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Minireview

How does the switch II region of G-domains work?

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The transition of guanine nucleotide binding proteins between the 'on' (GTP-bound) and 'off' (GDP-bound) states has become a paradigm of molecular switching after a chemical reaction. The mechanism by which the switch signal is transmitted to the downstream recipients in the intracellular signal pathway has been extensively studied by biochemical, biophysical and genetic methods, but a clear picture of this process has yet to emerge. Based on the similarities of ras-p21 and elongation factor Tu we propose here a model of the GDP state of ras-p21 that is in agreement with all relevant experimental evidence. The model provides important clues about: (1) a possible molecular mechanism for signal transmission from the site of GTP hydrolysis to downstream effectors; (2) a major conformational change during signal generation and a key residue involved in this process (Tyr-64); and (3) regions in ras-p21 that can be differentially recognized by binding to external partners in a GTP/GDP state dependent fashion, most notably residues D69, Q70, R73, T74, R102, K104, D105 at the end of the α-helices 2 and 3.

Guanine nucleotide binding protein; ras-p21; Elongation factor Tu; GTP hydrolysis; ras-p21 downstream effector; Protein modelling

1. THE FUNCTION OF G-DOMAINS

Guanine nucleotide binding proteins are involved in many important cellular functions. All of these proteins appear to have in common a GTPase domain, or G-domain, of similar three-dimensional structure, such as that seen in the crystal structures of ras-p21 proto-oncogene proteins and of elongation factor Tu [1–3]. The close structural similarity in spite of very low sequence similarity between EF-Tu and ras-p21 suggests that most, if not all, G-domains have a common underlying molecular mechanism. For ras-p21 proteins, extensively studied by a variety of biochemical, genetic and spectroscopic techniques, GTP hydrolysis appears to take the protein from an active to an inactive state, as if a molecular 'switch' were at work. The question of how this apparently ubiquitous switch works in molecular detail has aroused considerable interest [2,4,5].

2. THE MOLECULAR SWITCH HYPOTHESIS

For ras-p21 proteins, the following view has emerged. The switch is 'on' in the GTP-bound state of the protein and 'off' in the GDP-bound state. Hydrolysis of GTP, i.e. the transition between the two states, is accelerated (and in a sense controlled) by the binding of an external effector, GAP (GTPase activating protein) or NF1 [6,7]. The reverse transition, from the GDP-bound 'off' state to the GTP-bound 'on' state, is very slow and needs to be catalyzed by another protein, a guanine nucleotide exchange factor. The state of the switch is probably sensed by a downstream agent, which has not yet been clearly identified. By varying the effective concentration, covalent state and conformation of the participating molecules, the switch can be controlled in ways not yet completely understood.

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3. TWO REGIONS ARE INVOLVED

How does the switch work at the molecular level? What is the conformational change as a result of GTP hydrolysis and how is this change transmitted to the affected proteins or complexes? Time-resolved structural studies in solution, and crystallographic and genetic evidence indicate that a cluster of polypeptide segments located at one end of the nucleotide binding pocket, near the γ-phosphate, is directly involved in the switch mechanism (for a review see [8]). These functionally important segments are the 'effector' or L_2-loop that follows the first β-strand in the domain, also called switch I; and loop L_4-helix α_2-loop L_5, that follows strand β_3, also called switch II (Fig. 1). Approximate residue limits in ras-p21 are 30–38 for switch I and 61–77 for switch II.

