IgE Receptor Type I-dependent Regulation of a Rab3D-associated Kinase

A POSSIBLE LINK IN THE CALCIUM-DEPENDENT ASSEMBLY OF SNARE COMPLEXES*

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Following activation through high affinity IgE receptors (FceRI), mast cells release, within a few minutes, their granule content of inflammatory and allergic mediators. FceRI-induced degranulation is a SNARE (soluble N-ethylmaleimide attachment protein receptors)-dependent fusion process. It is regulated by Rab3D, a subfamily member of Rab GTPases. Evidence exists showing that Rab3 action is calcium-regulated although the molecular mechanisms remain unclear. To obtain an understanding of Rab3D function we have searched for Rab3D-associated effectors that respond to allergic triggering through FceRI. Using the RBL-2H3 mast cell line we detected a Ser/Thr kinase activity, termed here Rak3D (from Rab3D-associated kinase), because it was specifically co-immunoprecipitated with anti-Rab3 antibody. Rak3D activity, as measured by its auto- or transphosphorylation, was maximal in resting cells and decreased upon stimulation. The down-regulation of the observed activity was blocked with EGTA, but not with other degranulation inhibitors, suggesting that its activity functions downstream of calcium influx. We found that Rak3D phosphorylates the NH2-terminal regulatory domain of the t-SNARE syntaxin 4, but not syntaxin 2 or 3. The phosphorylation of syntaxin 4 decreased its binding to its partner SNAP23. Thus, we propose a novel phosphorylation-dependent mechanism by which Rab3D controls SNARE assembly in a calcium-dependent manner.

Mast cells are specialized immune cells able to secrete a variety of mediators stored in cytoplasmic granules and involved in inflammatory and allergic responses (1). Upon activation, they can discharge almost their entire granular content by compound exocytosis (2, 3). A potent physiological stimulus for degranulation is the antigen-dependent aggregation of IgE bound to high affinity IgE receptors (FceRI).† This activates both membrane-attached and cytoplasmic tyrosine kinases that pave the way for the intracellular calcium increase that is necessary for secretion (4–6).

The accumulated evidence demonstrates that during regulated exocytosis calcium can act directly on the molecular machinery involved in membrane fusion. An essential part of this machinery are SNARE proteins (7). During neurotransmitter release at the synapse, SNAREs assemble into an extremely stable multimeric core complex composed of one v-SNARE (synaptobrevin or vesicular-associated membrane protein = VAMP) and two t-SNAREs (syntaxin 1 and SNAP25) (8–10). Complex formation is thought to provide the necessary energy to bring together the two opposing membranes and drive bilayer mixing. SNARE complex formation is regulated by additional positive and negative effectors (8, 10). These include small GTPases of the Rab3 family (8, 11, 12). In mast cells, degranulation also depends on SNARE-mediated fusion but instead utilizes SNAP23, a SNAP25 homolog and syntaxin 4 (13, 14) as well as the calcium sensor synaptotagmin (15). Rab3D also plays an important role in regulating the fusion process in mast cells (16, 17) but the mechanism by which it exerts its role is unknown.

Rab proteins regulate membrane traffic in eucaryotic cells by organizing the assembly of effector molecules (18, 19). Rab-mediated functions include membrane tethering of organelles (20), calcium-dependent fusion (21, 22), and movement of vesicles along the cytoskeleton (23–25). While many Rab proteins are ubiquitous, members of the Rab3 family are enriched in regulated-secretion-competent cells (11, 12). They are comprised of four isoforms (Rab3A, -B, -C, and -D). Rab3A, the best studied family member is abundantly expressed in brain and neuroendocrine cells. Absence of Rab3A in knock-out mice led to an enhancement of neurotransmitter release (21). Rab3A appeared to act at a late step as the pool of fusion-competent vesicles was unchanged. Its absence also interfered specifically with calcium-regulated fusion as triggering via a calcium-independent stimulus was not affected. Similar conclusions for the polycrylamide gel electrophoresis; PI 3-kinase, phosphatidylinositol 3-kinase; PMA, phorbol 12-myristate 13-acetate; Rab3D, Rab3D associated kinase; RBL-2H3, rat basophilic leukemia cells; SNAP23 or 25, synaptosome-associated protein of 23 kDa or 25 kDa; SNARE, soluble N-ethylmaleimide attachment protein receptor; Syn, syntaxin; VAMP, vesicle-associated membrane protein; Pipes, 1,4-piperazinediethanesulfonic acid.

