

The emerging power of chemical genetics

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Chemical genetic methods allow signal transduction pathways to be probed in a domain-specific manner. This subtle perturbation of function, when combined with classical genetic and biochemical data, allows for a better understanding of protein function. This in turn is leading to elucidation of pharmacological maps of signaling pathways. Recent studies have focused on diverse pathways, including the initiation of actin polymerization, oncogenic tyrosine kinase control of cell transformation, and molecular motor involvement in adaptation of sensory cells of the inner ear.

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Current Opinion in Cell Biology 2002, 14:155–159

0955-0674/02/\$ – see front matter
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Published online 14 February 2002

DOI 10.1016/S0955-0674(02)00317-4

Abbreviations

Arp2/3	actin-related protein 2/3
N-WASP	neuronal Wiscott–Aldrich syndrome protein

Introduction

The maturation of powerful genetic methods such as efficient homologous recombination in mammalian cells, advances in the study of tightly regulated transcriptional systems, and the recent sequencing of many genomes, including that of humans, has brought with it a wealth of knowledge and new tools for the understanding of complex biological processes. With this new information has also come an appreciation for the limitations of classical genetic methods. Events that are regulated on a millisecond to hour timescale, processes requiring spatial and temporal regulation that are not amenable to biochemical purification, and processes involving proteins for which the functions can be compensated are not easily studied using classical genetics. The study of these systems requires tunable methods for selective modulation of single steps in complex pathways. Ideally, these methods would allow for the rapid and/or conditional inhibition of a single catalytic or protein–protein binding site of a given protein, without affecting its other functions.

Classical genetics has generally used the introduction of a secondary perturbation to study these events, such as overexpression, heat shock, temperature-sensitive mutations, or other conditional mutations. These secondary perturbations can have unwanted global side effects, which complicate the interpretation of the results. For example, a temperature shift from 25°C to 37°C was shown

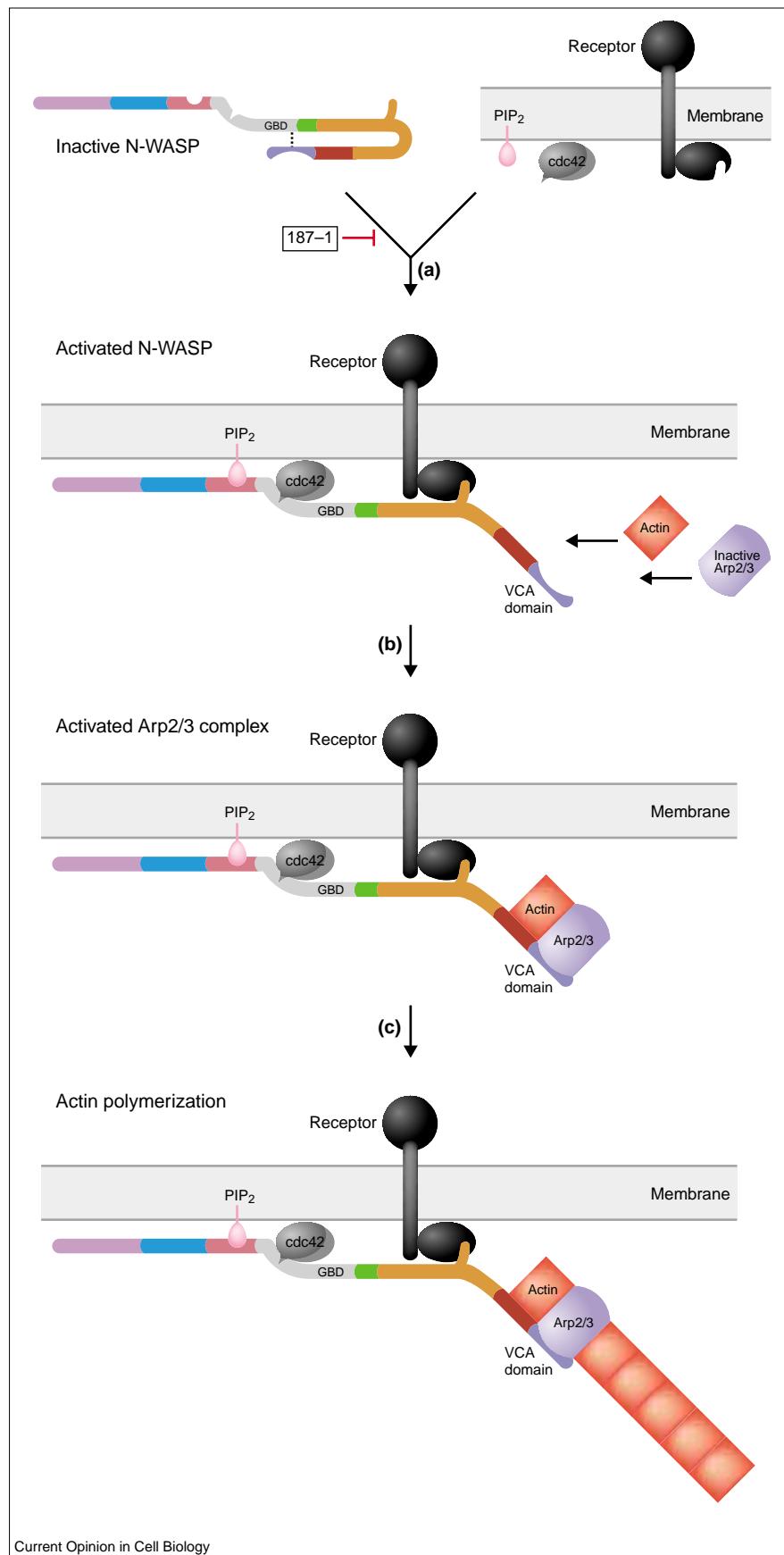
recently to be accompanied by a change in expression of 854 heat-responsive genes in yeast, 50% of which have undefined functions [1]. A small cell-permeable molecule with high specificity for the single protein site of interest would largely circumvent these effects [2] and seems to present an ideal solution. Although the human genome encodes fewer proteins than expected, the number of possible binding sites in a cell is enormous. Finding a mono-specific agent to bind to a desired target protein in a specific dynamic conformation presents a formidable challenge. In this review, we focus on two classes of solutions to the problem of developing specific small molecules to perturb and study cellular signal transduction systems. These methods highlight the growing field of chemical genetics.

