing Dominant-Active Cdc42\(^{\text{V12}}\) or Rac\(^{\text{V12}}\) upon Antigen Stimulation**

We next investigated the step immediately before the release of calcium from the ER stores, that is, production of the second messenger IP\(_3\), IP\(_3\) generated by the PLC-\(\gamma\)-mediated cleavage of PIP\(_2\)-4,5 in response to antigen stimulation, binds to the IP\(_3\) receptor, a ligand-gated calcium channel in the ER membrane. This activates channel opening and calcium efflux into the cytoplasm (Ferris et al., 1989; Mignery and Sudhof, 1990). The addition of DNP/BSA to uninfected cells and to cells infected with empty vector increased IP\(_3\) production by approximately twofold compared with nonstimulated cells (Fig. 5). The dominant-active mutants Cdc42\(^{\text{V12}}\) and Rac\(^{\text{V12}}\) further enhanced the antigen-promoted IP\(_3\) production, such that the stimulation was approximately threefold relative to nonstimulated cells. Wild-type Cdc42 or Rac displayed a modest (~1.5-fold) inhibition in antigen-stimulated IP\(_3\) production, while expression of the dominant-negative mutants Cdc42\(^{\text{N17}}\) and Rac\(^{\text{N17}}\) prevented any significant increase in IP\(_3\) levels upon antigen stimulation, compared with that of nonstimulated cells. We suspect that the levels of antigen-stimulated IP\(_3\) production in cells expressing dominant-negative Cdc42 or Rac are reduced relative to cells expressing wild-type protein and in fact, this appears to be the trend in any given experiment. However, when averaging the experiments, the deviations are sufficiently large to make it difficult to detect these differences and to make a definitive conclusion.

The exact mechanism by which release of calcium from the ER stores elicits calcium influx from the extracellular medium remains poorly understood. However, the enhancement in IP\(_3\) levels is consistent with the observed stimulation of the calcium response in cells expressing dominant-active Cdc42\(^{\text{V12}}\) or Rac\(^{\text{V12}}\), supporting our model that Cdc42 and Rac act upstream of IP\(_3\) production to regulate calcium signaling and ultimately, degranulation.

**Immunolocalization of the IP\(_3\) Receptor to Aggregates in the Cell Body Is Enhanced by Dominant-Active and Inhibited by Dominant-Negative Cdc42 or Rac**

The IP\(_3\) receptor has been demonstrated by immunolocalization studies to redistribute from a diffuse staining pattern to large, distinct aggregates after antigen stimulation in RBL-2H3 cells (Wilson et al., 1998). The functional significance of the IP\(_3\) receptor clustering is unclear, although models in which aggregation modulates channel opening or IP\(_3\) receptor interaction with resident ER calcium-binding proteins have been proposed. Formation of these clusters is highly dependent on elevated levels of intracellular calcium, since treatment of cells with thapsigargin or the ionophore ionomycin also produced cluster formation. We have used this immunofluorescence assay to determine whether the Cdc42 and Rac mutants and their concomitant effects on IP\(_3\) production and calcium signaling could also influence IP\(_3\) receptor clustering.

Upon antigen stimulation, permeabilized RBL-2H3 cells incubated with anti-IP\(_3\) receptor antibody displayed an average of 4.5 small brightly labeled aggregates per cell that localized to the periphery of the dense cell body (Fig. 6 b, see arrows and Table I), whereas nonstimulated cells exhibited an extremely low level of cluster formation (Fig. 6 a and Table I). It seems likely that the cellular structure outlined by the ring of aggregates represents the nuclear envelope and the contiguous ER membranes, the primary location of the IP\(_3\) receptor (Ross et al., 1989). This staining pattern differs from an earlier study in which the aggregates were more evenly distributed in the cytoplasm (Wilson et al., 1998). However, the discrepancy may be ex-
plained by the different sources of IP$_3$ receptor antibody used in both analyses.

Antigen stimulation also produced a dramatic increase in cell spreading, so that the flattened membrane extensions (Fig. 6 b, see arrowheads) are clearly delineated from the more central cell body (Fig. 6, compare a with b; Pfeiffer et al., 1985). This morphological change was not as pronounced in stimulated cells that underwent viral infection, presumably because the infection process itself induces a rounding-up effect on cells (Elroy-Stein and Moss, 1991). Thus, stimulated cells infected with the different forms of Cdc42 and Rac did not exhibit as flattened an appearance as stimulated, uninfected cells (Fig. 6, compare b with c–i).

