of apolipoproteins^{2,12–14}. However, our results show that the residues in the conserved acidic motif of LR5 are buried to participate in Ca²⁺ coordination, instead of being exposed on the surface of the domain (Fig. 4a). Although lipoprotein uptake by members of the LDLR family may involve an electrostatic component, perhaps through association of the lipoproteins with cell-surface proteoglycans¹⁵, the LR5 structure demonstrates that the primary role for the conserved acidic residues in LDL-A modules is structural. It has been noted previously that only lipid-associated apolipoproteins bind with high affinity to the LDLR¹⁶; the LR5 structure suggests an alternative model for LDLR function in which a hydrophobic concave face (Fig. 4b, c) provides a lipoprotein-binding surface. □

Methods

Protein crystallization. Protein was expressed, purified and refolded as described⁹. Protein stock solution contained 10 mg ml⁻¹ LR5 and 5 mM CaCl₂. Crystals of LR5 were grown by the hanging-drop method at 15 °C by equilibrating over a reservoir containing 2.1 M ammonium sulphate, adjusted to pH 5.0, and 25% sucrose. These crystals belong to the rhombohedral space group R3 ($a = b = c = 32.04 \text{ \AA}$, $\alpha = \beta = \gamma = 112.57^\circ$), with a single LR5 molecule in the asymmetric unit. Solvent content in the crystals is 28%.

Data collection, structure determination and structure refinement. Diffraction data were collected using a rotating anode source (Rigaku RU200) with an RAXIS IIC detector. All data were collected at -180 °C using an X-stream cryogenic crystal cooler (MSC). Crystals were mounted in a 20 μ m rayon fibre loop and flash-frozen in the gaseous nitrogen stream. Reflections were indexed and integrated with the program DENZO¹⁷ and were scaled using SCALEPACK¹⁷. Subsequent data manipulations were carried out using the CCP4 package¹⁸.

Heavy-atom derivatization was accomplished by collecting crystals into reservoir solution, adding solid heavy atom to 1 mM, and soaking overnight. Heavy-atom sites were located for each derivative by Patterson methods. Heavy-atom refinement and phasing were carried out with MLPHARE (CCP4). Density modification was done on the MIR map using the program DM¹⁹. The 3- $\text{\AA}$ density-modified map was displayed with the program O (ref. 20) for tracing of the polypeptide chain, using the NMR structures of rLB1 and rLB2 (refs 7, 8) as a guide. Phases from the initial polyalanine model were combined with MIR phases using SIGMAA weighting²¹; side chains were readily built into the phase-combined map. A set of reflections (~7%) were held aside before refinement to monitor a free R-factor²². The model was refined against the remaining data to 1.7 $\text{\AA}$ with the program XPLOR²³. B-factor refinement was done first, followed by simulated annealing. A bulk solvent correction was then applied and ordered waters were added.

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Crystal structure of a small G protein in complex with the GTPase-activating protein rhoGAP

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Small G proteins transduce signals from plasma-membrane receptors to control a wide range of cellular functions^{1,2}. These proteins are clustered into distinct families but all act as molecular switches, active in their GTP-bound form but inactive when GDP-bound. The Rho family of G proteins, which includes Cdc42Hs, activate effectors involved in the regulation of cytoskeleton formation, cell proliferation and the JNK signalling pathway^{3–9}. G proteins generally have a low intrinsic GTPase hydrolytic activity but there are family-specific groups of GTPase-activating proteins (GAPs) that enhance the rate of GTP hydrolysis by up to 10⁵ times^{10,11}. We report here the crystal structure of Cdc42Hs, with the non-hydrolysable GTP analogue GMPPNP, in complex with the GAP domain of p50rhoGAP at 2.7 $\text{\AA}$ resolution. In the complex Cdc42Hs interacts, mainly through its switch I and II regions, with a shallow pocket on rhoGAP which is lined with conserved residues. Arg 85 of rhoGAP interacts with the P-loop of Cdc42Hs, but from biochemical data and by analogy with the G-protein subunit G_{iα1} (ref. 12), we propose that it adopts a different conformation during the catalytic cycle which enables it to stabilize the transition state of the GTP-hydrolysis reaction.

