mechanism that ordinarily limits release to a single quantum: suppose that such regulation works by raising the energy barrier for neighbouring vesicle fusion as a result of rapid mechanical deformation of the active zone, caused by insertion of additional membrane by the fused vesicle²⁹. Rab3A or its effectors could secure such rapid deformation of the active zone by facilitating the complete and efficient fusion of the synaptic vesicle membrane through destabilizing the metastable intermediate fusion complex. A role for temporal regulation of the stability of the docking and/or fusion complex has also been proposed for the endosome-specific Rab family member Rab5 (ref. 30). Then, in the absence of Rab3A, the precise steps are slowed that lead to complete fusion of the synaptic vesicle upon binding Ca^{2+} , retarding the warping of the active zone sufficiently to prevent the energy barrier for ensuing neighbouring vesicle fusion from increasing fast enough, allowing multiple releases to occur. Our model accounts for the fact that release probability is independent of Rab3A function. □

Methods

Culture experiments. Low-density microisland cultures of hippocampal pyramidal neurons were prepared from E15–E17 wild-type, *rab3A*-mutant and synaptotagmin I mutant mouse embryos as described⁸; five to seven embryos from the same mother were used for every wild-type and *rab3A* mutant culture preparation. Independent cultures of the same genotype yielded reproducible results, and each set of experiments was performed on separate cultures. Some of the experiments were also done on cultures prepared from rat (Fig. 2), and were included in the wild-type data as there were no differences in synaptic properties of the cultures of the two rodent species. Cells were used 8–13 days after plating; coverslips were changed after each recording so that the number of cells/cell pairs given in the figure legends represents the number of coverslips used. extracellular recording solution contained (in mM): NaCl 137, KCl 5, CaCl_2 10, MgCl_2 0.5, D-glucose 10, HEPES–NaOH (pH 7.3) 5, and 100 μM picrotoxin was also added; concentrations of MgCl_2 and CaCl_2 were varied when indicated. The internal recording solution was as before⁸. Recordings were made with axopatch 200 (Axon Instruments). Signals were filtered at 2 kHz, digitized at 5 kHz and analysed with programs written in AXOBASIC, VisualBASIC and C.

Hypertonic solution was applied locally as described⁷, and the quantal release rate was normalized for the number of synapses. The upper limit of the releasable pool size was estimated by the total number of quanta released per synapse during the first 4 s of hypertonic solution application; the lower limit was defined by subtracting the steady-state pool size for the 4-s period. The releasable pool size for wild-type cells determined here was slightly higher than a previous estimate⁷: the discrepancy is probably due to underestimation of the number of synapses in the perfused area, because, depending on the intensity threshold of the fluorescence staining of the synapses (determined visually after immunofluorescence labelling against synapsin I; ref. 8) used for defining synapses, the number of synapses can readily vary by at least 2-fold (C.E.S. and J. F. Wesseling, unpublished observations). The wild-type and mutant culture experiments were nevertheless done under identical conditions; we observed no difference in the pool size among the three groups.

Slice experiments. Acute transverse hippocampal slices were prepared and whole-cell recordings were made as described^{26–28}. Concentrations of CaCl_2 and MgCl_2 were varied as indicated for each experiment. 1 mM QX314 (Sigma) was included in the recording pipette to suppress spike generation in the post-synaptic cell. MK-801 and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) were from Research Biochemicals International.

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C-terminal binding domain of Rho GDP-dissociation inhibitor directs N-terminal inhibitory peptide to GTPases

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The Rho GDP-dissociation inhibitors (GDIs) negatively regulate Rho-family GTPases^{1,2}. The inhibitory activity of GDI derives both from an ability to bind the carboxy-terminal isoprene of Rho family members and extract them from membranes^{3,4}, and from inhibition of GTPase cycling between the GTP- and GDP-bound states^{4,5}. Here we demonstrate that these binding and inhibitory functions of rhoGDI can be attributed to two structurally