Expression of dominant-active Cdc42$^{V12}$ and Rac$^{V12}$ induced a similar ring-like staining pattern of IP$_3$ receptor upon antigen addition, displaying an average of 5.12 and 5.60 IP$_3$ receptor clusters per cell, respectively (Fig. 6, e and h, see arrows and Table I). Nonstimulated cells that were infected with the dominant-active mutants did not exhibit this clustering phenotype, indicating that virus infection itself did not elicit a redistribution of the IP$_3$ receptor (data not shown). Brightly labeled clusters were also visible in cells infected with empty vector (3.74 clusters/cell), wild-type Cdc42 (3.22 clusters/cell), and wild-type Rac (3.04 clusters/cell), although to a lesser extent than seen for Cdc42$^{V12}$ or Rac$^{V12}$ (Fig. 6, c, d, and g and Table I).

Table I. Distribution of IP$_3$ Receptor Clusters in RBL-2H3 Cells Infected with Different Forms of Cdc42 and Rac

<table>
<thead>
<tr>
<th>Virus</th>
<th>DNP/BSA</th>
<th>Number of clusters/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>−</td>
<td>0.04</td>
</tr>
<tr>
<td>Uninfected</td>
<td>+</td>
<td>4.50</td>
</tr>
<tr>
<td>Vector</td>
<td>+</td>
<td>3.74</td>
</tr>
<tr>
<td>Cdc42</td>
<td>+</td>
<td>3.22</td>
</tr>
<tr>
<td>Cdc42$^{V12}$</td>
<td>+</td>
<td>5.12</td>
</tr>
<tr>
<td>Cdc42$^{N17}$</td>
<td>+</td>
<td>0.18</td>
</tr>
<tr>
<td>Rac</td>
<td>+</td>
<td>3.04</td>
</tr>
<tr>
<td>Rac$^{V12}$</td>
<td>+</td>
<td>5.60</td>
</tr>
<tr>
<td>Rac$^{N17}$</td>
<td>+</td>
<td>0.12</td>
</tr>
</tbody>
</table>

IP$_3$ receptor clusters were counted from ~50 cells.
Thus, these results indicate that PLC very similar binding profile (Fig. 7, a and b, lower panels) (Kuroda et al., 1996; McCallum et al., 1996), exhibited a panels, lane 1). Probing these Western blots with antibody panels, lane 5) nor to GST alone (Fig. 7, a and b, upper panels, lane 1). Probing these Western blots with antibody to IQGAP, a known target-effector protein for Cdc42 (Kuroda et al., 1996; McCallum et al., 1996), exhibited a very similar binding profile (Fig. 7, a and b, lower panels). Thus, these results indicate that PLC-γ1 is recognized by an anti-PLC-γ1 antibody on Western blots for both nonstimulated (Fig. 7 a, upper panel) or anti-IQGAP antibody (bottom panel).

Cdc42 Interacts with PLC-γ1 In Vitro in a GTP-dependent Manner

The combined effects on calcium signaling, IP₃ production, and IP₃ receptor aggregation strongly argue that Cdc42 and/or Rac function upstream of IP₃ formation. Additionally, the observation that PM-1 associates with A23187 to strongly stimulate degranulation in cells infected with the dominant-negative mutants suggests that Cdc42 and/or Rac also act upstream of diacylglycerol formation, which normally activates PKC. In an effort to begin to identify the target that Cdc42 and Rac may be modulating in the IP₃ pathway, we focused on PLC-γ, the enzyme responsible for cleaving PIP₂-4,5 into IP₃ and diacylglycerol. A recent study had demonstrated that both Cdc42 and Rac can stimulate the activity of phospholipase Cβ2 in a purified protein system (Illenberger et al., 1998), thus providing a precedent for small G proteins to interact with members of the PLC family.

Binding studies were performed by incubating lysates from nonstimulated (Fig. 7 a) and stimulated (Fig. 7 b) RBL-2H3 cells with GST fusions to wild-type and mutant forms of Cdc42. The dominant-active Cdc42V12, and to a lesser extent, wild-type Cdc42, interacted with a protein that migrated with the expected mobility (~145 kD) of PLC-γ1 and was recognized by an anti-PLC-γ1 antibody in vitro. Lysates from both nonstimulated (Fig. 7 a, upper panel) and stimulated (Fig. 7 b, upper panel, lanes 3 and 4) lysates. This protein did not bind to the dominant-negative mutant Cdc42N17 (Fig. 7, a and b, upper panels, lane 5) nor to GST alone (Fig. 7, a and b, upper panels, lane 1). Probing these Western blots with antibody to IQGAP, a known target-effector protein for Cdc42 (Kuroda et al., 1996; McCallum et al., 1996), exhibited a very similar binding profile (Fig. 7, a and b, lower panels). Thus, these results indicate that PLC-γ1 is recognized by an anti-PLC-γ1 antibody on Western blots for both nonstimulated (Fig. 7 a, upper panel) or anti-IQGAP antibody (bottom panel).

