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Structural Principles for the Multispecificity of Small GTP-Binding Proteins†

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ABSTRACT: The functional diversity of small GTP-binding proteins (G proteins) and their ability to function as molecular switches are based on their interactions with many different proteins. A wealth of structural data has revealed that their partners are often unrelated to each other in sequence and structure, but their binding sites are in general overlapping, notably at the so-called switch regions, whose conformation is sensitive to the nature of the bound nucleotide. We termed "multispecificity" this unique property of G proteins and investigated its structural principles by a database-implemented comparison of their protein−protein interfaces. Multispecific residues were found to be distributed throughout the G protein surface, with the highest multiplicity at the switch regions, each engaging interactions with 50−80% of the bound partners. Remarkably, residues involved in multiple interactions do not define consensus binding sites where all partners have convergent interactions. Rather, they adapt to multiple stereochemical and structural environments by combining the composite nature of amino acids with structural plasticity. We propose that not only the nucleotide switch but also multispecificity is the hallmark of the G protein module. Thus, G proteins are representative of highly connected proteins located at nodes of protein interactomes, probably the best structurally characterized member of this emerging class of proteins to date. This central functional property is also their Achilles' heal, facilitating their hijacking by pathogens, but may constitute an unexplored advantage in designing or screening novel therapeutic molecules.

Cyclic binding, hydrolysis, and release of guanine nucleotides are hallmarks of small GTP-binding proteins of the Ras superfamily (termed G proteins hereafter) by which they implement switch-type functions in processes as essential as the cell cycle, the dynamics of the cytoskeleton, and cellular traffic (reviewed in ref *1*). The spatio-temporal regulation of their GDP/GTP cycle and their activity in transmitting signals require different sets of partners. Schematically, these include guanine nucleotide dissociation inhibitors (GDIs)[1] and other partners of the GDP-bound form, guanine nucleotide exchange factors (GEFs) which stimulate the exchange of GDP for GTP through their interaction with the nucleotide-free form, effectors which bind to the GTP-bound form and receive action signals, and GTPase activating proteins (GAPs) which stimulate the hydrolysis of GTP to

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[1] Abbreviations: GTP, guanosine triphosphate; GDP, guanosine diphosphate; GEF, guanine nucleotide exchange factor; GDI, guanine nucleotide dissociation inhibitor; GAP, GTPase activating protein; Hs, *Homo sapiens*; Mm, *Mus musculus*; Cf, *Canis familiaris*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; St, *Salmonella typhimurium*; Pa, *Pseudomonas aeruginosa*; GNP, 5′-guanosyl imidodiphosphate; GSP, 5′-guanosine diphosphate monothiophosphate; BFA, Brefeldin A; RBD, Ras-binding domain; RanBD, Ran-binding domain; CRIB, Cdc42/Rac interactive binding.

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Table 1: Crystal Structures Used in This Study[a]

G protein	partner	partner type	nucleotide	resolution (Å)	PDB	ref
H-Ras	Hs-SOS1	GEF (cofactor site)	GTP	2.2	1NVU	*26*
	Hs-SOS1	GEF (active site)	none	2.2	1NVU	*26*
	Hs-PI3 kinase γ	effector (RBD)	GNP	3.0	1HE8	*27*
	Hs-RalGDS	effector (RBD)	GTP	3.4	*b*	*28*
	Hs-p120GAP	GAP	GDP·AlF_3	2.5	1WQ1	*29*
Ran	Cf-NTF2	GDP-specific partner	GDP	2.5	1A2K	*30*
	Hs-RCC1	GEF	none	1.8	1I2M	*15*
	Hs-karyopherin β2	effector	GNP	3.0	1QBK	*31*
	Hs-importin β	effector	GNP	2.3	1IBR	*32*
	Hs-RanBP2	effector (RanBD)	GNP	3.0	1RRP	*33*
	Hs-RanBP1	effector/GAP cofactor (RanBD)	GNP	2.7	1K5D	*34*
	Sp-RNA1	GAP	GNP	2.7	1K5D	*34*
Arf1	Hs-ARNO-BFA	GEF and inhibitor	GDP	1.9	1R8Q	*11*
	Sc-Gea2	GEF	none	2.8	*b*	*12*
	Hs-GGA1	effector	GTP	1.6	1J2J	*35*
Rac1	Hs-RhoGDI1	GDI	GDP	2.7	1HH4	*17*
	Mm-Tiam	GEF	none	2.8	1FOE	*36*
	Hs-P67−Phox	effector	GTP	2.4	1E96	*37*
	Hs-Arfaptin2	effector	GNP	2.6	1I4T	*38*
	St-Sptp	bacterial GAP	GDP·AlF_3	2.3	1G4U	*23*
	Pa-Exos	bacterial GAP	GDP·AlF_3	2.0	1HE1	*24*
Cdc42	Hs-RhoGDI1	GDI	GDP	2.6	1DOA	*18*
	Mm-Dbs	GEF	none	2.4	1KZ7	*39*
	St-SopE	bacterial GEF	none	2.3	1GZS	*40*
	Mm-Par6	effector (CRIB)	GNP	2.1	1NF3	*41*
	Hs-Cdc42GAP	GAP	GDP·AlF_3	2.1	1GRN	*42*
RhoA	Mm-Dbs	GEF	none	2.8	1LB1	*43*
	Hs-PKN	effector	GSP	2.2	1CXZ	*5*
	Hs-P50-RhoGAP	GAP	GDP·AlF_4	1.6	1TX4	*44*