In an attempt to understand the molecular mechanisms of the inhibitory activities of GDI, we have performed a series of structural and biochemical analyses of the archetypal protein in this family, rhoGDI. Investigations focused on two carboxy-terminal fragments of bovine rhoGDI, GDIΔ22 (residues 23–204) and GDIΔ59 (residues 60–204). GDIΔ22 is similar to a construct (residues 25–204) previously reported to be fully functional in both membrane-extraction and nucleotide-dissociation assays⁶, and is therefore used here as a model of the full-length protein. ¹⁵N-labelled samples of GDIΔ22 and GDIΔ59 were analysed by ¹H/¹⁵N heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectra⁷ (Fig. 1a,b). The wide ¹HN chemical shift dispersion (6.5–10.4 p.p.m.) and relatively uniform peak intensity in Fig. 1b suggest that GDIΔ59 forms a single folded

In order to understand the functions of the N- and C-terminal regions of rhoGDI, the affinities of the full-length protein, GDIΔ22, GDIΔ42 (residues 43–204) and GDIΔ59 for isoprenylated Cdc42 were measured by fluorescence resonance energy transfer between the GTPase (complexed with the fluorescent nucleotide 2'(3')-O-(*N*-methylanthraniloyl)GDP (Mant-GDP)) and the GDI constructs (covalently modified with fluorescein maleimide). The affinity of the constructs for Cdc42 decreases as increasing amounts of the N

changes >0.05 p.p.m. on addition of 0.9 equivalents of non-isoprenylated Cdc42(GDP) are yellow. **d**, Normalized $^1\text{H}/^{15}\text{N}$ HSQC peak intensity, $I_{0.9}/I_{0.0}$, versus residue number in GDIΔ22 (wild-type rhoGDI numbering). $I_{0.0}$ and $I_{0.9}$ are intensity measured for the free protein, and after addition of 0.9 equivalents non-isoprenylated Cdc42(GDP), respectively. Prolines and residues 26, 29, 30, 80, 107, 135, 153 and 169, which had amide peaks that were either badly overlapped or too weak for intensity to be measured, are not included.

terminus are removed (Fig. 2a). However, even GDIA59, which contains almost exclusively the folded domain of rhoGDI, still binds the GTPase in the high nanomolar range. In contrast, NMR studies with an N-terminal rhoGDI peptide (residues 23–58) show extremely low affinity ($K_D > 5$ mM) for Cdc42 (data not shown). Both domains of the regulator are required to partition Cdc42 from membranes, however, as GDIA42 and GDIA59 are inactive in membrane extraction assays (data not shown).

The different rhoGDI constructs were also examined for their ability to inhibit nucleotide dissociation from isoprenylated Cdc42. Although the GTPase is nearly saturated with regulator in all cases (93–98% based on the K_D in Fig. 2a; see Methods), GDP dissociation is slowed only in the presence of full-length rhoGDI and GDIA22 (Fig. 2b). Identical results were seen in assays performed at 1 μ M Cdc42 and 400 μ M GDIA59, strongly suggesting that the differences in activity are not simply a result of differences in binding affinity. Thus inhibitory activity resides in residues 23–42, with C-terminal residues primarily contributing binding energy rather than inhibitory effect. The combined NMR and biochemical data provide strong support for a model in which rhoGDI consists of a folded C-terminal binding domain that targets an inhibitory N-terminal peptide to GTPases.

In an attempt to develop a more detailed model of the rhoGDI–GTPase complex, we determined by NMR spectroscopy the three-dimensional solution structure of GDIA59. The structure was calculated using a hybrid distance geometry/simulated annealing protocol using a total of 1,992 distance restraints, and 110 ϕ , 52 ψ and 30 χ_1 torsional restraints. The structures agree well with the experimental data, and show good covalent geometry and non-bonded contacts (see Methods). A stereo view of a backbone superposition of residues 69–202 of the 20 lowest-energy structures of GDIA59 is shown in Fig. 3a. Residues 60–68 and 203–204 show no long-range nuclear Overhauser enhancements (NOEs), are disordered in the structure, and have been removed for clarity. The structure is well defined by the NMR data, with atomic root-mean-squared deviation (r.m.s.d.) from the mean coordinates of 0.64 ± 0.06 Å for backbone atoms and 1.18 ± 0.09 Å for all heavy atoms in residues 69–202.

