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Point of VIEW

A New Tool for G Protein Analysis

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ABSTRACT G proteins play a pivotal role in cellular signaling by acting as molecular switches that undergo conformational changes upon binding GTP. The primary sequence constituting the binding cleft among the >160 G proteins in the human genome is highly conserved, consistent with the fact that these proteins share similar guanine nucleotide-binding characteristics. Recent work has demonstrated the feasibility of designing new analogs of GTP that can specifically activate G proteins whose nucleotide-binding sites have been remodeled through mutagenesis. This strategy has the potential to provide new insights into how G proteins act as molecular switches that engage their downstream target/effector proteins to generate specific signaling outputs.

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Small-molecule design for the purpose of inhibiting or otherwise altering the activity of key cellular signaling events continues to be a cornerstone of modern pharmacology and, indeed, modern medicine. The search for the perfectly fitting key (*i.e.*, a small molecule) that provides the desired outcome of modulating the activity of a specific protein has more recently been extended to redesigning the lock (protein) as well. Here, the goal is to structurally distinguish the altered protein−small molecule pairing from the significant background of closely related proteins by the rational design of a new and sufficiently distinctive protein−small molecule interface. The highly specific binding interaction between a synthesized "unnatural" ligand and the target protein, which has been mutated in a manner that allows it to optimally bind the altered ligand, in principle, provides a method possessing great specificity for studying the downstream consequences of the targeted signaling protein.

Advances in the field of protein−small molecule interface remodeling, as a recent paper by Shah and colleagues (*1*) illustrates, begin to frame the possibilities of a chemical-genetic approach for studying G-protein-mediated cell signaling events. Nucleotide-binding proteins in general (*e.g.*, protein kinases and G proteins) offer some especially attractive possibilities for the interface engineering approach because the mammalian genome contains >500 genes encoding protein kinases (*2*) and ~160 genes that encode G proteins (*3*). Indeed, protein kinases, which use ATP for phosphoryl transfer, have previously been shown to be promising candidates for the chemical-genetics approach (*4*). Shah and colleagues (*1*) now turn the attention to G proteins, which use GTP for conformational switching.

In contrast to protein kinases, for which several cases exist where active-site-directed inhibitors have been used to obtain information about their function, no effective active-site inhibitors have been identified for G proteins. This may be due in part to their extremely high affinity for guanine nucleotides (K_d in the picomolar range). Thus, the development of new chemical-genetic strategies for modulating G protein function would seem to be especially worthwhile and timely. As Shah and colleagues (*1*) point out in their paper, G proteins have been the object of chemical-genetic strategies in the past, by mutation of H-Ras that allows it to use XTP instead of GTP (*5*). However, this approach has several shortcomings, not the least of which are the low intracellular concentrations of XDP and XTP and the tendency of H-Ras mutants designed to bind XTP to bind GTP instead (given its higher cellular concentrations) and thus to be constitutively active in cells. Therefore, Shah and colleagues (*1*) augment this approach by rationally designing an H-Ras mutant that maintains its natural nucleotide selectivity but is susceptible to specific orthogonal small molecules (nucleotide analogs). Their overall goal is to develop a system that allows for specific modulation (either activation or inhibition) of any G protein of interest. Toward this end, it was necessary that they satisfy various criteria. In particular, they needed to create an additional binding pocket in H-Ras within

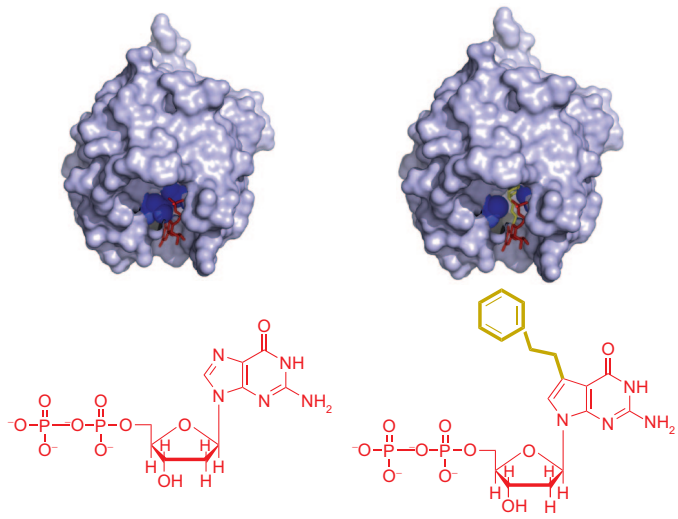

Figure 1. Surface drawings of H-Ras derived from the X-ray crystal structure (Protein Data Bank entry 1Q21) showing the space-filling differences adjacent to the nucleotide-binding pocket. Shown in blue are the side chains of amino acids 116 and 19 where these are asparagine and leucine in the wild-type structure (left) and the alanine substitutions at these positions that create room for the hydrophobic ethyl-phenyl moiety (yellow) present on the "unnatural" C(7)-deazaguanine inhibitor. The additional hydrophobic interactions provided by the dialanine—ethyl-phenyl interaction underlies the ~5-fold greater affinity of the mutant Ras protein for the inhibitor over GDP.

the vicinity of the active site, while still preserving the G protein's ability to bind GDP and GTP and to respond to its various regulators and target/effectors. In addition, they needed to design orthogonal molecules that possessed greater affinity than either GDP or GTP and did not bind to the wild-type protein (but only to the mutated G protein).