Introduction of the Y40 Effector Domain Mutation into Cdc42V12 Decreases the Enhanced Calcium Signaling Exhibited by Cells Infected with Cdc42V12 Alone

The GTPase family members contain a short effector binding domain from residues 32-40, termed the Switch I region, that mediates binding to a variety of target proteins in a GTP-dependent manner. To obtain further support that Cdc42 plays a role in degranulation through the signaling of downstream effector targets, we have constructed a virus expressing the Cdc42V12Y40 double mutant to determine whether disruption of Cdc42 binding to effector proteins would affect the enhanced calcium signaling and secretory response in cells infected with Cdc42V12.
Discussion

The Rho family of small GTP-binding proteins plays critical regulatory roles in a variety of cellular processes, including cytoskeletal organization, cell motility, transcriptional signaling, and cell cycle progression (for review, see Hall, 1998). In this study, we have implicated Cdc42 and/or Rac in a novel biological function to signal the degranulation of secretory vesicles in RBL-2H3 cells. By expressing Cdc42 and/or Rac alone. The Y 40 mutation has been demonstrated to prevent Cdc42 interaction with targets containing the CRIB domain, which leads to defective signaling in the JNK transcriptional activation pathway (Lamarche et al., 1996). The virus-expressed Cdc42V12,Y40 did not bind to the GST-PBD fusion protein (Fig. 8, lane 5), indicating that introduction of the Y 40 mutation disrupted the ability of Cdc42V12 to interact with GST-PBD (Fig. 8, lane 3). Upon antigen stimulation (Fig. 8 b, unfilled arrow), cells expressing Cdc42V12,Y40 exhibited a reduction in both the initial peak and sustained plateau phases of the calcium response, compared with cells expressing Cdc42V12 alone. The Y 40 mutation did not completely inhibit the stimulatory effects of the dominant-active V12 mutation, since cells expressing Cdc42V12,Y40 still displayed an enhancement of calcium signaling over cells infected with empty vector. Furthermore, expression of Cdc42V12,Y40 also dampened the release of calcium from ER stores compared with cells expressing Cdc42V12, suggesting that the Y 40 mutation most likely impaired a step before the initiation of calcium signaling (Fig. 8 c). Finally, expression of Cdc42V12,Y40 exhibited ~32% secretion, compared with ~40% in cells expressing Cdc42V12 and ~25% in cells expressing empty vector (data not shown). Taken together, the inhibition of calcium signaling and secretion produced by the Cdc42V12,Y40 double mutant are consistent with a defect in IP3 production and further supports our hypothesis that Cdc42/Rac interaction with downstream effectors is a key step in the pathway leading to calcium mobilization and degranulation.

Figure 8. Cells infected with the effector domain mutant Cdc42V12,Y40 exhibit impaired calcium signaling compared with cells infected with Cdc42V12. (a) Lysates from RBL-2H3 cells infected with vaccinia virus containing empty vector (lane 1), or a vector expressing Cdc42 (lane 2), Cdc42V12 (lane 3), Cdc42V12,Y40 (lane 4), and Cdc42V12,Y40 (lane 5) were treated as described in the legend to Fig. 1, a and b. (b) The calcium responses of RBL-2H3 cells infected with Cdc42V12,Y40 were compared with those of Cdc42V12 and empty vector upon antigen stimulation (unfilled arrows) in the absence or presence (c) of 4 mM EGTA (filled arrow). Traces shown are representative of three independent experiments.
The observed changes in IP₃ formation and calcium signaling are not merely secondary phenomena induced by Cdc42 and Rac effects on actin remodeling. Treatment of cells with the phorbol ester, PMA, induced cytoskeletal changes similar to those resulting from incubation with both PMA and the calcium ionophore A 23187. However, the combination of PMA and A 23187 strongly stimulated degranulation, whereas PMA alone did not, indicating that cytoskeletal rearrangement is insufficient to induce the signaling pathway leading to secretion (Ludowyke et al., 1994). In addition, our in vitro binding studies identifying an interaction between Cdc42 and PLCγ suggest that Cdc42 feeds directly into the IP₃/calcium signaling pathway.