The structure of GDIA59 consists of two largely antiparallel β -sheets that pack against each other in a parallel manner, forming a β -sandwich. The protein has an immunoglobulin-like fold, with a modified Greek-key β -strand topology. The top sheet in Fig. 3b consists of the antiparallel strands F, E, H and I, flanked by strand C, which aligns parallel to strand I (see Fig. 1c). The bottom sheet contains four antiparallel strands, B, A, D and G. The β -sandwich is closed at the top and bottom by loops, and is capped at one end by a short 3_{10} helix, and side chains from residues in strands B and C. The opposing end is open to solvent, and shows a deep pocket between the two sheets. The disordered N terminus extends from the top surface of the protein in Fig. 3b near loops H–I and D–E. The structure shows little resemblance to RabGDI, which selectively binds prenylated GDP-bound Rab GTPases and is involved in vesicle transport⁸. This discrepancy is not unexpected, given the functional differences between the two GDI families, including sensitivity to GTPase nucleotide state and isoprenylation patterns⁸.

The minimized average NMR structure of GDIA59 is shown in Fig. 3c, viewed into the pocket at the open end of the β -sandwich. Its base and inner walls are lined by several conserved hydrophobic residues, including Leu 104, Tyr 110, Ile 112, Thr 132, Ala 165, Pro 166, Tyr 175, Ile 177, Trp 194, Leu 196 and Ile 198. The side chain of a single conserved hydrophilic amino acid, Gln 130, extends into the pocket, and may form a hydrogen bond with the nearby hydroxyl group of Tyr 128. Two lines of evidence indicate that this is the isoprene binding site of rhoGDI. First, addition of the farnesylated peptide acetyl-KKSRRC(S-farnesyl) to GDIA59 as a readily available mimic of the isoprenylated C terminus of Rho-family GTPases (note that RhoB, which is farnesylated, is a target of rhoGDI (ref. 3)

and mGDI3 (ref. 9)) caused large, saturable changes to the backbone and/or side-chain amide (^1H , >0.1 , or ^{15}N , >1.0 p.p.m.) or ^{13}C methyl (>0.4 p.p.m.) resonances of many residues in and near the pocket (Fig. 1c). No changes were observed when non-isoprenylated peptide was added. However, a nearly identical set of residues was affected, although to a lesser degree, by addition of *N*-acetyl-S-farnesyl cysteine to the limit of its aqueous solubility. Second, it has been shown that the 20-fold difference in affinity for Cdc42 between rhoGDI and its homologue D4 (Fig. 1c) is due to a difference of isoleucine (rhoGDI) versus asparagine (D4) at residue 177 (ref. 6), which lines one side of the pocket.

In most of the structures in the final ensemble, the isoprene-binding site is approximately 4.5–5.5 Å wide, 9 Å long and 10 Å deep. The pocket is reminiscent of that found in fatty-acid binding proteins (FABPs), which sequester linear hydrophobic ligands in a cavity between two sheets¹⁰. However, the FABP pocket is much larger and contains many more polar and charged residues than that of rhoGDI, allowing promiscuous binding to a variety of ligands in many different conformations. A distinct feature of prenyl chains is their conformational restriction by local steric interactions, including allylic 1,3 ($\text{A}^{1,3}$) and pseudo-1,3 diaxial (*gauche*⁺, *gauche*[−]) strain^{11,12}. The hydrophobicity and small size of the binding pocket, combined with the geometric restrictions of the isoprene, probably impart selectivity for prenyl groups to rhoGDI.

Figure 2 **a**, Normalized change in fluorescence (F) of the (Mant-GDP)–Cdc42 complex on addition of fluorescein-conjugated GDI constructs. Binding affinity (K_D) was calculated as described¹⁶; wtGDI, wild-type GDI. **b**, Dissociation of Mant-GDP (indicated by decreased fluorescence²⁸) from isoprenylated Cdc42 following addition of EDTA (arrow) in the presence of nearly saturating amounts of GDI proteins.