Table 1 Summary of crystallographic analysis

Crystal resolution (Å)	R_{sym} (%)	Completeness (%) $N_{\text{total}}/N_{\text{unique}}$		α	β	γ	t1	t2	t3	c.c.*	$R_{\text{cyst}}^{\dagger}$
R3	7.7 (34.0)	91.4 (55.0)	GAP	14.73	85.23	212.7	0.802	0.058	0.709	38.1	38.8
15–3.2		18,552/9,250	Cdc42Hs	85.16	102.19	69.65	0.989	0.025	0.253		
P1	6.4 (24.7)	97.6 (96.5)	(1)	272.6	30.6	38.3	0.000	0.000	0.000		
25–2.7		246,060/49,357	(2)	239.4	91.3	310.5	0.416	0.088	0.505		
			(3)	178.5	59.4	221.2	0.505	0.582	0.087	42.1	37.2
Averaging and refinement statistics											
Resolution (Å)	Averaging c.c.*	Atoms	Solvent molecules	$R_{\text{crist}}(\%)^{\ddagger}$	$R_{\text{free}}(\%)^{\ddagger}$	R.m.s. ncs (Å) §	R.m.s. bond length (Å)	R.m.s. bond angle (deg)			
12–2.7	0.928	8,907	310	23.1	28.4	0.06	0.014	2.7			

Figures in brackets refer to the outermost resolution shell. $R_{\text{merge}} = \sum_i (|I_i| - \langle I \rangle) / \sum_i I_i$, where I_i is the observed intensity and $\langle I \rangle$ is the average intensity. α , β , γ , t1, t2 and t3 are the eulerian rotation angles and fractional translational shifts as defined in the AMORE package. (1), (2) and (3) refer to each of the three complexes present in the crystallographic asymmetric unit.

* c.c., Correlation coefficient; c.c. = $\sum (F_o - |F_c|)^2 / \sum (F_o^2 + |F_c|^2)$. $R_{\text{crist}} = \sum (|F_o| - |F_c|) / \sum (|F_o| + |F_c|)$ for all reflections, where F_o and F_c are the observed and calculated structure amplitudes respectively.

R_{free} is the same as R_{crist} , but calculated on the 5% of data excluded from refinement.

§ R.m.s. difference between non-crystallographic symmetry equivalents on all atoms.

The crystal structures of Ras and Rho proteins are similar^{13–16}, and of the 41 common residues, most are located around the guanine-nucleotide-binding site. It is likely, therefore, that the GTP-hydrolysis switch operates in the same way in both families. Sequence conservation within, but not between, the Rho and Ras families reflects the fact that they activate different targets. By contrast, there is no detectable tertiary structure or sequence similarity between the largely helical GAP domains that act on Rho family proteins and those that act on Ras proteins^{17,18}. The heterotrimeric G-protein family share structural and functional parallels with small G proteins. Members of this family, which include transducin and the closely related G-protein subunit $G_{i\alpha 1}$, are important in physiological responses to neurotransmitters, hormones and sensory stimuli^{12,19,20}. They too act as GTP-modulated molecular switches and contain a Ras-like GTPase domain. Heterotrimeric G proteins also possess, an extra, largely helical, domain that is important for enhancing their intrinsic GTPase rate, which is 10^2 fold faster than for Ras proteins. In addition, there are GAPs specific for heterotrimeric G proteins. The structure determination of RGS4, a $G_{i\alpha 1}$ -specific GAP, again reveals it to be both largely helical and of a different tertiary structure to either Ras- or Rho-specific GAPs²¹. It is intriguing that for the three different families of GAPs whose structures are known, all are mostly helical but share no sequence or tertiary structure similarity. They all appear to perform the same function, but it remains to be seen whether they share a related mechanism of action.

The crystal structure of the Cdc42Hs-GMPPNP/p50rhoGAP complex was solved by molecular replacement using crystallographic models for both the G protein¹⁶ and GAP domain¹⁸. The initial electron density maps at 2.7 Å were of good quality because of the availability of well refined models for both components, the fact that there were only limited rearrangements of the two starting models, and the presence of non-crystallographic symmetry. An example of the averaged electron density map where the model has not been included in the calculation of starting phases is shown in Fig. 2. The current resolution of 2.7 Å does not enable us to make detailed mechanistic proposals for GAP-enhanced GTPase activity, but it does allow us to identify the key residues and structures involved in the formation of the complex.