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The abbreviations used are: FceRI, type I high affinity IgE receptor; Ca2+/CaM, calcium/calmodulin; CaMKII, Ca2+/calmodulin-dependent protein kinase II; DNP, dihydroethidium; GST, glutathione S-transferase; HSA, human serum albumin; IGKA, in-gel kinase assay; IVK, in vitro immune complex kinase assay; MBP, myelin basic protein; PAGE,
Modulation of Rab3D-associated Kinase and SNARE Assembly

calcium-dependent action of Rab3 have been drawn from studies in adrenal chromaffin cells and Aplysia cholinergic neurons (22, 26). In mast cells, the predominant isoform is Rab3D, although Rab3A and Rab3B have also been detected (16, 27, 28). Overexpression of WT Rab3D inhibited FceRI-triggered degranulation, an effect that was enhanced with preferentially GFP-bound mutants (16, 17).

Several Rab3 effectors have been described. Some, like calmodulin (29, 30), Rabphilin (31), Nox2 (32), or Rim (33), may be components of the calcium-dependent mechanism of exocytosis. However, a molecular understanding of their function in conjunction with Rab3 has not been achieved. In the present study we report the discovery of a Rab3D-associated kinase activity in RBL-2H3 mast cells. The activity or possibly the association of this kinase, named here Rak3D, was calcium regulated. Rak3D specifically phosphorylates syntaxin 4 thus decreasing its capacity to bind SNAP23. Therefore, this kinase may provide the link between Rab3D and the calcium dependence of SNARE complex formation in regulated-secretion competent cells.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Transfections—**RBL-2H3, COS-7, and C57 cells (kindly provided by Dr. D. Stachel, International Institute of Molecular and Cell Biology, Poland) were maintained in Dulbecco’s modified Eagle’s medium-Glutamax medium (Life Technologies, Inc., Egargny, France) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, G, and 100 μg/ml streptomycin (Life Technologies, Inc.) at 37 °C in a humidified 5% CO2 incubator. COS-7 cells were transfected by electroporation with 30 μg of Rab3A-SRemeo, Rab3D-SRemeo, and Sronove vectors, as previously described (16). Cells were harvested 48 h later and processed for immunoprecipitation.

**Antibodies and Other Reagents—**Anti-dinitrophenyl (DNP)-specific immunoglobulin E (IgE), rabbit anti-syk antibodies and affinity purified rabbit antibodies to Rab3D, syntaxin 4, and glutathione immunoglobulin E (IgE), rabbit anti-syk antibodies and affinity purified vectors, as previously described (16). Cells were harvested 48 h later and processed for immunoprecipitation.

**Antibodies and Other Reagents—**Anti-dinitrophenyl (DNP)-specific immunoglobulin E (IgE), rabbit anti-syk antibodies and affinity purified rabbit antibodies to Rab3D, syntaxin 4, and glutathione S-transferase (GST) have been described (14, 16, 34). Rabbit anti-syntaxin 4 was also purchased from Alomone Labs (Jerusalem, Israel). F(ab’)2 fragment of peroxidase-conjugated anti-rabbit and normal rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The pharmacological agents 4-μmol 12-myristate 15-acetate (PMA), wortmannin, EGTA, the kinase substrates myelin basic protein (MBP) and enolase, glutathione-Sepharose beads, as well as dinitrophenyl-human serum albumin (DNP-HSA) were purchased from Sigma. Ionomycin and bisindolylmaleimide I were obtained from Calbiochem (San Diego, CA). Protein A beads were from Amersham Pharmacia Biotech (Oxford, France).

**Cell Stimulation and Immunoprecipitation—**RBL-2H3 cells were either harvested by trypsinization or left adherent. For stimulation experiments, RBL-2H3 or C57 mast cells were sensitized at 1 × 107 cells/ml (nonadherent cells) or by directly adding 1 ml of ice-cold phosphate-buffered saline containing 10% fetal bovine serum, 100 IU/ml penicillin, G, and 100 μg/ml streptomycin to adherent cells before harvesting them by scraping. Postnuclear supernatants were prepared by centrifugation at 15,000 × g for 30 min and added to 5 μg of anti-Rab3D antibody or normal rabbit IgG prebound to protein A-Sepharose beads and immunoprecipitated for 2 h at 4 °C.