The authors outline in their study how all of these criteria were met. Thus, they were able to demonstrate a high degree of specificity for the regulation of the well-studied oncogenic G protein H-Ras by pairing a synthetic guanosine nucleotide analog with an altered GTP-binding site that better accommodates the analog's bulkier hydrophobic guanosine ring substitution.

Figure 1 shows the optimized result of mixing and matching mutated Ras proteins with the unnatural GTP molecule that provides the highest specificity based on the preference of the H-Ras mutants for this analog when compared with the naturally occurring GTP. Although the selectivity provided by the newly described GTP/H-Ras interface is significant (~5-fold), one can imagine that even greater affinity differences (and therefore selectivity) may well be possible by using the iterative approach outlined in this work. By systematic examination of different *in silico* docking approaches, coupled with the custom synthesis of new nucleotides to fit precisely sculpted binding sites, it should be possible to achieve highly selective stimulation of the G protein of interest, thereby providing a new tool for the study of G-protein-mediated cell signaling networks.

One might ask what advantages this approach holds over current methods for G protein pathway analysis. The authors show that their approach can be applied to other G proteins, by demonstrating the applicability of their strategy using the Ras-related protein, Rap1. They also suggest that their chemical-genetic tool will help to uncover novel H-Ras effectors and, in fact, use the approach to identify a new putative effector protein, Nol1 (proliferating cell nucleolar protein p120). Time will tell whether in fact Nol1 turns out to be an important player in Ras-dependent signaling events. However, it is less obvious just how advantageous the chemical-genetic approach will be for identifying target/effectors, given that epitope-tagging specific G proteins that possess either an activating or deactivating point mutation has been an extremely powerful tool in the hands of cell biologists for some time (*6, 7*). Ectopically expressed, tagged G proteins can be observed in fixed or living cells and can be coimmunoprecipitated with binding partners, providing a straightforward way to isolate and identify novel downstream effectors (*8*). Similarly, bead-immobilized recombinant G proteins and, in particular, GST-coupled G proteins specifically loaded with either nonhydrolyzable GTP analogs or GDP or depleted of nucleotide have provided a wealth of information when used as specific affinity resins or in "pull-down" assays (*9*). At least in these experimental formats, it is hard to make a case that selective interface engineering will uncover new G-protein-binding interactions that have been undetectable and undiscovered by the aforementioned approaches.

Nonetheless, one can imagine some exciting possibilities for the chemical-genetic approach in studying G-protein-coupled signaling events. One potentially interesting avenue may be through the monitoring of important conformational transitions that accompany the binding of a G protein to one of its regulators. For example, in the case of the heterotrimeric G proteins that are coupled to seven-membrane spanning (heptahelical) receptors, little is known about the complex set of interactions occurring in G protein α-subunits that are triggered by their interaction with an activated receptor and that initiate the intramolecular switch communication that precedes nucleotide exchange (*i.e.*, the G protein activation event). Custom design of guanosine triphosphate nucleotides that interact with different critical binding pocket residues in predicted ways might offer an extremely

sensitive tool for reading-out the conformational dynamics that occur prior to nucleotide dissociation. For this purpose, one might design a fluorophore "alcove" adjacent to the nucleotide-binding pocket that provides an appropriate microenvironment for enhancing the emission from a fluorescent nucleotide analog. This could offer a valuable method for assaying directly the switch communication that accompanies receptor binding and results in G protein activation. The limits of this approach may lie in the degree that the nucleotide pocket can be deformed before its binding capability is severely impaired. Nevertheless, a small fluorescent moiety such as *N*-methylanthraniloyl, which has been used successfully as a fluorescent GTP analog when attached to the ribose hydroxyls (*10*), could instead be coupled to the purine ring. This, together with some fine-tuning of the nucleotide-binding pocket through mutagenesis, might then provide a viable strategy for monitoring conformational changes in real time that occur within specific G proteins as an outcome of their activation.

Ultimately, one can even imagine that these strategies might be applied to studies of G protein activation and signaling in cells. Admittedly, cell studies with the engineered G protein interface present some technical barriers that will likely have to be overcome before this approach can have broad-scale use in monitoring G protein activation and accompanying signaling events in cells. In particular, in order to induce the selective activation of the engineered proteins expressed in cells by unnatural nucleotide, these analogs must be cell-permeable. In principle, this problem could be circumvented by detergent permeabilization, or perhaps through a whole cell patch configuration, prior to the addition of the nucleotide analog of interest, allowing for the selective activation of a given G protein. Thus, it is not difficult to imagine that in the not-too-distant future, it will be possible to follow G-protein-mediated cellular signaling activities on a very short time scale by using the appropriate pairings of nucleotide and protein. Selective activation using interface engineering coupled with the rapid addition of nucleotide to permeabilized cells might ultimately provide a way of monitoring the sequence of events leading to G protein activation (as an outcome of nucleotide exchange) or the steps that follow and lead to a specific signaling output. With the rapid rate in the development of customized nucleotide analogs, together with better molecular modeling approaches, the future development of these molecular tools holds promise for expanding our knowledge of G-protein-coupled cell signaling pathways.

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