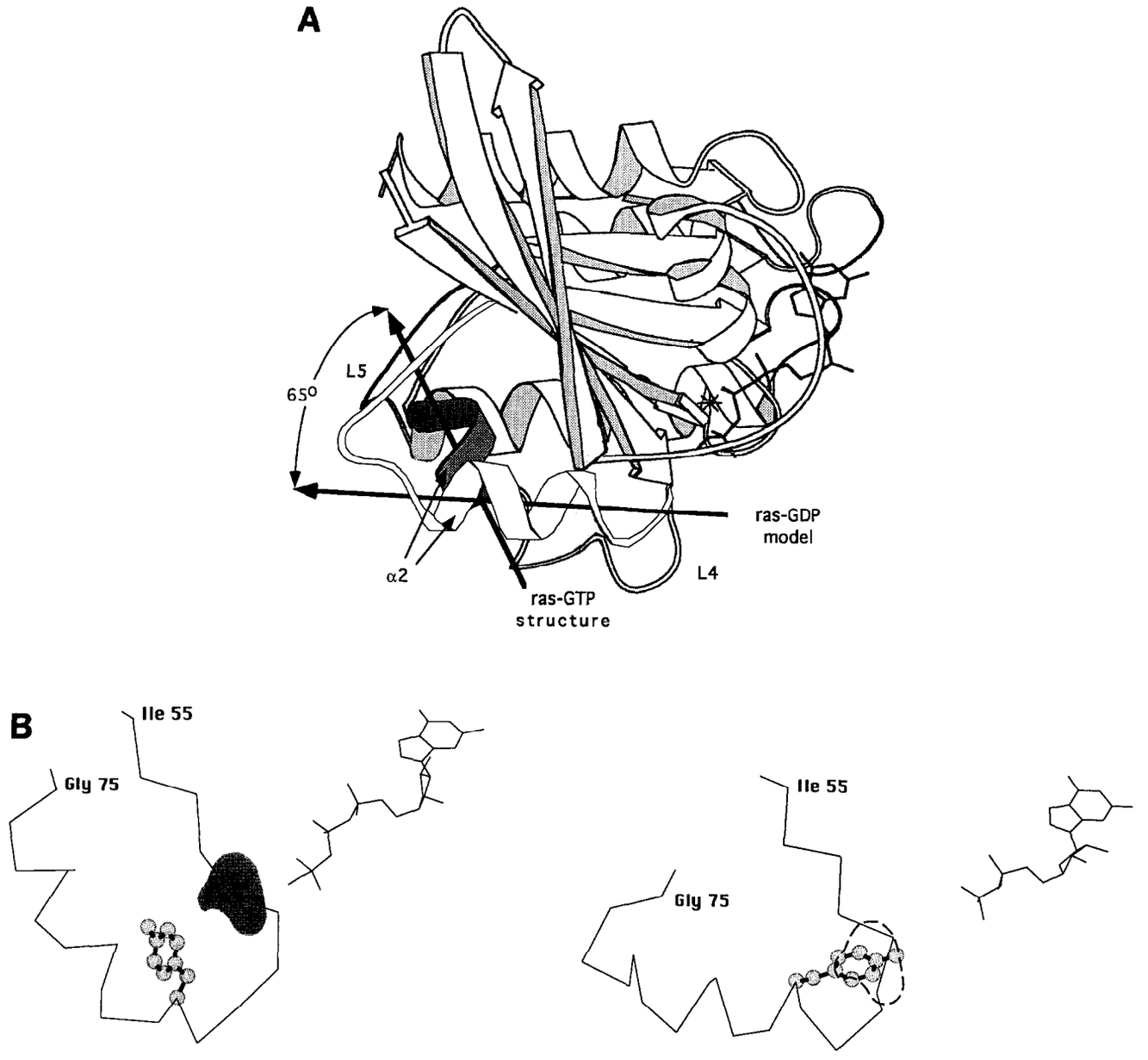

Fig. 1. The ras-p21:GTP crystal structure and the ras-p21:GDP model. (a) Ribbon plot of the ras-p21:GTP structure [1] with the model of the region L_4-α_2-L_5 in ras-p21:GDP. Helix α_2 is dark in the ras-p21:GTP structure and light in the ras-p21:GDP model. The helical axes in the structure and in the model and the angle between them are shown. Positions of GTP (stick representation) and the magnesium ion (asterisk) in the ras-p21:GTP structure are indicated. (b) Cα trace of the Y64 region in the ras-p21:GTP structure (left) and the ras-p21:GDP model (right), showing the cavity present in the ras-p21:GTP structure (grey in the structure, dashed outline in the model). GTP, GDP and the Y64 side chain are shown as well. The ras-p21:GDP L_4-α_2-L_5 region was modelled by replacing the entire L_4-α_2 backbone (residues 59–74) in the ras-p21:GTP structure [1] by its counterpart in the trypsinized version of the G-domain of EF-Tu:GDP [3]. In order to accommodate the bulky ras-p21 residues at the end of α_2 and to ensure that R73 is exposed the conformation of the stretch D69-T74 was taken from the original ras-p21:GTP structure. Helix α_2 makes different angles with respect to the rest of the protein in EF-Tu:GDP and ras-p21:GTP resulting in a gap between the end of α_2 and the beginning of β_4 in the ras-p21:GDP model. This gap was bridged by regularizing the backbone from residues 69 to 77. Reasonable initial positions for the side chains of residues 72–76 (C-terminal part of α_2 and L_5) were obtained in an iterative process of scanning through all κ-angle rotamers. This model building procedure was carried out using the WHAT IF program [13]. The resulting crude structure (including GDP in the GTP binding pocket) was further optimized using the GROMOS molecular simulation package [14]. The model passed several sensitive tests of 'normality,' based on analysis of packing, surface polarity, side chain torsion angles, and energy. The main energetic term that favours the model appears to be the internalization of Y64 which fills the cavity present in the ras-p21:GTP structure and makes additional hydrogen bonds.