**In Vitro Immune Complex Kinase Assay (IVK)—**Immunoprecipitates were performed as above and washed additionally two times in kinase buffer (25 mM Pipes, pH 7.3, 150 mM NaCl, 5 mM KCl, 5 mM MgCl2, 1% Triton X-100, 1 mM sodium orthovanadate (Sigma), 1000 units/ml aprotinin (Sigma), 10 μg/ml pepstatin, 20 μg/ml leupeptin, 2 μM 4-(2-aminoethyl)benzenesulfonyl fluoride (from all with Alexis Inc., San Diego CA) at 5 × 106 cells/ml (nonadherent cells) or by directly adding 1 ml of lysis buffer to adherent cells before harvesting them by scraping. Postnuclear supernatants were prepared by centrifugation at 15,000 × g for 30 min and added to 5 μg of anti-Rab3D antibody or normal rabbit IgG prebound to protein A-Sepharose beads and immunoprecipitated for 2 h at 4 °C.

**In Vitro Immune Complex Kinase Assay (IVK)—**Immunoprecipitates were performed as above and washed additionally two times in kinase buffer (25 mM Pipes, pH 7.3, 150 mM NaCl, 5 mM KCl, 5 mM MncI, 5 mM MgCl2, 0.25% Triton X-100). IVK was performed in 50 μl of kinase buffer containing 10 μCi of [γ-32P]ATP. Beads were washed twice and eluted in 2 × SDS sample buffer. When exogenous substrates were added, the reaction mixture was incubated in the presence of 10 μM substrate and following the IVK the total mixture was resolved after addition of SDS sample buffer without a further washing step. Quantitation of phosphorylated species was carried out on a PhosphorImager (Molecular Dynamics) using ImageQuant software.

**In-gel Kinase Assay (IGKA)—**Anti-Rab3D or normal rabbit IgG immunoprecipitates were electrophoresed in a SDS-polyacrylamide gel containing MBP (0.5 mg/ml). IGKA was performed as described (35). Specific bands, staining electroretrogreen, SDS was removed by repeated washings in 50 mM Tris-HCl, pH 8, containing 20% propa- rol-2. Proteins were denatured in 8 uT urea 1 h at room temperature and renatured overnight in 50 mM Tris-HCl, pH 8, containing 5 mM β-mercaptoethanol and 0.04% Tween 20. The kinase assay was carried out by incubating the gel for 1 h at room temperature in kinase buffer containing 15 mM ATP and 200 μCi of [γ-32P]ATP. The gel was washed with a 5% trichloroacetic acid solution containing 1% sodium pyrophosphate and analyzed on a PhosphorImager.

**Immunoblotting—**Proteins resolved on SDS-PAGE were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and blocked by incubation in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk. Blots were then incubated for 1 h at room temperature with primary antibodies (anti-Rab3D at 1 μg/ml and anti-syntaxin 4 at 1/600 dilution). After several washes, peroxidase-labeled anti-rabbit IgGs (1:30,000 dilution) were used as secondary antibodies and incubated for 1 h at room temperature. The blots were developed using the enhanced chemiluminescence assay (Amersham Pharmacia Biotech).

**Modulation of β-Hexosaminidase Secretion—**Granule secretion of the marker β-hexosaminidase in the presence of pharmacological agents was analyzed as previously described (16). Pharmacological agents were added 15 min before stimulation and were kept during the 30-min stimulation period. The net percent inhibition was calculated in comparison to cells incubated in the presence of vehicle only.

**Recombinant Proteins—**The cytoplasmic domains of rat syntaxin 3, syntaxin 4, and syntaxin 4 were expressed as GST fusion proteins using the pGEX-2TK vector (Amersham Pharmacia Biotech) and purified as described (14). Thrombin (Roche Molecular Biochemicals, Meylan, France) was used to cleave GST from soluble recombinant syntaxin 4. Recombinant human GST-SNAP23 was a gift of Thierry Galli (Institut Curie, Paris, France). Deletion mutants of syntaxin 4 were generated by polymerase chain reaction amplification to obtain Ha– (aa 4–68), Ha/Hb– (aa 4–114), and Ha/Hb/Hc– (aa 4–168) domains fused to GST.

**In Vitro Binding Assay—**Soluble recombinant syntaxin 4 (10 μM) was phosphorylated in an IVK by Rak3D present in anti-Rab3D immunoprecipitates (107 cell equivalents). For binding to recombinant GST-SNAP23, soluble phosphorylated S4 (0.3 μM) was mixed in 300 μl of kinase buffer with glutathione-Sepharose beads containing 2 μg GST-SNAP23. The mixture was incubated 2 h at 4 °C with end-over-end rotation. Beads were centrifuged and the supernatant (unbound syntaxin 4) was recovered and an equal amount of 2 × SDS sample buffer was added. Beads were washed twice with 1 ml of kinase buffer and 60 μl of sample buffer was added to recover bound syntaxin 4. Samples (bound and free syntaxin 4) were boiled for 3 min and equal amounts of protein were simultaneously resolved on two separate SDS-PAGE. Gels were stained for PhosphorImager analysis or subjected to immunoblotting with anti-syntaxin 4 antibody. Quantitation of phosphorylated and immunoblotted syntaxin 4 species was carried out using ImageQuant and NIH Image software, respectively. No binding was detectable to two other GST fusion proteins (GST-VAMP3 and GST-VAMP8) in control experiments.

