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Functional analysis of protein kinase networks in living cells: Beyond "knock-outs" and "knock-downs"

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Abstract

The identification of over 500 protein kinases encoded by the human genome sequence offers one measure of the importance of protein kinase networks in cell biology. High throughput technologies for inactivating genes are producing an awe-inspiring amount of data on the cellular and organismal effects of reducing the levels of individual protein kinases. Despite these technical advances, our understanding of kinase networks remains imprecise. Major challenges include correctly assigning kinases to particular networks, understanding how they are regulated, and identifying the relevant *in vivo* substrates. Genetic methods provide a way of addressing these questions, but their application requires understanding the nuances of how different types of mutations can affect protein kinases. The goal of this article is to provide a brief introductory primer into these issues using examples from yeast MAPK cascades and to motivate future systematic genetic analysis focusing on individual residues of protein kinases.

Keywords: Kinase; Signaling; Genetics; Yeast

1. Introduction

The generation of gene "knock-outs" and the application of RNAi technology have been the most widely used methodologies for assessing the functions of protein kinases in cells. Less generic but of major clinical importance are small-molecule inhibitors of protein kinases. Although highly useful, each method has its drawbacks. Knock-out mutations can fail to yield effects because of genetic redundancy. RNAi can be incomplete and display off-target effects. Small-molecule inhibitors can affect both the intended target and unintended targets. Beyond these technical issues, there are more fundamental limitations to these often-utilized approaches because of the inherent sophistication of protein kinases as regulatory enzymes.

2. Thinking about the genetics of protein kinases

Several properties of protein kinases are important for the interpretation of mutations in the corresponding genes. These include (but are not limited to) the following (Fig. 1):

(A) Protein kinases often have multiple states, such as an enzymatically active phosphorylated state and an enzymatically inactive unphosphorylated state.
(B) The enzymatically inactive state may have "kinase-independent" functions.
(C) Protein kinases often contain so-called "autoinhibitory" domains that inhibit the activation of the enzymatic kinase domain. Activation of kinases can result from inactivation of the autoinhibitory domain.
(D) Protein kinases frequently have multiple substrates. For some kinases, associated proteins control substrate specificity.

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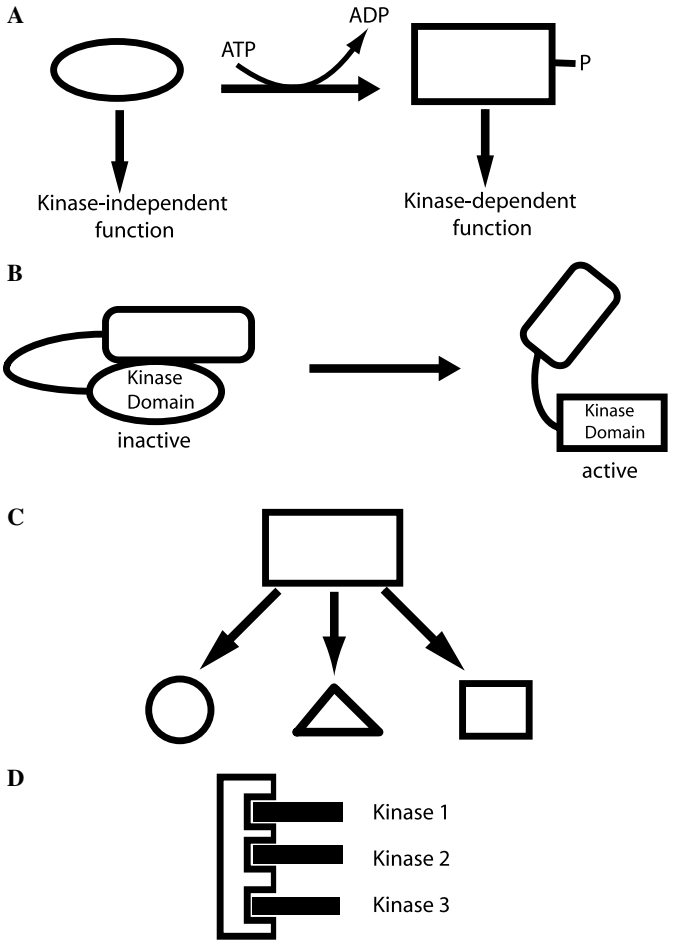

Fig. 1. Protein kinases are sophisticated enzymes. (A) Kinases can have kinase-independent functions. (B) Kinases can be regulated by autoinhibitory domains. (C) Kinase can have multiple substrates. (D) Kinases can be controlled by interactions with scaffolding proteins.

(E) Many protein kinases bind to scaffold proteins that can influence their activities.

The complexity of regulation and substrate specificity of protein kinases means that mutations that eliminate a protein kinase from a cell will have different effects from those that affect a particular state of a kinase, an inhibitory domain, or a residue in the kinase involved in substrate specificity. This means that considering only the effects of reducing the levels of a protein kinase will lead to an incomplete picture of regulation and function.

3. Classes of mutations: terms and meanings

Placing mutations into categories is helpful for interpreting the results of genetic experiments. The categories that follow were originally described by H.J. Muller based on his analysis of mutants in *Drosophila* in the early part of the 20th century (Table 1). Note that I use the term "mutation" to refer to a change in the DNA sequence of a gene, whereas "mutant" refers either to a cell containing a mutation or a protein encoded by the gene harboring that change.