[a] The first two letters in the partner name indicate the species. The resolution is that of the crystallographic structure. The nucleotide is the type bound to the G protein. [b] Not deposited in the Protein Data Bank; gift from the authors.

GDP. Evidence that in many cases regulators and effectors outnumber their G protein targets and that an individual G protein has often more than one regulator or effector in each set is accumulating. Thus, G proteins are able to interact with many partners in the course of their function, in many cases unrelated to each other.

Extensive structural studies over the past decade have shown how G proteins recognize their partners. Notably, two segments called switch 1 and 2 make a critical contribution. They recognize the nature of the bound nucleotide via invariant residues that bind to the γ-phosphate of GTP, they change their conformation accordingly, and they signal the nucleotide status to G protein partners by their direct involvement in protein−protein interactions, with almost no exception to this rule (reviewed in ref *2*). In many cases, the interface between G proteins and their partners also comprises residues outside the switch regions such that, if all complexes are taken together, the whole surface of the G protein module is susceptible to the formation of interactions (reviewed in ref *3*). However, although a crude clustering could be established on the basis of which switch region was more central to the interfaces (*3*), the modes of interaction of G proteins display a variety of patterns. Crystallographic, NMR, and other spectroscopic studies show that the flexibility of their switch regions makes a major contribution to the adaptability of G proteins to their various partners, including disorder-to-order transitions upon binding to their partners, and structural polymorphism in their different complexes and in the unbound forms (reviewed in ref *4*).

Remarkably, the binding sites of regulators and effectors for an individual G protein are largely overlapping, the two switch regions playing a central role in this process in most, if not all, cases. We call "multispecificity" this property of G proteins, and analyze here its fundamental structural basis at the atomic level. Taking advantage of a wealth of structural information, we have developed a web-interfaced database that allows the simultaneous comparison of multiple protein−protein interfaces. This was applied to the Ras, Ran, Arf, and Rho/Rac/Cdc42 G proteins, for which, besides their unbound structures, complexes have been determined with up to seven different partners. This allowed us to quantify the multiplicity of the interactions of amino acids inside and outside the switch regions and to characterize the physicochemical nature of their multiple interactions and the contribution of structural plasticity.

MATERIALS AND METHODS

Data Included in the Survey. We selected 27 crystal structures of binary or ternary complexes of G proteins of medium to high resolution (Table 1 and Figure 1A). All G proteins are from human sequences, with in some cases point mutations and/or truncation at the C- or N-terminus. In the two ternary complexes (Ran-GNP−RanBP1−RanGAP and Ras-GTP−SOS−Ras), each subcomplex was considered individually. For a given G protein, two to five partners were unrelated, while homologous but nonidentical partners were retained for Ran (RanBP1 and RanBD1, and karyopherin and importin), Ras (RalGDS and PI3 kinase), Rac (ExoS and SPTP), and Arf (BFA-inhibited and nucleotide-free