4. SWITCH I REGION

In the switch I region the changes between the GTP- and GDP-bound forms are visible in the crystal structures of ras-p21. The transition mainly involves flips of some peptide units and reorientation of side chains [2,9]. Key residues are Thr-35 that coordinates the magnesium ion of the Mg-GTP complex and can thus sense the presence of the γ-phosphate; and Tyr-32 on the protein surface that drastically changes its side chain orientation and is therefore likely to interact with an effector. Indeed, the region 30–38 has been identified as a GAP binding region [10,11]. Unfortunately, it is impossible to compare this region of ras-p21 with the analogous one in EF-Tu, as the latter was proteolytically excised before crystallization.

5. SWITCH II REGION

In ras-p21, the switch II segment starts at the end of the functionally important 57-DTAGQE-62 consensus motif (D57 is coupled to GTP/GDP through interaction with the magnesium ion) and includes loops L_4 and L_5, and helix α_2. Its precise limits are a matter of definition, but it can be considered to start with Q61 (which is probably involved in catalysis) and to end with G77. There is considerable conformational flexibility in this region, especially in the GDP state [2,9]. Flexibility is particularly high in the first few residues following Q61, as measured by crystallographic B-factors.

In elongation factor Tu, so far only the GDP state is known crystallographically. The switch II region appears to play an important role in the function of EF-Tu. The exterior face of helix α_2 (along with that of α_3) is the main zone of contact between the G-domain and the third domain of EF-Tu, closing an approximate ring structure of three globular domains [3]. This region is not in contact with the bound nucleotide, yet contains several very conserved residues that appear to be important for specific interaction of the G-domain with the C-terminal globular domain, possibly involved in the switch transition. The principal difference between the ras-p21:GTP and EF-Tu:GDP structures is an apparent shift of the axis of helix α_2 [12].

6. MODEL OF THE GDP STATE OF ras-p21

The transition in the switch II region can, therefore, be understood by taking the high resolution structures of the ras-p21:GTP analogue complex as representing the 'on' state and that of the EF-Tu:GDP complex as representing the 'off' state. This is best done by building a detailed molecular model of the GDP state of ras-p21 assuming that the switch II region in ras-p21:GDP has a conformation similar to that observed in EF-Tu:GDP. After grafting loop L_4 and helix α_2 from EF-Tu:GDP [3] onto the framework of ras-p21:GMPPNP [1], small adjustments were made consistent with the criterion of proper stereochemistry and the model was refined using simulation techniques (for details see Fig. 1). Coordinates of the ras-p21:GDP model are available from the authors via anonymous FTP (internet address: ftp.embl-heidelberg.de, directory: /pub/databases/protein_extras/models).

7. CONFORMATIONAL CHANGES IN THE SWITCH II REGION

When comparing the switch II region in the GDP (model) state of ras-p21 with its GTP (crystal structure) counterpart, five major differences are observed.

(1) The backbone of A59 flips so that its Cβ atom occupies the position vacated by the γ-phosphate of GTP.

(2) Residues 63–66 (which form the C-terminal end of loop L_4 in the GTP state) rearrange into a helical conformation in the GDP state, thus extending helix α_2 by one turn at its N-terminal end. The extension is stabilized by a hydrogen bond between the side chain of E63 and the backbone NH of G60.

(3) The side chain of Y64 fits into a hydrophobic cavity (Fig. 1B) that is already present in the GTP state. This cavity is lined by residues from strand β_1 (V9), from the rearranged loop L_4 (58-TAGQ-61) and from helix α_3 (Y96). The Y64 side chain is further stabilized in its new position by several hydrogen bonds. In EF-Tu:GDP this tyrosine is in a very similar environment.

(4) The four C-terminal residues of helix α_2 (71–74) unravel, thus extending loop L_5. The necessary rearrangement of L_5 exploits the backbone flexibility at G75 and G77. In ras-p21:GTP the α_2-L_5 region is in contact with the end of α_3 and L_7. The conformational changes in the α_2-L_5 region induce also changes in α_3 and L_7.

(5) Apart from partly winding and unwinding, helix α_2 also rotates about its own helical axis and by approximately 65° about an axis perpendicular to this axis (Fig. 1A). The positional shift during the conformational change is smallest for D69 and Q70 and largest for E63, M72 and T74 (Cα displacements ranging from 2 to 7 Å).