**In Vivo Labeling with [32P]Orthophosphate—**RBL-2H3 cells were treated as described (36). Briefly, 2 × 106 RBL-2H3 cells were incubated in phosphate-free medium for 3 h at 37 °C before addition of 1 μg/ml IgE for 1 h at 37 °C, then labeled with 330 μCi of [32P]Orthophosphate for 3 h at 37 °C. Cells were washed with phosphate-free medium and stimulated with 100 ng/ml DNP-HSA for 5 min or left unstimulated. Ice-cold phosphate-buffered saline was added to stop stimulation and cells were lysed as described above. Syntaxin 4 and p72Hem were immunoprecipitated from stimulated and unstimulated cells. After separation by SDS-PAGE and transfer onto a nitrocellulose filter, the membrane was autoradiographed and subsequently blotted with anti-syntaxin 4.

**Phosphoamino Acid Analysis—**Phosphoamino acid analysis was carried out according to a published protocol (37). Briefly, Rab3D was immunoprecipitated and proteins were subjected to an IVK. The sample was run on SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Hybond™, Amersham Pharmacia Biotech). After a short autoradiography the portion corresponding to labeled species was excised. Bound protein was hydrolyzed in 6 M HCl for 1 h at 110 °C. The hydrolysate was dried, washed, and redissolved in acetic acid/pyridine/water (10:1:189, v/v/v). Phosphoserine, phosphothreonine, and phospho- tyrosine standards (3 μg of each) were dissolved in 2 μl of acetic
FIG. 1. Presence of a kinase activity in Rab3D immunoprecipitates. A, specificity of an antibody to Rab3D. Proteins from lysates of COS-7 cells transfected with empty, Rab3A- or Rab3D-containing SRneo vector were immunoprecipitated with anti-Rab3D, resolved on 12% SDS-PAGE, transferred to nitrocellulose, and probed with anti-Rab3D. The arrow indicates a specific band corresponding to the expected molecular weight for Rab3D. B, a kinase activity is associated with Rab3D immunoprecipitates (IP). Proteins from lysates of resting RBL-2H3 cells (10^7 cells/sample) were immunoprecipitated with anti-Rab3D or normal rabbit IgG (rIgG) and subjected to an IVK in the presence of [γ-32P]ATP. After washing, labeled proteins were resolved on 10% SDS-PAGE, and analyzed using a PhosphorImager. The arrow indicates the presence of a Rab3D-associated 130-kDa phosphoprotein (pp130).

acid/pyridine buffer. The phosphoamino acids were separated at 1600 V for 20 min on a cellulose TLC in the same buffer used to dissolve samples. Unlabeled phosphoamino acid standards were detected with ninhydrin and labeled amino acids were visualized by autoradiography.

RESULTS

A Ser/Thr Kinase Is Associated with Rab3D in RBL-2H3 Cells—Several functional links between protein kinases and small GTPases have been demonstrated making the search for protein kinases as potential effectors of Rab3D particularly important. To identify such a putative protein kinase we first examined whether the antibody we generated to the Rab3D carboxyl-terminal divergent region could specifically immunoprecipitate the Rab3D isoform, as both Rab3A and Rab3D are expressed in RBL-2H3, the mast cell line used in these studies. COS-7 cells were transfected with cDNAs encoding Rab3A, Rab3D, or the empty expression vector. Cell lysates were prepared and proteins were immunoprecipitated and immunoblotted using the anti-Rab3D antibody. As shown in Fig. 1A, a band corresponding to the expected molecular mass of 27 kDa was only detected in the Rab3D-transfected cells demonstrating that our antibody could indeed immunoprecipitate Rab3D in a specific manner. We next examined whether immobilized Rab3D was associated with a protein kinase activity by performing an IVK. A phosphorylated molecular species of about 130 kDa (pp130) was consistently detected in anti-Rab3D immunoprecipitates but not in those immunoprecipitates where normal rabbit IgG was used (Fig. 1B). To determine whether this phosphoprotein could represent a kinase, we first analyzed whether the kinase activity associated with Rab3D was able to phosphorylate enolase and MBP, which are generic substrates for protein tyrosine and Ser/Thr kinases, respectively. Fig. 2A shows that MBP was strongly phosphorylated in this assay while enolase remained unphosphorylated suggesting that the identified kinase activity corresponds to a Ser/Thr kinase. This was further confirmed by direct phosphoamino acid analysis that revealed that pp130 is phosphorylated on Ser/Thr residues but not on Tyr (Fig. 2B). The MBP protein was then used as a substrate in an IGKA to directly assay whether pp130 has by itself kinase activity. Fig. 2C shows the phosphorylated band that could be detected at a molecular mass of around 130 kDa. This suggests that the pp130 protein has itself kinase activity. Although this kinase activity appeared to be weak, when compared with IVK, denaturation, and renaturation of the kinase could likely affect the fraction of active kinase remaining in the IGKA. Indeed, as stronger exposures revealed a background activity in lanes of control IgG and anti-Rab3D in the p130 area by PhosphorImager analysis, quantitation of the optical densities (arbitrary units) between normal IgG and anti-Rab3D in the p130 area by PhosphorImager analysis revealed a 7.4 ± 2.3 (mean ± S.D.) difference in three independent experiments (experiment 1 = 10.0; experiment 2 = 5.8, and experiment 3 = 6.8).