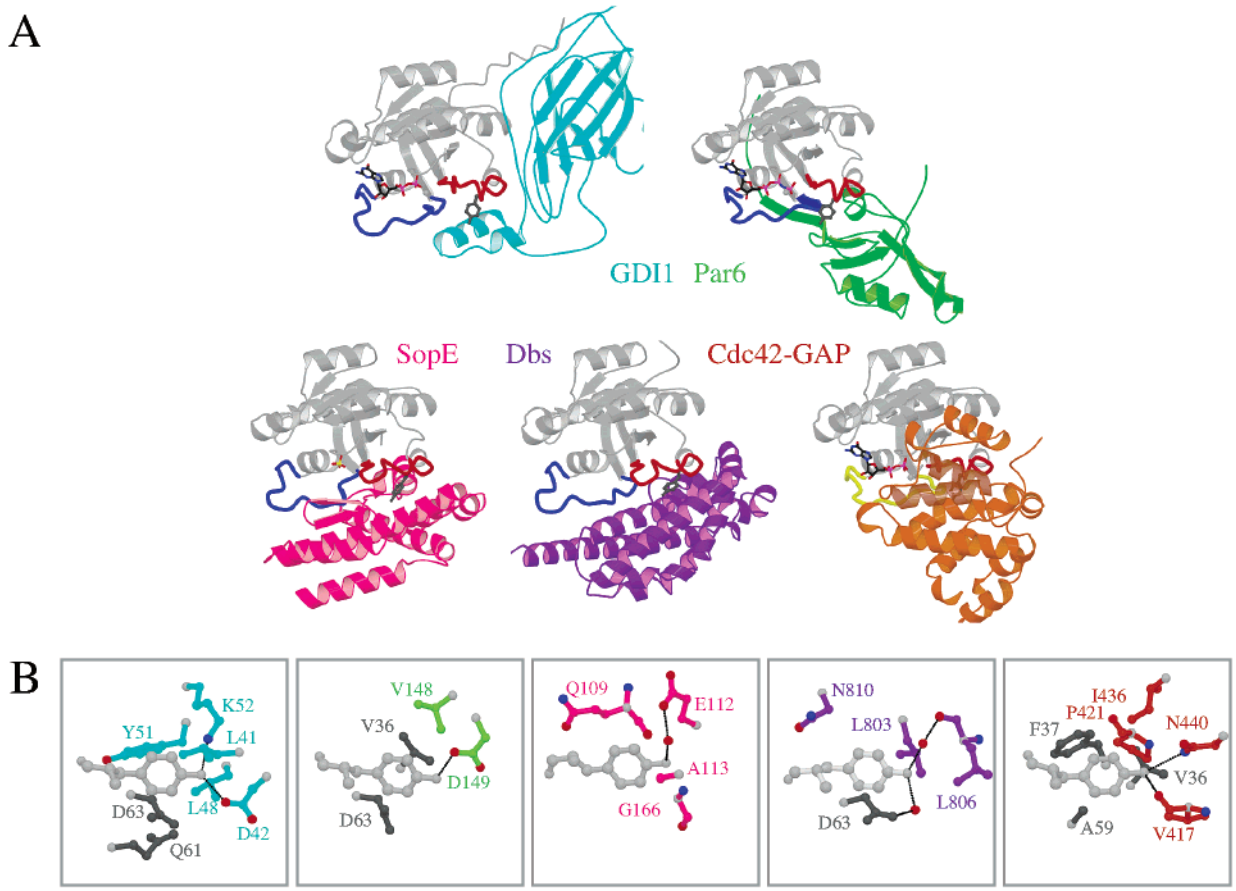

FIGURE 1: Multispecificity of Cdc42. (A) Cdc42 (in gray) is in the same orientation in the five complexes (see Table 1). Switch 1 is shown in blue, switch 2 in red, and the bound nucleotide as a ball-and-stick model. The side chain of Tyr84 is shown in black. (B) Close-up view of the 4 Å interaction sphere of Tyr64 in the complexes from panel A. The five views are selected to show Tyr64 with the same orientation. Residues from Cdc42 forming intramolecular interactions are shown in dark gray and marked with an asterisk. Residues from the partners are color-coded as in panel A. Water molecules are shown as red spheres. Cα atoms are shown in light gray, oxygens in red, and nitrogens in blue. Hydrogen bonds are represented with dotted lines.

complexes with GEFs). In addition, Rac, Cdc42, and Rho shared related GDIs and cellular GEFs and GAPs, while their effectors were unrelated. In the case of PKN, an effector of Rho, two potential interfaces equal in surface area were identified in the crystal (*5*), of which we selected the one generated by crystal symmetry. Finally, we did not include NMR studies, because very large discrepancies with X-ray data at the G protein core compromised a detailed pairwise comparison.