These observations suggest the following balance of interactions: GTP hydrolysis causes loss of interactions between the leaving phosphate and L_4. This loss is compensated for by the transfer of the Y64 side chain into a cavity (where it engages in both hydrophobic and hydrophilic interactions) and N-terminal extension of helix α_2 (accompanied by reorientation of this helix). This model suggests that a signal is transmitted from the L_4 region (in contact with the phosphate) to the other side of the molecule (C-termini of α_2 and α_3 and following loops L_5 and L_7).

The changes described here are qualitatively different from those proposed by Jurnak et al. [4], who report a displacement (rather than rotation) of the helix and an N-terminal extension of the helix in the 'on' state (rather

than in the 'off' state). However, these earlier conclusions were derived from approximate (digitized from printed stereo views) and incomplete (missing trace for residues 61–65 in switch II) coordinates of ras-p21:GDP and GTP-analogue complexes. The model presented here is based on more accurate data, i.e. high resolution structures of EF-Tu:GDP and ras-p21:GTP analogue complexes. We agree with the earlier study in one aspect: switch II is inherently flexible and a crucial element in the switch transition. This was explained in detail by Milburn et al. [2], who analyzed several crystal structures of ras-p21 in the two structural states, showing that it is difficult to distinguish features that result from crystal packing from those due to the presence or absence of the γ-phosphate.

There is an apparent contradiction regarding the conformational flexibility of the switch II region. In ras-p21 crystallographic B-factors are high in this region and there is an ambiguity in the orientation of Gln-61. In comparison, the region is much less flexible in the EF-Tu crystal. The contradiction is resolved by the observation that domain 3 of EF-Tu makes a tight contact with the switch II region, locking it into place. We predict that a similar complex is formed with proteins that interact specifically with ras-p21. Such proteins would probably be structurally analogous to domain 3 of EF-Tu, a six-stranded anti-parallel β-barrel that in the crystal structure interacts strongly with the α_2-L_5/α_3-L_7 region [3].

8. DATA THAT SUPPORT THE ras-p21:GDP MODEL

Support for the model comes from the functional effect of mutations of the key residue Y64, which fits into a hydrophobic pocket in the proposed ras-p21:GDP state. Substitution of Y64 by polar residues eliminates GTP hydrolysis and the transforming activity of G12V oncogenic ras-p21 [15]. In contrast, hydrophobic substitutions are tolerated, with activities similar to the wild type protein. In the EF-Tu family the tyrosine at this position is completely conserved.

The GTP->GDP transition has also been probed by time resolved fluorescence measurements using Y64W mutants. A large increase in tryptophan fluorescence is observed accompanying GTP hydrolysis [16]. This is consistent with the fact that Y64 (and W64, by analogy) is completely shielded from solvent in the ras-p21:GDP model, while it is partially exposed to solvent in the GTP state.

Mutation of S65 (just before helix α_2 in the GTP state) to proline strongly suppresses GAP-stimulated GTP hydrolysis, while the S65A control mutant has little effect (A. Scheidig, P. Gideon and A. Wittinghofer, to be published). Most likely, N-terminal extension of helix α_2 is an important aspect of the switch transition, which is energetically less favourable in the S65P mutant, as proline cannot be accommodated in a helix without losing a hydrogen bond and without causing the helix to bend.

9. HOW IS THE STATE OF THE SWITCH SENSED AND THE SIGNAL TRANSMITTED?

The striking feature of the scenario presented here is the direct coupling of the departure of the γ-phosphate after GTP hydrolysis to the movement of the switch II region. In the transition, helix α_2 moves with respect to the rest of the protein, but residues on its exterior face remain exposed (E62, E63, S65, A66, D69, Q70, R73, T74 – Fig. 2). At the same time residues R102, K104, D105 at the end of helix α_3 become less exposed to solvent as a result of the rearrangement of the end of helix α_2. Proteins interacting specifically with these two sets of residues and the surrounding protein surface are able to sense the conformational change between the GTP and GDP complex.

What are these proteins? Specific and direct interaction with ras-p21 in the GTP state has been established for GAP (NF1, etc.), accelerating GTP hydrolysis. The switch II region probably is involved in this interaction as some mutations that affect the ras-p21-GAP interaction, e.g., in residues E62 and E63 [21], map to this region. For EF-Tu, the GTP-state specific partner is the ribosome and the tRNA; for receptor-coupled G-proteins, it is the effector.

The proven specific partners in the GDP state are the nucleotide dissociation inhibitors (GDI [22]). Specific interaction in the GDP state is also clearly established for the association of the α and $\beta\gamma$ subunits of trimeric G-proteins. For these proteins, GDP exchange is accompanied by release of the $\beta\gamma$ subunits.