The specificity of GDIs for Rho-family members indicates that, although the isoprene is important for binding, other protein-protein contacts are necessary for high-affinity interactions. To identify these regions, ^{15}N -GDI Δ 22 was titrated with unlabelled, non-isoprenylated Cdc42 loaded with GDP. At subsaturating GTPase levels, differential exchange broadening (as manifested by decreased peak intensity) in $^1\text{H}/^{15}\text{N}$ -HSQC spectra can be used to identify residues with chemical shifts that are most perturbed by binding. A plot of peak intensity at 0.9 equivalents of Cdc42 (40–50% saturation based on peak volumes for four resonances that show distinct bound and free species) versus amino-acid sequence

of GDI Δ 22 is shown in Fig. 1d. N-terminal residues 23–63 are strongly affected. Four additional regions of the folded C-terminal domain, spanning the D–E and H–I loops at the apex of the molecule, and adjacent parts of strands H, E, F and G, are also affected. Most of the residues in these regions, in particular 119–128, 147–150 and 181–186, are conserved throughout the GDI family. The protein-binding residues form a continuous surface immediately adjacent to the isoprene-binding site of rhoGDI (Fig. 3d). The N-terminal inhibitory peptide extends from strand A between the D–E and H–I loops at the back of this surface, where it could easily contact the bound GTPase to impart GDI activity.

The combined structural and biochemical data suggest a model for the full rhoGDI–Cdc42 complex in which the GTPase sits on top of the C-terminal domain of the regulator, with its attached isoprene buried in the GDI hydrophobic pocket. These interactions anchor the inhibitory N-terminal peptide to the GTPase, allowing it to block exchange of GDP, and also probably binding of GAPs and GNRFs. The combined interactions of the GDI N and C termini then provide sufficient thermodynamic driving force to compete GTPases away from membranes.

We observed that GDIΔ42, which binds Cdc42 only 100-fold more weakly than full-length protein, is unable to extract the GTPase from membranes. Also, the binding energy of rhoGDI for Cdc42 ($\sim 12 \text{ kcal mol}^{-1}$) is very similar to the membrane–water partitioning energy reported¹³ for model geranylgeranylated peptides ($\sim 12 \text{ kcal mol}^{-1}$). These data suggest that there is a delicate thermodynamic balance in the partitioning of rhoGTPases between membranes and rhoGDI family members in the cell. In this case, small changes in GTPase affinity for either species could result in large changes in distribution between membranes and the cytoplasm. Carboxymethylation of Rac2 in neutrophils in response to various signals correlates with migration of the GTPase from a rhoGDI complex in the cytoplasm to the plasma membrane¹⁴. This modification, which is known to increase the partitioning of prenyl peptides into negatively charged liposomes by a factor of at least 10–20 (refs 13, 15), could thus be used in the cell to release GTPases from inhibitory GDIs by tipping the thermodynamic balance in favour of membranes.

It has been reported that in T cells the N-terminal region of the rhoGDI homologue D4 is specifically cleaved at two positions (residues 18–19 and 54–55) by two different ICE-like proteases following induction of apoptotic signalling pathways^{16,17}. The larger of the two resulting C-terminal proteolytic fragments is likely to retain inhibitory activity, but the smaller will be unable to bind GTPases in the environment of the cell. Our finding that the N terminus of rhoGDI is disordered in solution suggests that this region of all GDI family members will be proteolytically sensitive. Thus proteolysis may be a general mechanism for regulating the activity of these proteins. Moreover, given that GDIΔ22 retains membrane extraction and nucleotide-exchange inhibition activity, but that GDIΔ42 and GDIΔ59 do not, and that the N-terminal extension of mGDI3 (Fig. 1c) seems to mediate membrane localization of this protein (R.A.C., unpublished observations), it is possible that proteolysis at different sites in the GDI N terminus could lead to fragments with different inhibitory properties. Our data now provide a structural and thermodynamic framework for continued analysis of the regulatory functions of GDI proteins in the cell.