Parameters of the Analysis. To perform the simultaneous comparison of multiple interfaces, we devised a MySql database to store and analyze the chemical and structural characteristics of individual interfaces computed from their three-dimensional coordinates from the Protein Data Bank. Computations were carried out with the CCP4 program suite (*6*) or by ad-hoc algorithms interfaced with the database. These include a computation of interatomic contacts (maximal distance set to 4 Å), indirect contacts mediated by water molecules or ions, dihedral angles at contact residues, and their accessible surfaces. Comparative analysis of the stereochemistry and conformation of multispecific residues was performed through a web-based interface, either for the complexes of an individual G protein or collectively for all G protein complexes. Cutoffs were set to 4 Å for hydrophobic interactions, 3.5 Å for van der Waals contacts, 3.4 Å for polar interactions, and 3 Å for salt bridges. Additional graphical analysis was carried out on protein complexes superimposed on the G protein core using TURBO (*7*).

RESULTS

Quantitative Description of Multispecificity. We defined multispecificity as the ability of a residue to form interatomic interactions in at least two complexes, and counted the multiplicity for this residue as the number of complexes in which this residue was in contact. We found that highest multiplicities ranged from 3 to 5, a figure that is a minimum estimate given that none of the G proteins in our survey has been crystallized with all of its partners, and some partners were crystallized as fragments and may lack complementary interactions. A total of 185 of 365 interacting residues (51%) are involved in multiple contacts, with the distribution shown in Figure 2A. Switch regions 1 and 2 are multispecific in all the systems that have been studied, with an average multiplicity that is significantly higher than in nonswitch regions (Figure 2C). This is also the case for the unique C-terminal switch of Ran. The distribution on either switch is different for each G protein subfamily, some using one switch preferentially (switch 1 for Ras for instance) and others having bimodal interactions. We do not exclude, however, the possibility that this results from the incomplete coverage of protein−protein interactions by the available crystal structures. Surprisingly, multispecific residues are also