Forward chemical genetics

One form of chemical genetics takes the approach of using chemicals as mutagens; that is, a given chemical induces a given phenotype. This is akin to forward genetics, in that a phenotype-based screen is used to identify chemicals that can perturb known or unknown elements of the pathway, and these can be used to further dissect the pathway. The advantage with this approach is that specificity is not always strictly required. If the compound induces an interesting phenotype in a given system, it can prove useful as long as its other effects are minimal or are at least in non-overlapping pathways.

A recent example of such forward chemical genetics illustrates the utility of small molecules for probing dynamic systems. The Kirschner and Mitchison groups generated a synthetic library and screened for compounds that inhibit actin polymerization [3•]. Previously, most small molecules found to perturb molecular-motor-dependent cellular phenomena have bound directly to the structural components [4]. This library was designed to inhibit upstream components in the signaling pathway by being biased toward protein–protein interface disruption. In this screen, a 14-amino-acid cyclic peptide that binds to the neuronal Wiscott–Aldrich syndrome protein (N-WASP) was identified. N-WASP is a signal-integrating protein that activates the Arp2/3 complex, which directly promotes formation of new actin filaments (Figure 1) [5]. The binding of the cyclic peptide to N-WASP specifically stabilizes the inactive form and inhibits protein–protein interactions that lead to actin polymerization. Interestingly, the N-WASP targeting peptide identified also binds to other cellular targets, as demonstrated by a whole-cell photo-affinity labeling test using the compound. Several other small molecules were identified in the same screen, and the authors are pursuing these. They may be more specific or potent for N-WASP or may target other interesting steps in the pathway.