but not on Tyr (Fig. 2B). The MBP protein was then used as a substrate in an IGKA to directly assay whether pp130 has by itself kinase activity. Fig. 2C shows the phosphorylated band that could be detected at a molecular mass of around 130 kDa. This suggests that the pp130 protein has itself kinase activity. Although this kinase activity appeared to be weak, when compared with IVK, denaturation, and renaturation of the kinase could likely affect the fraction of active kinase remaining in the IGKA. Indeed, as stronger exposures revealed a background activity in lanes of control IgG we quantitated the difference to Rab3D immunoprecipitates using PhosphorImager analysis, which was found to be more than 7-fold (see figure legend). Taken together, these results indicate that a Ser/Thr kinase of ~130 kDa is associated with Rab3D that we refer to in the following as Rak3D.

FcεRI-dependent Modulation of Rab3D—Rab3D has been shown to inhibit mast cell degranulation stimulated via FcεRI (16, 17). As the signaling pathways between the initial stimulation event and Rab3D are presently unknown we wished to analyze whether receptor triggering could affect the kinase activity associated with Rab3D. IgE-sensitized RBL-2H3 cells were challenged with antigen for varying times and p130 autophosphorylation was determined. Fig. 3A shows a represent-
RBL-2H3 cells were stimulated with anti-Rab3D.

*Fig. 3.* Rak3D activity is FcεRI-stimulation-dependent. FcεRI-modulation of Rak3D activity as revealed by the phosphorylation of p130 (A) and MBP (B). IgE-sensitized RBL-2H3 cells were stimulated with DNP-HSA (100 ng/ml) for the indicated times. Proteins in cell lysates were immunoprecipitated with either anti-Rab3D or rabbit IgG as indicated and subjected to an IVK in the presence of \(^{32}\)P-ATP. A PhosphorImager analysis of the 130-kDa and 50-kDa bands in the autoradiograph is shown (A). Surprisingly, the phosphorylation pattern of the different deletion mutants as well as a quantitative analysis summarizing several experiments. The results from a representative IVK in the presence of 10 μM substrate are shown in Fig. 4A. Surprisingly, these results revealed a unique specificity for Rak3D, in that it effectively phosphorylated GST-syntaxin 4 while not phosphorylating GST-syntaxin 2 or 3. Because of the specificity of Rak3D we tested a variety of other SNARE fusion proteins expressed in RBL-2H3 cells including GST-SNAP23, GST-VAMP2, GST-VAMP3, and GST-VAMP8. With the sole exception of syntaxin 4, all other tested proteins failed to be phosphorylated by Rak3D under the conditions used (data not shown). We also found that comparable to its autophosphorylation and the phosphorylation of the exogenous substrate MBP, the phosphorylation of GST-syntaxin 4 followed the same receptor-dependent kinetics, with a pronounced down-regulation at 3 min of stimulation (Fig. 4B). To shed some insight on its unusual specificity for syntaxin 4 we wished to determine the site of phosphorylation in syntaxin 4. We constructed a series of deletion mutants (Fig. 4C) that allowed examination of the various helices (Ha, Hb, and Hc) present in the NH\(_2\)-terminal regulatory domain as well as the SNARE motif (H3 domain) as predicted from sequence alignment and the resolved crystal structure of syntaxin 1 (42) (Fig. 4C). Ten μM of the various deletion mutants were subjected to an IVK in the presence of Rak3D. Fig. 4C shows the phosphorylation pattern of the different deletion mutants as well as a quantitative PhosphorImager analysis summarizing several experiments. The results demonstrate that Rak3D is able to phosphorylate syntaxin 4 at more than one site in the NH\(_2\)-terminal regulatory domain. Significant differences in relative amount of phosphorylation were observed for both Hb and Hc in the NH\(_2\)-terminal regulatory domain. Sequence alignment comparisons among the three syntaxins suggest that Ser-78 could be one of the phosphorylated residues in Hb as it was the only serine or threonine residue which was absent in syntaxin 2 and 3. These data also corroborate our previous conclusion that Rak3D is a Ser/Thr kinase because tyrosine residues are not present in Hb. Several candidate residues that could account for specific phosphorylation of Hc in syntaxin 4 are Thr-129, Ser-143, and Ser-146, because these are absent in the corresponding syntaxin 2 and 3 sequences.