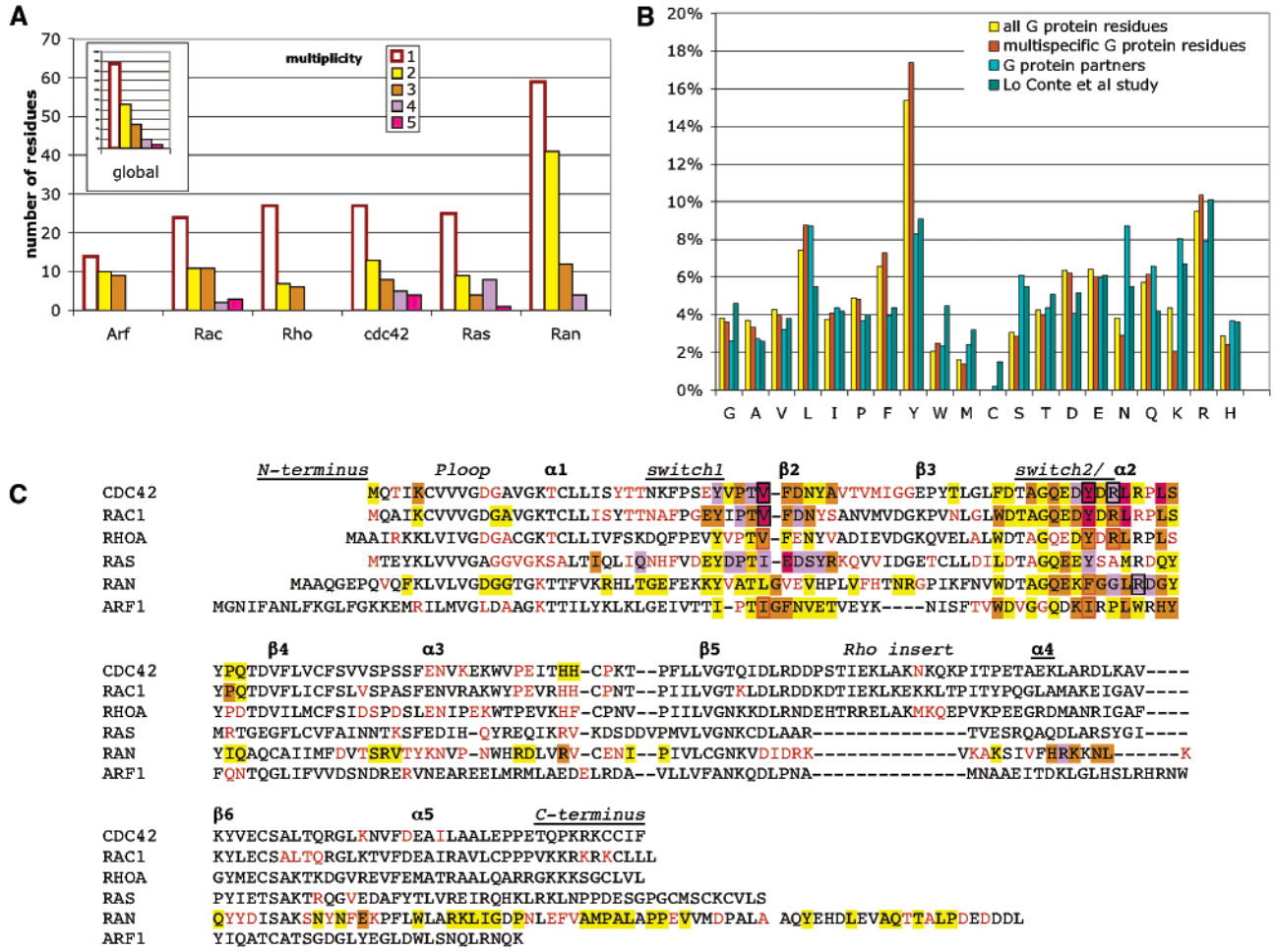

FIGURE 2: Distribution and stereochemistry of multispecific interactions. (A) Multiplicity histogram for individual G proteins. The cumulative histogram is shown in the inset. Color coding is as follows: red box for 1, yellow for 2, orange for 3, mauve for 4, and magenta for 5. (B) Distribution of contacts per amino acid type. Residues from our survey found in several contacts are counted only once. Color coding is as follows: yellow for all contacts on the G protein side, orange for multispecific contacts on the G protein side, cyan for contacts on the partner side, and green for statistics from general protein−protein complexes, taken from ref *14*. (C) Sequence alignment showing multispecific residues and their multiplicities. Residues with contacts in one complex are shown in red, and multiple contacts are boxed using the same color code as in panel A. The sequence alignment is adjusted to reflect the three-dimensional structures of the GTP-bound forms. The top line indicates regions names (in italics) and secondary structure numbers (bold letters). Regions that have poor structural alignment are underlined. Residues shown in Figures 1 and 3 are boxed in black.

found in various places outside the switch, with "hot spots" located at β1, the α1−switch 1 loop, the β2−β3 interswitch (these three regions forming an extended patch of surface near switch 1), and helix α3 near switch 2 (Figure 2C). Ran departs most from the other G proteins studied here in this regard, with a large number of multispecific residues but a low multiplicity average, coupled with an almost entire coverage of its surface with protein−protein interactions. This possibly reflects the fact that it is the sole G protein in our survey which is acting in the soluble phase, thus vacating a part of its surface that is shielded by the proximity of the membrane in the other G proteins. We note that multispecific residues can also be unbound at the protein surface in some complexes, indicating that although they form preferable binding sites, they can also tolerate a lack of interactions.

Stereochemistry of Multiple Interactions. Multispecific residues with a multiplicity of >3 were then compared at the atomic level according to the following criteria: side chain versus main chain contributions, the nature of the interatomic interactions, and the nature of interacting residues. Remarkably, we find that according to each criterion, multispecific residues tend to explore their entire potential of interactions. Contributions of the main chain are, however, less abundant, except in the cases where the central β-sheet of the G protein extends into the β-sheet of the partner (Ras−PI3kinase, Ras−RalGDS, and Rac−Par6). Side chains from the G protein amino acids contribute the essential part, interacting with either main chains or side chains of the interacting partner. Bridging water molecules are also found in a significant number of cases (see Dbs and SopE in Figure 1B). Hydrophobic, van der Waals, polar, and charged interactions, according to the nature of the amino acid, are used in various combinations, not all possibilities being used in a particular complex as illustrated in Figure 1B. Remarkably, the spatial distribution and the nature of the residues in the interacting sphere were heterogeneous and barely overlapping in the multiple contacts (Figures 1B and 3). Residues with high multiplicity tend to have the more closely packed interactions within the 4 Å sphere used here, with two to four residues in contact in each complex, and a total of as many as 12 different types of amino acids in contact (for instance, Tyr64 in Figure 1 is in contact with Gly, Ala, Val, Leu, Ile, Tyr, Asn, Asp, Glu, Gln, Lys, and Pro). In