Guanine nucleotide release proteins (GNRPs) also possibly recognize the switch state by binding to helices α_2 and α_3. Nucleotide exchange stimulated by GNRP is impaired by mutations in these regions (63, 71, and 73–75 in α_2-L_5 and 103–108 in α_3-L_7 [18–20]). GNRPs apparently are able to interact with both the GTP and GDP states. However, the interaction of GNRPs with the two states differs in detail. This was shown by Verrotti et al. [19], who found that mutations in positions 73–74 and 75 have a different effect on the nucleotide exchange rates in the two states.

How are the two states exploited functionally? The general view of the GTP-state as the only active state of G-domains is obsolete, as there are cases where the released $\beta\gamma$ subunits of trimeric G-proteins act as regulators of effector proteins [23], e.g. in pheromone signaling in yeast. By analogy, one arrives at the interesting possibility that ras-p21 passes on an active message through a protein that is inhibited by specific binding to the ras-p21:GDP state and becomes active after GDP exchange. Such a molecule has not yet been identified, but ras-p21 GDIs may well be such active messengers.

ras:GDP MODEL																					
Secondary structure	e	-	-	s	t	t	h	h	h	h	h	h	h	h	s	t	t	-	-	-	e
Accessibility	0	0	1	4	9	14	6	0	4	5	2	1	9	14	4	8	13	10	1	6	0
ras:GTP STRUCTURE																					
Secondary structure	e	e	-	s	s	s	s	-	s	g	g	g	h	h	h	h	h	h	-	s	e
Accessibility	0	0	0	1	9	11	13	3	8	8	9	1	4	15	1	0	12	8	0	7	0
Sequence position	57			60					65					70					75		77
ras	D	T	A	G	Q	E	E	Y	S	A	M	R	D	Q	Y	M	R	T	G	E	G
	*			*				*													*
EF-Tu	D	C	P	G	H	A	D	Y	V	K	N	M	I	T	G	A	A	Q	M	D	G
Sequence position	80					85					90					95					100
Secondary structure	e	-	s	s	h	h	h	h	h	h	h	h	h	s	s	t	t	-	-	s	e
Accessibility	1	0	10	2	4	6	2	0	4	5	0	2	10	8	5	3	4	13	2	7	0
EF-Tu:GDP STRUCTURE																					

Fig. 2. Sequence alignment of ras-p21 and EF-Tu in the region L_4-α_2-L_5, and secondary structure classification and solvent accessibility [17] of ras-p21:GDP (model), ras-p21:GTP (crystal structure [1], energy-minimized) and EF-Tu:GDP (crystal structure [3]). The ras-p21:GTP crystal structure was energy minimized prior to the accessibility calculation in order to allow for a meaningful comparison with the ras-p21:GDP model. An asterisk between the sequences indicates sequence identity. Solvent accessibilities are in 10 $Å^2$; secondary structure codes are e: β-strand; s and t: turn; h: α-helix; g: 3_{10}-helix.

This hypothesis could be checked by looking for an active function of ras-p21 GDI (or GNRP) or by searching for another protein that specifically binds to ras-p21 in the GDP state.

10. BIOLOGICAL CONSEQUENCES

Guanine nucleotide binding proteins feature a delicate balance between two states corresponding to the GTP and GDP-bound forms. Based on comparison of the crystal structures of ras-p21:GTP and EF-Tu:GDP and the model structure of ras-p21:GDP a detailed view emerges of the conformational changes accompanying GTP hydrolysis. These changes probably occur as part of the signaling pathway of all G-domains, including the α-subunits of the receptor-coupled trimeric G-proteins.

The conformational differences in the two distinct states of G-domains are most pronounced in the switch II region. This region, relatively far away from the nucleotide binding site, can be sensed by external partners in a manner specific to the GTP or GDP state of the protein. The switch sensor is formed by the ends of helices α_2 and α_3 and loops L_5 and L_7. The most prominently exposed residues in both states in this region are: E62, E63, S65, A66, D69, Q70, R73, T74, R102, K104, D105. Most of these residues are conserved in a subfamily-specific way [24] and, therefore, probably constitute an externally sensed specificity patch. Detailed study of molecules interacting with these residues will provide clues about the remaining elusive messengers that are directly involved in the control and effect of the G-domain switch.

NOTE ADDED IN PROOF

After preparation of this manuscript strong experimental support for the model presented here was obtained by Rolf Hilgenfeld et al. (personal communication) who determined the crystal structure of an EF-Tu:GTP complex. The five key structural changes predicted here for the ras-p21:GTP->GDP transition are in essence mirrored in the differences between the EF-Tu:GDP and EF-Tu:GTP crystal structures.

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