Syntaxin 4 Phosphorylation by Rak3D Affects Its Interaction with the t-SNARE SNAP23—Although not located in the SNARE motif, phosphorylation in the NH\(_2\)-terminal regulatory domain of syntaxin 4 may indirectly affect SNARE assembly due to induced conformational changes or steric hindrance. We therefore assessed whether phosphorylation by Rak3D could...
Fig. 4. Rak3D specifically phosphorylates the Q SNARE syntaxin 4. A, syntaxin 4 is a specific substrate of Rak3D. Protein from lysates of resting RBL-2H3 cells (10⁷ cells/sample) were immunoprecipitated with anti-Rab3D and subjected to an IVK in the presence of [γ-³²P]ATP and 10 μM GST-syntaxin 2 (GST-syn2), GST-syntaxin 3 (GST-syn3), or GST-syntaxin 4 (GST-syn4). Reaction products were resolved on 10% SDS-PAGE and analyzed using a PhosphorImager and Coomassie Blue staining. B, phosphorylation of GST-syntaxin 4 by Rak3D is FcR1-regulated. IgE-sensitized RBL-2H3 cells were stimulated with DNP-HSA (100 ng/ml) for the indicated times. Proteins from cell lysates were immunoprecipitated with anti-Rab3D and subjected to an IVK in the presence of [γ-³²P]ATP and 10 μM GST-syntaxin 4 fusion protein. Phosphorylated proteins were resolved on 10% SDS-PAGE and analyzed using a PhosphorImager and Coomassie Blue staining.

C. Proteins in the supernatant of resting RBL-2H3 cells (10⁷ cells/sample) were immunoprecipitated with anti-Rab3D and subjected to an IVK in the presence of [γ-³²P]ATP and 10 μM GST-syntaxin 4 or the syntaxin 4 deletion mutants (GST-Ha, GST-Ha/Hb, and GST-Ha/Hb/Hc). The relative location of each mutation is shown in the schematic representation of syntaxin 4 modeled after the structure of syntaxin 1. The histogram shows a quantitative determination of relative phosphorylation levels for the different mutants (mean ± S.E.) from five independent experiments using ImageQuant Software. The asterisk indicates statistical significance between mutants (p < 0.05, Wilcoxon test).

DISCUSSION

Rak3D and [γ-³²P]ATP. The assay was designed to measure total syntaxin 4 or phosphorylated syntaxin 4 binding to SNAP23. Recombinant soluble syntaxin 4 devoid of GST was incubated with Rak3D to induce phosphorylation. An aliquot of the reaction was incubated with GST-SNAP23 prebound to glutathione-Sepharose. The bound and unbound syntaxin 4 was determined by immunoblotting (total syntaxin 4) and by PhosphorImager analysis (phosphorylated syntaxin 4). Fig. 5 shows the results of a representative experiment and Table I summarizes the data obtained from five independent experiments where different concentrations of syntaxin 4 were added to the binding reaction. Based on these results we determined that the binding of phosphorylated syntaxin 4 to SNAP23 was inhibited by 70.6 ± 13.1% (mean ± S.E.). This demonstrates that the phosphorylation of syntaxin 4 by Rak3D affects its ability to assemble with SNAP23.

Syntaxin 4 Is Phosphorylated in RBL-2H3 Cells—We finally asked whether syntaxin 4 could be phosphorylated in vivo in resting cells and whether this phosphorylation decreased as observed in our in vitro experiments. RBL-2H3 cells were labeled with [³²P]orthophosphate and syntaxin 4 was immunoprecipitated from resting and stimulated cells. Experiments shown in Fig. 6 revealed that syntaxin 4 is indeed phosphorylated in resting cells in agreement with our in vitro data. Its overall phosphorylation status, however, did not seem dramatically affected by receptor engagement although stimulation was successful as indicated by the increased phosphorylation of syk tyrosine kinase.