Note added in Proof: Preliminary NOE analyses of a GDIΔ59–farnesyl peptide complex suggest that the isoprene binding pocket may deepen on ligation through structural changes that allow it to merge with a small adjacent cavity seen in some members of the free protein ensemble. □

Methods

Protein expression and purification. ^{15}N labelled or $^{15}\text{N}/^{13}\text{C}$ labelled GDIΔ22 and GDIΔ59 were overexpressed in *Escherichia coli* strain BL21(DE3) grown in minimal media containing $^{15}\text{NH}_4\text{Cl}$ or $^{15}\text{NH}_4\text{Cl}/^{13}\text{C}_6\text{-glucose}$, respectively, and purified chromatographically. NMR samples were 2 mM GDI protein in 20 mM sodium phosphate buffer, pH 6.8, except for titrations where protein concentration was 0.5 mM. Non-isoprenylated and isoprenylated Cdc42 were expressed in bacteria and insect cells, respectively, and purified^{18,19}.

GDI binding assay. GDI proteins were conjugated at Cys 79 with fluorescein by reaction with a 10-fold excess of fluorescein maleimide for 1 h at room temperature in the dark. Cdc42 was loaded with Mant-GDP as described¹⁸. Energy transfer between the two chromophores in the GDI–GTPase complex results in quenching of Mant fluorescence ($\lambda_{\text{excitation}}$ 350 nm; $\lambda_{\text{emission}}$ 440 nm).

Nucleotide dissociation assay. Cdc42 (25 nM) prelabelled with Mant-GDP

was incubated in the presence of 125 mM GDP and either 100 nM wild-type rhoGDI, 250 nM GDIΔ22, 2 μM GDIΔ42, 10 μM GDIΔ59, in the absence of any GDI construct, or in 10 mM MgCl_2 . Nucleotide dissociation was initiated by addition of EDTA to 6 mM. Mant fluorescence was monitored as above.

NMR spectroscopy. NMR spectra were collected at 30 °C on a Varian UNITY+ 600 MHz spectrometer. Backbone sequential assignments of GDIΔ59 and GDIΔ22 were completed using standard methods through HNCO, HNCA, Cb, CbCoNHH, HNCAHa, HCC-TOCSY-NNH and ^{15}N -edited TOCSY spectra²¹. Side-chain assignments were completed through analysis of CCC-TOCSY-NNH, HCCH-TOCSY and HCCH-COSY spectra²¹. Stereospecific assignments of 9 Val and 11 Leu methyl groups were obtained from constant time- $^1\text{H}/^{13}\text{C}$ HSQC data acquired on a 10% ^{13}C -labelled sample²².

Structure determination. Structures were determined through a standard hybrid distance geometry/simulated annealing protocol implemented in X-PLOR²³. Distance restraints were obtained from three-dimensional ^{15}N , simultaneous $^{15}\text{N}/^{13}\text{C}$ - and ^{13}C -edited NOESY spectra⁷, and classified into four categories: 1.8–2.7 Å (2.9 Å for amides), 1.8–3.3 Å (3.5 Å for amides), 1.8–5.0 Å and 1.8–6.0 Å, based on peak intensities in spectra recorded with mixing times of 50 or 60 ms. A further 98 restraints were included for 49 slowly exchanging backbone amides (1.5–2.8 Å (H–O) and 2.4–3.5 Å (N–O)). A total of 110 ϕ (± 30 – 50°) and 52 ψ ($\pm 50^\circ$ for helix, $\pm 90^\circ$ for sheets) torsional restraints were based on $^3J_{\text{HN-H}\alpha}$ coupling constants measured in an HNHA experiment²⁴, and on the correlation between $\text{C}\alpha$ and $\text{C}\beta$ chemical shifts and backbone torsion angles²⁵, respectively. At the final stages of the calculations, 30 χ^1 restraints ($\pm 30^\circ$) were added for Val, Ile, Thr, Leu and aromatic residues based on intra-residue and sequential NOEs²⁶. For the final ensemble of 20 structures, the r.m.s.d. values from experimental restraints are: NOE, 0.017 ± 0.001 Å; torsional, $0.22 \pm 0.07^\circ$. No structures showed distance violations greater than 0.3 Å or dihedral violations greater than 5° . X-PLOR energies are (kcal mol^{-1}) E_{NOE} , 30 ± 5 (force constant, $50 \text{ kcal mol}^{-1} \text{Å}^{-2}$); E_{cdih} , 0.6 ± 0.4 (force constant, $200 \text{ kcal mol}^{-1} \text{rad}^{-2}$); E_{total} , 349 ± 14 ; E_{repeb} , 63 ± 5 . A quartic repulsion potential (force constant, $4 \text{ kcal mol}^{-1} \text{Å}^{-4}$) was used with atomic radii set to 0.80 times their actual values. R.m.s.d. values from ideal geometries are: bonds, 0.00 ± 0.00 Å; angles, $0.56 \pm 0.01^\circ$; impropers, $0.38 \pm 0.01^\circ$.