The structure of the complex shows that the Cdc42Hs contacts with rhoGAP are mainly mediated through its switch I and II regions and the P-loop (Fig. 1a). Together, these regions contact residues of rhoGAP that are primarily located in and around a

shallow pocket formed between the B and F helices, the A–Al loop and the F–G loop. As described previously, there is a preponderance of conserved residues associated with this rhoGAP pocket region¹⁸. The two proteins pack together so that the switch I and II regions of the Cdc42Hs slot into this pocket, making polar and apolar interactions with the rhoGAP domain (for ease of description, residues will be identified as belonging to Cdc42Hs and rhoGAP by using the subscripts 'c' and 'r' respectively). Contacts mediated through the switch II region include salt-bridging interactions between Asp 63_c and Lys 122_r, which is conserved in rhoGAP proteins, and Arg 126_r on the B-helix. Tyr 64_c hydrogen-bonds with both the side chain of Asn 220_r on the G-helix and the main-chain C=O of Val 197_r, whereas Leu 67_c packs against the F–G loop (Fig. 1b). Although structural data are only available for the GMPPNP form of a Rho protein¹⁶, it is interesting that the switch II region in Ras, including Tyr 64, has been implicated in conformational rearrangements following GTP hydrolysis^{22,23}. Such a conformational change in Cdc42Hs would disrupt many of the interactions with p50rhoGAP observed in this complex structure. In common with the structure of Rac1-GMPPNP, three residues (29–31) of the switch I region are not ordered in this complex. However, the remainder of this loop is well ordered and contributes significantly to the nonpolar interactions at the molecular interface (Fig. 1b). In particular, residues Val 36_c and Phe 37_c, towards the end of switch I, make hydrophobic packing interactions with residues in the F–G loop of rhoGAP. This probably accounts for the F–G loop being ordered in this complex, but not in crystals of p50rhoGAP alone. As residues in the F–G loop of the rhoGAPs are not conserved, it seems that these interactions contribute stabilization, rather than specificity, to complex formation.

We have previously argued that Arg 85_r is likely to be important in G-protein interaction¹⁸. In this complex, Arg 85_r makes hydrogen-bonding interactions with the main-chain carbonyl of the conserved Gly 12_c on the P-loop. However, the role of this residue in rhoGAP-activated hydrolysis of GTP may well be similar to the roles of arginines in transducin, $G_{i\alpha 1}$ and Ras/rasGAP. Transducin-GDP and $G_{i\alpha 1}$ -GDP both bind to AlF_4^- , which mimics the terminal phosphate of GTP. In contrast Ras-GDP can only form this so-called transition-state analogue in the presence of p120GAP or neurofibromin²⁴. This hypothesis is supported by both biochemical and structural data. Changes in fluorescence anisotropy have shown that rhoGAP binds tightly to rhoA-mantGDP (where mantGDP is fluorescently labelled 2'(3')-O-(N-methylanthraniloyl)GDP) in the presence, but not in

Figure 3 Comparison of the Cdc42Hs-GMPPNP/p50rhoGAP complex with the structure of transducin- α -GTP γ S. Here, Cdc42Hs is shown in blue, with rhoGAP in red and transducin- α in white. Transducin- α consists of two domains: one is structurally homologous to the small GTPase family and the other is a smaller α -helical domain unique to the heterotrimeric G-protein family. Least-squares overlap²⁸ of Cdc42Hs with the GTPase domain of transducin gives an r.m.s. fit of 1.7 Å for 122 structurally equivalent C α atoms. Arg 174 has been proposed to be involved in transition-state stabilization during GTP hydrolysis in transducin. Its C α position is shown in yellow and is positioned within a piece of extended structure corresponding to the effector or switch I region of Cdc42Hs (magenta). In contrast, Arg 85_r, which appears to fulfil a similar role in GTPase activation by p50rhoGAP, is situated on the A–Al loop on the opposing face of the triphosphate moiety of the nucleotide analogue.

the absence, of AlF_4^- . Furthermore, mutation of Arg 85_r to lysine abolishes the formation of the rhoGAP/rhoA-GDP- AlF_4^- complex and reduces the rhoGAP activation of GTP hydrolysis by a factor of $\sim 10^4$ (D. Graham, P. N. Lowe, K. R. and J. F. E., unpublished results). These data suggest that Arg 85_r interacts with the GDP- AlF_4^- transition state analogue. An alternative side-chain rotamer of Arg 85_r to that observed in the current model would enable this interaction to occur. In this context, when $G_{i\alpha 1}$ is complexed with GTP- γ S, Arg 178 is not well ordered but does interact with the transition state analogue in the complex with GDP- AlF_4^- . However, in the case of transducin, the equivalent residue Arg 174 is ordered in both types of guanine-nucleotide-analogue complex^{19,20}.