Rab3 GTPases function in regulated-secretion competent cells by limiting calcium-triggered membrane fusion mediated by SNARE proteins (12, 21, 22). The relationship between the calcium signal obtained after cell stimulation and Rab3 action remains poorly understood. Nevertheless, recent evidence from studies both in yeast and mammalian cells indicates that protein phosphorylation and dephosphorylation represents an important mechanism that regulates SNARE assembly (40, 41, 43–45). Furthermore, it was demonstrated that small G-proteins from the Ras, Rho, and Rab families function together with protein kinases to facilitate their actions (46–48). A well known example is the Ras-dependent recruitment of Raf leading to the activation of the mitogen-activated protein kinase cascade and gene regulation (46). This prompted us to search for IgE-triggered phosphorylation events that could be associ-
nant GST-Rab3D fusion proteins have been unsuccessful,3 sug-

Attempts to isolate Rak3D using GTP or GDP-bound recombi-

between the kinase and the nucleotide-bound state of Rab3D.

Rak3D, as inhibition with bisindolylmaleimide I had no effect

is important in mast cell secretion (49), did not seem to regulate

sary for regulation of Rak3D. Similarly, PKC activation, which

data using wortmannin suggest that PI 3-kinase is not neces-

providing membrane attachments sites for effectors (18), our

though evidence exists in several systems that the action of

the critical role of calcium influx in regulating Rak3D. Al-

was reproduced after stimulation with PMA/ionomycin indicat-

ing that the granule membrane-localized Rab3D (28) most

likely requires lipid modification for this interaction, as is the

case for several Rab3 effectors (29, 50–52). Similarly, when we

tried to immunoprecipitate Rab3D from cells stably transfected

with GTP-bound mutant forms (N135I and Q81L) no changes

were observed in the FcεRI-dependent downmodulation.3 The

easiest explanation would be that the association of the kinase

may not necessarily depend on the nucleotide-bound state of

Rab3D. In this context, it has been recently demonstrated that

lipid-modified Rab3A can bind to calcium/calmodulin (Ca2+/ CaM) in the presence of either GDP or GTP via a Rab3-con-
served basic amino acid sequence (29). This interaction caused

the dissociation of Rab3A from its target membrane (29, 30).

Thus, it is possible that FcεRI engagement induces the disso-

ciation of Rab3D from Rak3D by a similar calcium/CaM-de-

pendent mechanism.

Although we have not formally demonstrated that the pp130

protein (Rak3D) is the kinase responsible for phosphorylation of

the t-SNARE syntaxin 4 we found that the activity present in

Rab3D immunoprecipitates specifically phosphorylates this t-

SNARE and the activity was similarly down-regulated after

stimulation. Thus, as this was the only identifiable activity in

Rab3D immunoprecipitates it is likely that pp130 represents

the syntaxin 4 kinase. The t-SNAREs syntaxin 2 and 3 were

not phosphorylated, although the latter clearly have been shown to be substrates of different kinases (40, 41). Likewise, we did not detect phosphorylation of other SNARE proteins expressed in RBL-2H3 cells by Rak3D, including the t-SNARE SNAP23 or several v-SNAREs (VAMP2, VAMP3, and VAMP8). Thus, this unique selectivity for syntaxin 4 suggests an important role for Rak3D in regulating SNARE complex assembly. Interestingly, the plasma-membrane localized syntaxin 4 (53, 54) was recently demonstrated to be a key component of the fusion complex in mast cells and a variety of other cells (14, 55, 56). Using a series of deletion mutants we established that syntaxin 4 becomes phosphorylated at two different helices (Hb and He) in the NH2-terminal regulatory domain (42, 57). Comparison of sequence alignments with syntaxin 2 and 3 suggest several phosphorylatable residues (Ser-78, Thr-120, Ser-143, and Ser-146) that are solvent accessible in the homologous syntaxin 1 structure (42). However, it appears that some of these sites (Ser-143 and Ser-146) may become inaccessible when another regulator, the Munc18-3 protein is bound to syntaxin 4 (58), whereas Thr-120 upon phosphorylation could affect the interaction with the COOH-terminal SNARE motif. Phosphorylation of syntaxins in the regulatory domain is not unique to syntaxin 4 (40, 41). Syntaxin 1 was shown to be phosphorylated in the regulatory domain, but unlike syntaxin 4 its phosphorylation is located close to the NH2 terminus before the Ha domain (59).