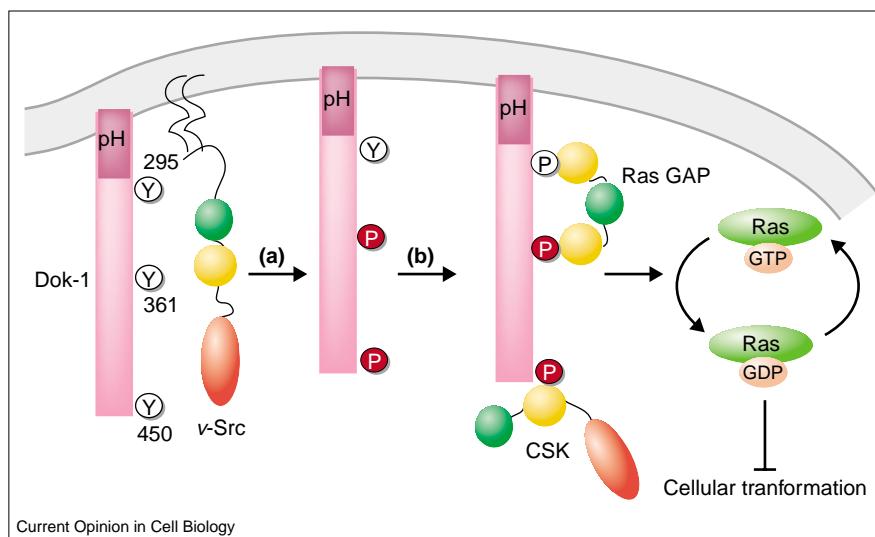
Figure 1



Signaling to actin polymerization through the N-WASP protein. (a) N-WASP is recruited to the membrane and activated by the GTPase cdc42, and PIP₂ (phosphatidylinositol 4,5-bisphosphate) exposing the VCA (verprolin, cofilin, acidic) domain. (b) The exposed VCA domain binds to and activates the Arp2/3 complex, which (c) initiates actin polymerization. The cyclic amino acid 187-1 (cyclo[L-Lys-D-Phe-D-Pro-D-Phe-L-Phe-D-Pro-L-Gln]₂) binds to and stabilizes inactive N-WASP, thus inhibiting activation. GBD, G-protein-binding domain.

Figure 2

A proposed model for retrograde signaling pathway by *v-Src*, mediated by the adapter protein Dok-1. (a) *v-Src* phosphorylates both Tyr361 and Tyr450 on Dok-1, even though the latter site was not predicted to be an optimal consensus phosphorylation site for Src. (b) Phosphorylation of all three sites recruits the Ras GAP and CSK (carboxy-terminal Src kinase) proteins, which negatively regulate cellular transformation. (Adapted from and reproduced courtesy of K Shah.) pH, pleckstrin homology domain.



One of the most difficult aspects of forward chemical genetics is identification of the target(s) of the small molecule, especially if the target is novel. This difficulty must be overcome if the method is to be a highly successful engine for discovery of new biology. One exciting development in this area is a recent report utilizing a standard genetic screen to identify mutants that confer resistance to a small molecule. In the past few years, Kahne and co-workers have searched for novel antibiotics with new modes of action, to attack vancomycin-resistant bacterial strains. One particularly interesting set of vancomycin analogues was shown to target a step in cell-wall biosynthesis distinct from that of vancomycin [6•]. In order to identify the pathway targeted by this new molecule, they screened for genetic mutations that conferred resistance to drug-induced lysis. Resistant mutants in *Escherichia coli* contained mutations in *yfgL*, a gene of unknown function. That this gene functioned in the death pathway was only observed upon treatment with the small-molecule inhibitor. Thus, by using their drug as a specific mutagen, Kahne and co-workers showed that the combination of chemical and classical genetics can be extremely powerful for elucidation of new pathways in bacteria that can be exploited for treatment of severe bacterial infections. Importantly, this was demonstrated in an organism that has been studied exhaustively.

Reverse chemical genetics

The second type of chemical genetic experiments has taken a conceptually very different approach. In reverse chemical genetics, genetic methods are used to introduce a mutant allele of the target protein of interest that is specifically sensitive to treatment with a designed small molecule. The designed small molecule contains a substituent capable of ‘complementing’ the mutation site and is unable to bind to wild-type targets of similar

structure. The approach has been applied successfully in a portable form to three main classes of signaling proteins: the GTPases, protein kinases, and molecular motors [7•].