Figure 3 shows the Cdc42Hs-GMPPNP/rhoGAP complex and transducin-GDP- AlF_4^- structures in which the cores of the Cdc42Hs and Ras-like domain of transducin have been aligned. Two points emerge from this comparison. First, although the non-Ras-like domain of transducin and the GAP domain of the complex are both extensively helical, they have entirely different folds and occupy exclusively different positions with respect to the G-protein component. Second, the C α positions of Arg 174 of transducin and Arg 85 of rhoGAP, both of which apparently bind to the GDP- AlF_4^- transition-state analogue, are 14 Å apart. Nevertheless, the guanidinium moiety of Arg 174 is in contact with the nucleotide analogue in the crystal structure of the transducin-GDP- AlF_4^- complex, whereas in our GMPPNP complex, a simple side-chain rotation of Arg 85 would be sufficient to bring its guanidinium group into a similar position and fulfil the same biochemical function.

The structure of RGS4 bound to AlF_4^- -activated $G_{i\alpha 1}$ has been solved²¹ and shows that this GAP domain is composed of nine α -helices arranged into two subdomains. The RGS4 makes extensive contacts with the switch I, II and III regions of the Ras-like domain of $G_{i\alpha 1}$, but very limited contacts with the α -helical domain. Not surprisingly, the RGS4 occupies the same overall position as rhoGAP does in the superposition shown in Fig. 3. However, rhoGAP and RGS4 have quite different helical folds and RGS4 does not contribute catalytic residues for the GTP-hydrolysis reaction. Instead, it stabilizes the switch regions of $G_{i\alpha 1}$, thus enhancing the intrinsic GTP-hydrolysis reaction. Therefore, although rhoGAP and RGS4 both interact with their respective G proteins through the switch regions, the contacts are quite different in detail. It is as though rhoGAP combines the function of RGS4 and the $G_{i\alpha 1}$ helical domain in one protein.

It was previously uncertain whether GAP acts allosterically, or if it participates directly in catalysis. Our results suggest that Arg 85_r is important in transition-state stabilization, which is in keeping with similar roles ascribed to specific arginine residues in Ras/rasGAP

and heterotrimeric G proteins. Nonetheless, rhoGAP still enhances GTPase activity by 30-fold when this arginine is mutated to lysine. It is likely that this remaining activation is provided by rhoGAP stabilizing the switch I and II regions of the G protein in a manner similar to that suggested for RGS4 acting on $G_{i\alpha 1}$. A docking model has been suggested for Ras/p120rasGAP (ref. 17); if this model proves to be correct then a detailed comparison of these two systems will provide insight into the apparently similar catalytic chemistry achieved by these two different GAP structures. □

Methods

Crystallization. Cdc42Hs (fragment 1–184) was cloned via pBluescript (Stratagene) into the expression vector pGEX-4T1 and the protein purified by standard protocols²⁵, the p50rhoGAP (fragment 198–439) was expressed and purified as described previously¹⁰. Crystals were grown by vapour diffusion at 4°C from 20% PEG 4000, 100 mM sodium cacodylate, pH 5.9, 10 mM MgCl_2 , 3 mM DTT, 3 mM NaN_3 . Initial crystals belonged to space group R3 ($a = b = c = 78.2$ Å, $\alpha = \beta = \gamma = 90.1^\circ$), with one Cdc42Hs/rhoGAP complex in the crystallographic asymmetric unit. Better diffracting crystals were subsequently obtained from similar conditions which belonged to space group P1 with three complexes in the unit cell ($a = 78.0$ Å, $b = 78.1$ Å, $c = 78.3$ Å, $\alpha = 90.1^\circ$, $\beta = 90.2^\circ$, $\gamma = 90.3^\circ$).

Data collection and processing. Diffraction datasets were collected from single crystals at 100 K using an Oxford Cryosystems device. Crystals were prepared for flash cooling by immersion in mother liquor made up to 15% PEG 400. Native datasets for both crystals were collected on an Raxis II image plate mounted on a Rigaku RU200 generator. Datasets for the P1 crystal form were also collected at station 5.2R at Trieste and BM14 at ESRF but were subsequently superseded by a largely complete and highly redundant dataset collected at Daresbury 7.2 on a large MAR image detector (statistics for which are shown in Table 1). Data were processed with DENZO²⁶ and SCALEPACK²⁶; all subsequent calculations being performed with the CCP4²⁷ program suite.