Our work also revealed that phosphorylation by Rak3D re-

sults in a decrease of syntaxin 4 binding to its t-SNARE part-

ner SNAP23. Several arguments suggest that a phosphoryla-


d 

c 

I. Pombo and U. Blank, unpublished data.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Concentration of syntaxin 4 addeda</th>
<th>Total syntaxin 4b</th>
<th>Phospho syntaxin 4c</th>
<th>% Inhibition of phospho syntaxin 4 bindingd</th>
</tr>
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<tr>
<td>1</td>
<td>0.15</td>
<td>0.131</td>
<td>0.050</td>
<td>61.8</td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td>0.410</td>
<td>0.170</td>
<td>58.5</td>
</tr>
<tr>
<td>3</td>
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<td>0.342</td>
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</tr>
<tr>
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<td>0.6</td>
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<td>0.041</td>
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<td>0.9</td>
<td>0.590</td>
<td>0.099</td>
<td>83.2</td>
</tr>
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</table>

a Binding reaction was performed by adding the indicated concentration of soluble phosphorylated syntaxin 4 to 1.5 μM immobilized GST-

SNAP23.

b Total bound and unbound syntaxin 4 was quantified by densitometry after immunoblotting with anti-syntaxin 4. No binding was detectable to two other GST fusion proteins (GST-VAMP3, GST-VAMP8) in control experiments.

c Bound and unbound phospho syntaxin 4 was quantified by PhosphorImager analysis.

d Percentage of inhibition was calculated according to the formula [1-(phospho syntaxin 4/total syntaxin 4)] × 100.

![Figure 6: Syntaxin 4 is phosphorylated in RBL-2H3 mast cells.](image)
be relevant in mast cell exocytosis. (i) Clearly, the down-modulation of Rak3D can be observed in intact cells and this correlates with the initiation of secretion (16). (ii) In vivo labeling of RBL-2H3 cells with $^{32}$P orthophosphate has shown that syntaxin 4 is indeed phosphorylated in unstimulated cells. (iii) There is also evidence that secretion in mast cells is coupled to dephosphorylation by the protein phosphatase 2A, which is recruited to the plasma membrane following FceRI triggering (60). Although the molecular targets of this phosphatase recruitment are not known it could serve to dephosphorylate syntaxin 4 thereby facilitating membrane fusion. In our in vivo labeling experiments we were, however, unable to observe an apparent change in the syntaxin 4 phosphorylation status following stimulation. A probable explanation may be that dephosphorylation could concern only a small fraction of phosphorylated syntaxin 4 molecules at a given time. Previous analysis of SNARE-mediated-fusion processes in yeast have indeed suggested that regulatory mechanisms occur locally, are transient in nature, and include only a small proportion (<2%) of all SNARE molecules (61). An additional reason may be that syntaxin 4 can be phosphorylated at multiple sites and that dephosphorylation at a precise residue might occur following stimulation and be functionally important without dramatically affecting the overall phosphorylation level of the total cellular protein. How can these studies be interpreted in the context of the proposed differential localization of Rab3D and syntaxin 4? Indeed, in unstimulated cells, syntaxin 4 is plasma membrane localized while Rab3D is localized to the granule compartment (28, 53, 54). It is possible that the kinase is only active toward syntaxin 4 when mediator-containing granules become tethered at the plasma membrane. Thus, the tethered granule membrane is actively restricted from fusing with the plasma membrane by Rak3D-dependent phosphorylation of syntaxin 4 (29). After stimulation Rab3D is found at the plasma membrane (28) possibly as a Rab3D-Ca$^{2+}$-CaM complex that continues to control exocytosis, as previously suggested (30). In conclusion, mast cells tethering of granules to the plasma membrane has been observed (2, 3). Moreover, the compound exocytosis observed in mast cells suggest that tethered granules are functionally granules for fusion as they are first to release their content upon encountering an appropriate stimulus. Therefore, we suggest that phosphorylation of syntaxin 4 in resting mast cells by Rak3D represents one of the mechanisms that limits the assembly of closely tethered SNARE proteins in resting cells. Cell activation and intracellular calcium increases may counteract this Rab3D phosphorylation-dependent fusion clamp. Preliminary analysis suggest that Rak3D can be detected in other secretion-competent tissues such as rat adipocytes and rat pancreas. However, the identity of the kinase remains as of yet unknown. Inhibitors of PKC as well as a variety of other kinases (CaMKII, casein kinase, myosin light chain kinase, and protein kinase A) did not affect Rak3D activity in our IVK. This of course differs from the PKC-dependent syntaxin 4 phosphorylation, observed in activated human platelets (62) and thus we are actively engaged in the identification of this kinase. 

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REFERENCES

Modulation of Rab3D-associated Kinase and SNARE Assembly