Protein kinases, as a class of proteins, are difficult to study using classical genetics because redundancy and high homology in their active sites results in functional compensation in gene knockout experiments [8,9]. Our laboratory has devised a strategy for direct identification of kinase substrates. A designed ATP analogue, A*TP, interacts uniquely with an engineered mutant kinase of interest. When radiolabeled, this [γ -³²P] A*TP only transfers the radiolabel to direct substrates of the mutant kinase. This strategy has been applied to the study of the direct substrates of c-Jun amino-terminal kinase (JNK) [10], cyclin-dependent kinase 2 (CDK2) [11], and, most recently, the *v-Src* protein [12•]. Using the specific radiolabeling reagent [γ -³²P]-N⁶-(benzyl)-ATP, several novel substrates were identified, leading to a model for a retrograde signaling pathway in cellular transformation by *v-Src* (Figure 2). This study further revealed that some models of substrate selection by protein kinases (such as phosphorylation-site specificity, using peptide libraries) can be misleading when compared with intact protein signaling complexes. Since the mutation that allows [γ -³²P]-N⁶-(benzyl)-ATP acceptance by a kinase is conserved across the entire kinase superfamily, this method is being applied to many key kinases in order to identify unambiguously their direct substrates.

An equally challenging problem in the kinase field is identification of mono-selective inhibitors for kinases that can be used in whole cells and animals. This problem has been addressed using mutation of the same conserved residue as described above, together with a non-selective kinase inhibitor modified to be selective for the mutant

Figure 3

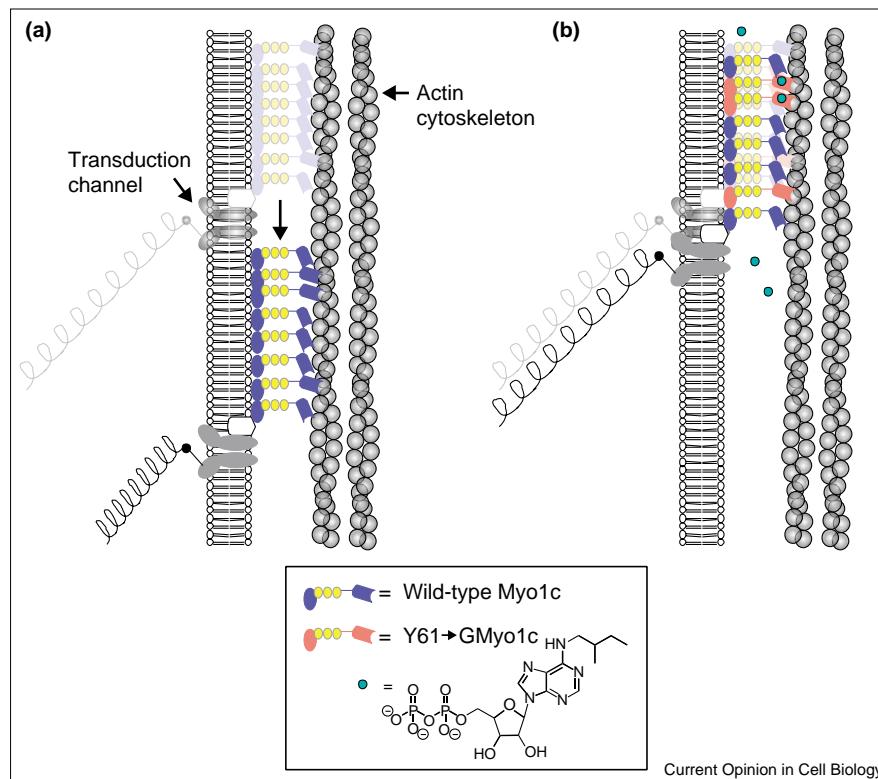


Illustration of the role of the myosin Myo1c in the adaptation motor in hair cells. (a) The adaptation motor, composed of a cluster of wild-type Myo1c (blue) anchored to the transduction channel, moves along actin in response to a stimulus and permits adaptation. (b) A cluster of Myo1c containing the Y61→G Myo1c mutant (red) binds to the inhibitor *N*⁶-(2-methylbutyl)-ADP (green circles). This binding locks the myosin motor preventing adaptation in response to the stimulus. (Adapted from Holt *et al.* [15•].)