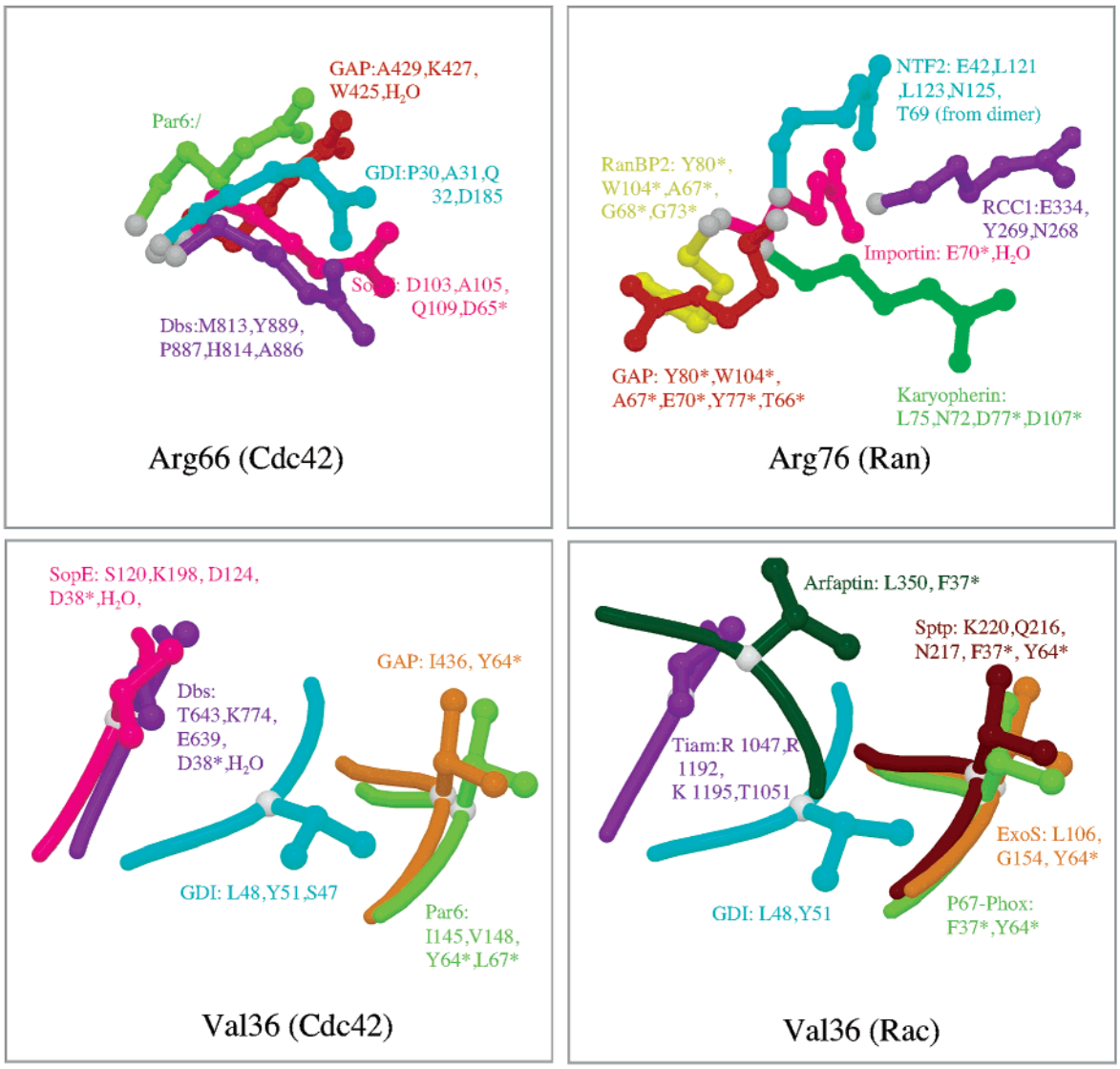

FIGURE 3: Interaction sphere and conformational space of multispecific residues. Color coding for Cdc42 is the same as that in Figure 1. Contact residues labeled with an asterisk are from the G protein. Orientations are obtained by superposition of all complexes on the G protein cores. Only the side chain (Arg66 in Cdc42 and Arg76 in Ran) or the side chain with the Cα trace (Val36 in Cdc42 and Rac) is shown for clarity. Cα atoms are shown in light gray.