PLCγ hydrolyzes PIP₂, to form the two second messengers, IP₃, which binds to the IP₃ receptor in the ER membrane to release internal calcium stores, and 1,2-diacylglycerol, which stimulates PKC activity. Thus, activation of PLCγ is instrumental in initiating a signaling cascade in a wide variety of cell types that responds to receptor–ligand interactions at the cell surface. Three major families of PLC have been identified: β, γ, and δ (for review see Rhee and Bae, 1997). PLCβ and PLCγ stimulation are linked to receptors coupled to heterotrimeric G proteins and tyrosine kinases, respectively, whereas a role for PLCδ activation by cell surface receptors remains unclear. PLCβ2 has been characterized as a target for Cdc42 and Rac in a study describing the isolation of a complex between Cdc42 and LyGDI, which was found to mediate GTP-dependent activation of PLCβ2 in vitro (Illenberger et al., 1998). Rac, but not Rho, was also capable of stimulating PLCβ2 activity. This activation was further observed in a system composed of purified recombinant proteins, suggesting that the interaction between Cdc42/Rac and PLCβ2 was direct. Cdc42/Rac activation of PLCβ2 appeared to be very specific since the GTPases could not enhance the activity of PLCδ1 or a second PLCβ isoform, PLCβ1 (Illenberger et al., 1997).

We had originally focused on PLCγ1 as a possible target for the Rho GTPases in stimulating the IP₃/calcium pathway based on the work examining Cdc42/Rac-mediated activation of PLCβ2. We have established an in vitro interaction between purified, recombinant GST-Cdc42 and a protein from RBL-2H3 cell lysates that closely matched the expected molecular mass (∼145 kD) of PLCγ1 and was recognized by a polyclonal antibody raised against amino acids 1249–1262 at the COOH terminus of PLCγ1, but migrated slightly faster than the majority of the PLCγ1 purified from insect cells. The purified PLCγ1 contained a component protein that both comigrated with the interacting protein from RBL-2H3 cell lysates and bound to GST-Cdc42 in a GTP-dependent manner. This suggests that it may be a modified form of PLCγ1 that binds most effectively to activated Cdc42, and perhaps results in a specialized regulatory function that links Cdc42 to signaling events that lead to secretion. We attempted to determine whether the small increase in mobility observed for the PLCγ-immunoreactive species that binds Cdc42 may have
beaten by hypophosphorylation of PLC-γ1. In both quiescent NIH-3T3 cells and A 431 human epidermoid cells, PLC-γ1 in its basal state has been demonstrated to contain phosphoserine (M. Eisenhelder et al., 1989). Thus, it seemed possible that a percentage of the total purified, intact cell-expressed PLC-γ1 may contain fewer phosphate groups on serine residues, and thus account for the faster mobility in protein gels. However, treatment of purified or immunoprecipitated PLC-γ1 with potato acid phosphatase to remove susceptible phosphate groups did not yield a lower molecular mass protein (data not shown). There are a number of other possibilities for Cdc42/PLC-γ1 interactions that remain to be addressed. For example, it is possible that the association between activated Cdc42 and an unmodified PLC-γ1 is transient, as thus far, we have been unable to detect a stable in vivo complex between PLC-γ1 and Cdc42 using coimmunoprecipitation methods.

In addition to possibly regulating PLC-γ1 directly and remodeling the cytoskeletal architecture, the Rho GTPases may also modulate the activities of other signaling molecules that play a role in triggering degranulation. Some potential candidates belong to the lipid kinase family, which generates lipid products that perform key signaling tasks after antigen stimulation. First, phosphoinositide 3-kinase (PI3-K) phosphorylates PIP2-4,5 to form phosphatidylinositol-3,4,5-trisphosphate (PIP3-3,4,5), which can induce PLC-γ1 translocation to the plasma membrane via interactions with its pleckstrin homology and SH2 domains (Bae et al., 1998; Falasca et al., 1998). Since Cdc42 and Rac have been shown to stimulate PI3-K activity in a GTP-dependent manner (Zheng et al., 1994; Tolas et al., 1995), the GTPases may be exerting their influence on PLC-γ1 indirectly by stimulating PI3-K to generate higher levels of PIP3-3,4,5. Furthermore, treatment of RBL-2H3 cells with the PI3-K inhibitor, wortmannin, attenuated the secretory responses that remain to be addressed. For example, it is possible that the association between activated Cdc42 and an unmodified PLC-γ1 is transient, as thus far, we have been unable to detect a stable in vivo complex between PLC-γ1 and Cdc42 using coimmunoprecipitation methods.

In summary, we now present findings that highlight an important role for the Cdc42 and/or Rac GTPase in mediating calcium changes that are critical for the exocytosis of secretory granules. These regulatory events occur at a step immediately upstream of calcium release from stores and appear to impact on IP3 production and on the interplay between this second messenger and the aggregation of its ER receptor/calcium channels. The ability of activated Cdc42 and/or Rac to bind PLC-γ1 may underlie a key step in what is likely to be a complex regulation of calcium signaling via antigen binding and the resultant activation of Rho GTPases. Future efforts will be directed at better understanding the mechanistic basis by which Cdc42 and Rac influence calcium changes and whether other aspects of antigen-mediated secretion are also affected by these GTPases.

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