Structure solution and refinement. Initial phases for the complex were calculated by molecular replacement. The starting models were the p50rhoGAP fragment structure (Brookhaven accession code 1rgp) and the Rac1 homologue of Cdc42 (accession code 1mh1). Using the AMoRe²⁷ package, rotation/translation parameters were calculated for the R3 dataset using all data between 15 and 3.2 Å. After rigid-body refinement, the p50rhoGAP molecular replacement produced a correlation coefficient of 0.26; the next highest peak was 0.18. The rhoGAP fragment was then fixed while translation function searches were carried out with the Rac1 model. The molecular replacement solution with both p50rhoGAP and Rac1 gave a correlation coefficient of 0.38, with the next highest peak being 0.26. The same unique solution was found if the molecular replacement was carried out in the reverse order: first determining and then fixing the parameters for the Rac1 model, followed by translation searches with the rhoGAP model. The two-component model for the complex (without

refinement) was then used for molecular replacement calculations against the P1 dataset. Three solutions were found, related by a local three-fold axis approximately aligned with the body-diagonal of the unit cell, producing a correlation coefficient of 0.42 (the next highest solution being 0.28). Electron density for parts of the complex missing from the starting model coordinates, including some residues at the C terminus and in the F–G loop of p50rhoGAP, were readily interpretable. Electron density maps were 3-fold averaged using the program DM²⁷. The subset of reflections flagged for omission from the refinement process were also omitted from DM phase refinement. Manual model adjustment was carried out using the O program²⁸; reciprocal space refinement was done with REFMAC²⁷ using DM-modified phases as additional restraints. Non-crystallographic positional and thermal restraints were applied in PROTEIN/REFMAC, with tight targets for both main and side-chains. Water molecules were built into peaks greater than 3.5 σ in averaged difference electron density maps using the program ARP²⁷ and retained only if they met strict B-factor (less than 1.5 greater than average for protein) and stereochemical requirements.

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corrections

A new chromosomal protein essential for mitotic spindle assembly

Jing-Ping Yeo, Frank Alderuccio & Ban-Hock Toh

Nature **367**, 288–291 (1994)

In this Letter, we identified a chromosomal antigen and its corresponding cDNA by using a human autoimmune serum. We named this 47K autoantigen RMSA1 (for regulator of mitotic spindle assembly 1). Tugendreich *et al.*¹ and Margalit *et al.*² observed that the open reading frame (ORF) we ascribed to the cDNA of RMSA1 contains two Alu repeat sequences. Although truncated Alu repeats have been identified in the ORFs of messenger RNAs, Tugendreich *et al.* suggested that our reported cDNA may be an expressed pseudogene or a cloning artefact. We have now repeated the experiments (but without J.-P.Y., who has left our laboratory) and find that the reported RMSA cDNA is indeed a cloning artefact and that the identity of the chromosomal protein, which we have confirmed reacts by immunofluorescence with the autoimmune serum, remains unknown. □

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RNA recognition and translational regulation by a homeodomain protein

Josh Dubnau & Gary Struhl

Nature **379**, 694–699 (1996)

In this Article, we presented genetic and biochemical evidence that Bicoid (Bcd) protein binds and regulates the translation of *caudal* (*cad*) messenger RNA through direct interactions between the Bcd homeodomain and a discrete target sequence in the 3' UTR of *cad* mRNA, the Bcd response element (BRE). Although our main findings have been verified by additional experiments (see page 634 in this issue)¹, we cannot reproduce a subset of the results under the conditions initially reported.

The results we cannot repeat are: (1) the ultraviolet crosslinking and band-shift experiments which provided evidence for sequence-specific interactions between the Bcd homeodomain and the BRE (Figs 4c and 5); and (2) a bicistronic transcript experiment (Fig. 6) which provided evidence that the BRE mediates Bcd-dependent regulation of *Cad* at the level of translational initiation. However, biochemical evidence that the Bcd homeodomain can bind the BRE in a sequence-specific fashion has now been obtained in independent experiments using different binding conditions¹. In addition, we have successfully repeated the key genetic experiments indicating that Bcd regulates *Cad* expression in a way that depends on both the Bcd homeodomain and the *cad* BRE (see Figs 1, 2c i, v and vi, 2d vi (bcd⁺, bcd^{E1}), and 3 of our Article).

These errors do not alter our main interpretation that the homeodomain of Bcd mediates RNA recognition and translational regulation by the intact protein, but we apologize for any confusion they may have caused. □

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