many cases, intramolecular interactions contributed to the interaction sphere and were themselves subject to structural variations (for instance, compare GDI and GAP in Figure 1B). Similar patterns could be recognized locally between complexes taken by pair (for instance, Gln109 in Dbs and Asn810 in SopE in Figure 1B), but only a small number of multispecific residues formed similar interactions in all their complexes. Thus, multispecific residues adapt to stereochemical environments of various natures rather than define a consensus site where different partners make similar interactions.

Plasticity of Multispecific Residues. Structural polymorphism at the switch regions was analyzed as a major contributor to the ability of G proteins to bind to various partners (*4*), and it also distinguishes related G proteins within a subfamily (*8*). Here we analyzed the conformational space occupied by multispecific residues with multiplicities of ≥3. Largest amplitude differences resulted from rigid body rearrangements at switch 2, or rearrangement of the loop from switch 1. Smaller differences included medium-range changes initiated one to two residues from the multispecific residue (for example, GDI and GAP complexes), and the frequent use of different side chain rotamers associated with small main chain movements (up to 1 Å). Remarkably, although the interaction of G proteins with their partners generally results in more ordered structures compared to their unbound structures, in a few cases this flexibility was maintained or even increased (for instance, switch 1 in GEF complexes). Typical volume occupancies for highly multispecific residues are illustrated in Figure 3. However, not all the positions of a given multispecific residue were found to be different. Some conformations, often corresponding to a particular class of partner, were sometimes clustered in subgroups, yet these similar conformations were not always associated with an identical interaction sphere from the bound partners (for example, Val36 in Figure 3). An explanation for the plasticity of the switch regions could be that they have an overall deficiency in intramolecular interactions in their unbound forms, which would potentiate their avidity for intermolecular interactions.

Conserved versus Unique Switch Residues. Switch sequences comprise consensus residues that bind guanine

nucleotides and the associated Mg^{2+}, conserved residues found in many subfamilies but not involved in nucleotide binding, and unique sequences that define subfamilies and their individual members (*9*). Each category was involved in multiple interactions, but highest multiplicities were mostly associated with conserved residues (for example, Tyr64 in Figure 1 and Val36 or Arg66 in Figure 3). Consensus and conserved residues involved in multispecific interactions frequently formed alternative intramolecular interactions with either other residues in the G protein (Figures 1B and 3) or the guanine nucleotide (for example, Tyr32 in Ras, Cdc42, and Rac proteins). An interesting extension of the alternation between intramolecular and intermolecular interactions is found in Arf, where Phe51 is successively buried in the protein core in Arf-GDP (*10*), a target for a drug in the Arf-GDP−Brefeldin A−GEF complex (*11*), and interacts with the GEF in the nucleotide-free Arf−GEF complex (*12*). Conserved multispecific residues, however, contributed to different interactions for different families, as exemplified by Val36 in Figure 3. Thus, their conservation in the switch region, unlike that of nucleotide-binding residues, does not correspond to a consensus binding site.

Sequence and Structure of G Protein Partners. Our set of G proteins is too small to analyze the amino acid composition of the multispecific surfaces with statistical confidence. We note, however, that tyrosines in switches 1 and 2 make an important contribution, reminiscent of interfaces in antibodies (Figure 2B) (*13*). The diversity of their partners, on the other hand, allows such an analysis to be performed. We compared their cumulative amino acid composition to those computed by Lo Conte and co-workers on a set of protein−protein complexes from various families (*14*) (Figure 2B). We did not observe a significantly different distribution, except for the somewhat more frequent occurrence of leucines, glutamines, and asparagines. Thus, multispecificity encoded by G proteins does not select for a biased amino acid composition on the surface of their partners. The secondary structures of the G protein partners were also surprisingly diverse. The example of Cdc42 in Figure 1A, which is representative of the other G proteins in our survey, shows that the multispecific surface can accommodate multiple combinations of α-helices, loops, and β-strands in various orientations. Comparison of the different G proteins in our survey also shows that the ensemble of recognized secondary structures varies from one G protein to the other. Thus, the fold of partners in the multispecific interface is not unequivocally defined by the G protein. Finally, the few cases in which a G protein partner has been crystallized both alone and in complex suggest that structural flexibility can, although not necessarily, assist their interaction with the G protein. Conformational changes include small local changes as in RCC1 (*15*), moderate domain motion as in Sec7 domains (*11*), and large disorder-to-order transitions as in GDIs (*16*−*18*).