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Correspondence and requests for materials should be addressed to M.K.R. (e-mail: rosen@mrnmrl.ski.mskcc.org). Coordinates of the minimized average GDIA59 structure and of the final ensemble of 20 structures have been deposited in the Brookhaven Protein Data Bank (accession numbers 1gdf and 1ajw, respectively).

Synergistic activation of transcription by CBP and p53

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The tumour suppressor p53 is a transcriptional regulator whose ability to inhibit cell growth is dependent upon its transactivation function^{1–3}. Here we demonstrate that the transcription factor CBP, which is also implicated in cell proliferation and differentiation^{4–14}, acts as a p53 coactivator and potentiates its transcriptional activity. The amino-terminal activation domain of p53 interacts with the carboxy-terminal portion of the CBP protein both *in vitro* and *in vivo*. In transfected SaoS-2 cells, CBP potentiates activation of the *mdm-2* gene by p53 and, reciprocally, p53 potentiates activation of a Gal4-responsive target gene by a Gal4(1–147)–CBP(1678–2441) fusion protein. A double point mutation that destroys the transactivation function of p53 also abolishes its binding to CBP and its synergistic function with CBP. The ability of p53 to interact physically and functionally with a co-activator (CBP) that has histone acetyltransferase activity^{15,16} and with components (TAFs)^{17,18} of the general transcription machinery indicates that it may have different functions in a multistep activation pathway.

CBP functions as a transcriptional coactivator through interactions with a number of cellular activators^{4–14} and, possibly, with components of the basal transcription machinery⁶. CBP has at least three domains that bind transcription factors: a region containing residues 451–662 (designated CBP1) which is required for interactions with CREB (refs 6, 7), c-Jun (ref. 7) and c-Myb (ref. 11); a region containing residues 1,680–1,891 (designated CBP2) which is essential for interactions with E1a (refs 8, 9), c-Fos (ref. 10) and TFIIB (ref. 6); and a carboxy-terminal region containing residues 1,990–2,441 (designated CBP3) which is important for interactions with SRC-1 (ref. 12) and Jun-B (ref. 14) and which contains a

Figure 1 p53 association with CBP. **a**, Schematic representations of CBP and its functional/protein interaction domains, the hybrid activator Gal4–CBP(1678–2441), and the GST–CBP fusion proteins used for affinity chromatography. C/H: cysteine/histidine-rich region. **b**, Affinity purification of p53 on immobilized GST–CBP3. Western blot analysis with anti-p53 (antibody DO-1) of input HeLa nuclear extract (0.5% of 4 mg total protein) and the total 1 M KCl eluates from the indicated GST–CBP affinity columns. **c**, **d**, Transactivation domain of p53 interacts with CBP and p300, respectively. Western blot analysis with anti-CBP3 (**c**) or anti-p300 (**d**) of input HeLa nuclear extract (5% of 1 mg total protein), and half of the total eluates from the GST, GST–p53 affinity columns and from the control column (the GST–p53 column without HeLa nuclear extract loading).

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