allele [2,13]. Most recently, this approach has been used to reveal an unexpected regulation of glucose metabolism by a kinase (Pho85), identified as a key regulator of the phosphate response pathway [14•]. O’Shea and co-workers were able to use a designed small-molecule inhibitor complementary to and specific for a *PHO85* mutant allele to show the full extent of Pho85-dependent gene expression not previously observed using a knockout. The adaptation of gene expression over time was observed and was consistent with the phenotypic differences between the chemical inhibition and the knockout, illustrating the usefulness of this strategy in studying processes regulated on a rapid time scale.

The designed inhibitor/mutation strategy has been used to study another class of highly conserved proteins critical in a wide variety of signal transduction processes, the molecular motor myosins. The study of individual molecular motors is complicated by the mechanical nature of the signal transduction event, which does not involve amplification of the signal and therefore requires studying a single molecular event in a cell. Hair cells adapt to sustained stimuli and it is thought that a molecular motor involving myosin (Figure 3a) carries out this adaptation. A large number of individual myosin subtypes are expressed in each cell. Application of the designed inhibitor/mutation strategy to molecular motors by Gillespie and co-workers [15•] showed a direct involvement of a specific myosin, Myo1c, in the adaptation motor in hair cells. The use of a mutant allele of *Myo1c* that is completely functional

but uniquely susceptible to the designed inhibitor, *N*⁶-(2-methylbutyl)-ADP, allowed for the study of an adaptation event that takes place over tens of milliseconds (Figure 3b). The question of which myosin is the adaptation motor had previously eluded extensive immuno gold labeling techniques, biochemical reconstitution efforts, and exhaustive patch/clamp studies of hair follicles [16,17].

Conclusions

Recent advances in chemical genetics and new results from such studies offer more detailed pictures of cellular signal transduction pathways. From our current views, largely based on genetic and biochemical analyses, the most interesting differences arise from the domain-specific nature of chemical inhibitors contrasted with the more global effects of knockout experiments. One of the most profound insights into signal transduction to emerge in the past 15 years is the fact that signaling proteins are made up of multiple domains designed to integrate and deliver signals from different inputs. When one speaks of the target of a small molecule, it is likely that a given agent only binds to and disrupts the function of one of the domains of a signaling protein. This is illustrated by the cyclic peptide identified to bind to only the auto-inhibitory conformation of N-WASP. The ability to perturb the function of a single domain in a large signaling pathway can have dramatic effects on the entire pathway, and can reveal that protein to be integrated into other unexpected pathways, as was shown in the study of the kinase Pho85.

The second new insight into signal transduction comes from the ability to rapidly inactivate a target by a small molecule. Importantly, the ability of cells or whole animals to adapt to genetic perturbations via transcriptional upregulation of closely related genes or to regulate whole pathways in unexpected ways is a significant complication in the interpretation of many gene knockout studies [9]. The approach of using small molecules to rapidly inactivate a given target in a cell is likely to lead to a better appreciation of these adaptation mechanisms and to a more complete understanding of the dynamic nature of cell signaling in general. This new understanding is likely to be key to the development of better therapeutics, because current views of signaling pathways defined by genetic experiments have sometimes led to the development of drugs that fail to recapitulate genetic phenotypes.

With this new vision of signal transduction comes the requirement for new methods to probe the dynamic states of cell signaling. Experiments such as those carried out recently by Remy and Michnick [18•], who combined the results of specific enzymatic inhibition with a determination of protein–protein interactions, lead to ordering of a specific event in a signaling cascade. This results in a ‘pharmacological map’ of biologically relevant interactions spatially and temporally organized in a pathway.

The use of chemical genetics as a rapid and selective tool is ideally suited for the profiling of signal transduction pathways. The ability to manipulate single binding sites allows for subtle perturbation and probing of discreet steps in a pathway. Only the combination of new information about specific domain interactions and dynamic events with classical genetic and biochemical data will lead to a fully integrated picture of cellular processes.

Acknowledgements

We thank the National Institutes of Health for funding and a postdoctoral Biochemistry Training Grant for KM Specht.

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