DISCUSSION

A remarkable property of G proteins is their ability to perform interactions with multiple partners that can be at the same time overlapping and highly specific such that a single amino acid change can invert individual specificities (*19*). To define the fundamental structural basis for this unique property, which we call multispecificity, we performed a quantitative comparison of the stereochemical and conformational characteristics of the protein−protein interfaces in the crystal structures of individual G proteins in complex with different partners. We find that both switch and nonswitch regions contribute residues that form interatomic interactions in more than one complex. However, in the switch regions, the multiplicity of individual residues reaches very high values, with some residues participating with almost all complexes. Comparison of the interaction spheres of highly multispecific residues reveals that they are extremely versatile in the nature of their partner residues and the structures that carry them. Detailed atomic analysis shows that residues from different partners do not build up, in general, a conserved stereochemical environment, ruling out the possibility that the switch regions define "consensus" sites in their partners such as those reported for immunoglobulins (*20*). G proteins and their switch regions are thus tailored to adapt to stereochemical and conformational environments that are both specific and multiple. Therefore, we propose that both the nucleotide switch and multispecificity form the hallmarks of the small G protein structural module. From the experimental point of view, a critical consequence of multispecificity is that engineered mutations in the switch regions are likely to impair more than one functional interaction, and must be interpreted in this context. Overlapping interactions at the multispecific sites also address the issue of the spatial alternation between effectors and regulators that are mutually exclusive. It is possible that the bimodal switch region allows some partners to bind in tandem, possibly using their own modular and/or flexible structures to facilitate the transition from one partner to the other.

On the basis of its biological and structural multispecificity, the G protein module is thus a candidate to be a member of the emerging families of highly connected proteins located at nodes of protein interactomes (*21*, *22*), and probably the best-characterized at the structural level. This is in line with theoretical analysis of interactome network topologies, which predict that such proteins should be more essential to organisms than less connected ones while at the same time introducing fragility in the network (*21*, *22*). Our study allows us to analyze how multispecific interactions are achieved at the atomic level, and whether they require a special structure to bind many different proteins as suggested (*21*). We found that multispecific interfaces take advantage, on one hand, of the composite chemical nature of amino acids, which allows them to form polar interactions at the main chain and various combinations of charged, polar, van der Waals, and hydrophobic interactions at their side chains. Additional interactions can also be mediated by buried water molecules filling up cavities at the protein−protein interfaces, and by intramolecular interactions that assist and/or alternate with intermolecular interactions. On the other hand, structural changes of various amplitudes rearrange the relative amino acid positions in various combinations, thus yielding distinct binding landscapes. Altogether, the characteristics of the multispecific interfaces appear, however, to use general properties of protein structures and surfaces and do not depart significantly from those of other protein−protein interfaces. This raises the possibility that protein surfaces may be intrinsically more permissive to multiple interactions than has been previously

appreciated. As a consequence, proteins with selective specificity may have evolved from the introduction of determinants that eliminate undesirable partners rather than solely improve individual specificity. In the case of G proteins, the above analysis predicts that multispecificity should have important consequences from a pathological point of view. On one hand, it should make G proteins vulnerable to pathogens, both because they function at critical nodes of cellular networks and because their adaptability to multiple interactions should facilitate the establishment of novel connections. Pathogens have indeed developed strategies for diverting G protein cellular functions for their own profit, for instance, by providing regulators unrelated in nature to the cellular regulators but still recognizing the proteins at the same site and competing with host partners as exemplified by two bacterial regulators and a fungal toxin in our survey (*11*, *23*−*25*). The adaptability of G proteins to multiple interactions, on the other hand, may also turn out to be an unappreciated advantage for therapeutic strategies aimed at the interruption of G protein functions in human diseases, as the screening or design of inhibitors should be facilitated by the multispecific nature of their protein−protein interfaces.

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