Table 1
Classes of mutations

Loss-of-function (*recessive*)	
Null	No gene product synthesized
Hypomorph	Reduced levels of normal gene product and/or reduced activity of gene product
Gain-of-function (*dominant*)	
Hypermorph	Increased amount of normal gene product and/or increased activity of gene product
Neomorph	Abnormal activity of gene product and/or expression in wrong time or place
Antimorph	"Dominant-negative" inhibits activity of normal gene product

3.1. Loss-of-function mutations

A mutation in a gene that precludes synthesis of the encoded polypeptide is termed a "null allele" or "deletion allele." In recent years, the boxing term "knock-out" has been used synonymously. A mutation that reduces (but does not eliminate) the levels of a protein or its activity is termed a "reduced-function" or "hypomorphic" allele. RNAi techniques induce degradation of mRNAs, reducing protein levels. The reduced-function effect of RNAi is sometimes called a "knock-down." These classes of mutations are generally recessive to wild-type; that is, in the presence of the normal or wild-type allele of a gene, the mutation has no effect. This is interpreted to mean that the mutation reduces or eliminates the function of protein, hence the name "loss-of-function" mutation.

3.2. Gain-of-function mutations

These mutations are "dominant" to the wild-type allele meaning that they produce a phenotype even when the wild-type allele is present. They can be classified as follows. Those that increase the levels of the wild-type protein or the activity of the wild-type protein are called "increased wild-type function" or "hypermorphs." A second class comprises mutations that produce a protein with an abnormal activity (a protein that does something that the normal protein does not do or does it at the wrong time or place)—these mutations are termed "neomorphs." A third class comprises mutations that produce a phenotype similar to a null or hypomorphic allele, yet are dominant to the wild-type allele: these are called "antimorphs" or, more commonly, "dominant-negative" mutations.

This vocabulary falls short when a protein has more than one function, a common occurrence in biology. In these cases, the effect of a mutation is generally considered with respect to a particular function.

4. Interpreting mutants of protein kinases: three examples from yeast MAPK cascades

The considerations outlined above are not merely theoretical. Below I describe examples that illustrate how the analysis of specific alleles of protein kinases (as opposed to

the "knock-out") has illuminated a particular aspect of function or regulation. These are taken from three *Saccharomyces cerevisiae* MAPK cascades: the mating pheromone response pathway, the MAPK pathway that controls filamentous growth, and the high osmolarity or HOG MAPK pathway. These three pathways share a number of signaling components but utilize different MAP kinases.

4.1. Kss1 (MAPK)

Kss1 is the MAPK for the filamentous growth pathway of yeast. Studies of this kinase have provided an example of where there are significant differences between the phenotype of the null mutant versus those of cells expressing a mutant protein.

When activated by still-undetermined environmental signals, this MAPK leads to a morphological switch from budding oval-shaped yeast cells to filaments of elongated cells called pseudohyphae. Surprisingly, in diploid strains containing a homozygous knock-out of the *KSS1* gene, cells still undergo pseudohyphal growth [1,2]. This occurs for two reasons. First, Kss1 plays both positive and negative roles during filamentous growth—in the knock-out allele, both activities that promote and inhibit filamentous growth are eliminated, resulting in cells that can still undergo filamentation (Fig. 2). Second, other signaling pathways act in parallel to the Kss1 MAPK pathway to promote filamentous growth.

In its enzymatically active form, Kss1 activates its downstream transcription factor Ste12-Tec1 (Fig. 2). When unphosphorylated, Kss1 inhibits the transcription factor, in part by directly binding to Ste12 [2,3]. The isolation and analysis of mutations in *KSS1* was fundamental to understanding how the Kss1 MAPK functions. Mutants were isolated through a targeted genetic screen in which the *KSS1* gene was subjected to random mutagenesis [2]. Mutants that were either defective in filamentous growth or hyperfilamentous were identified. Those that were defective were found to be deficient in kinase activity *in vitro*. Those that displayed increased filamentation fell into two categories. One set was dependent on the kinase activity of Kss1 for their phenotype. These were most likely mutations that were resistant to inactivation of the kinase by phosphatases. The second set was independent of the kinase activity of Kss1 for their hyperfilamentous phenotype. These disrupted the ability of the inactive form of Kss1 form interacting and inhibiting its target transcription factor Ste12 [2]. Together, these results illustrate how the null phenotype of a gene encoding a kinase provided an incomplete picture of kinase function and regulation—the isolation of specific loss-of-function alleles yielded invaluable information.

4.2. Ste20 (PAK)

Ste20 is a p21-activated kinase (PAK) family member that is a MAP4K for three different yeast MAPK cascades. Like other members of the PAK family, its N-terminal noncatalytic domain binds to and inactivates its kinase domain (Fig. 3). Binding of the GTP form of Cdc42 to the CRIB domain within the N-terminal domain of Ste20 overcomes the intramolecular inhibition, allowing the kinase to become activated (Fig. 3). A deletion of the N-terminal domain results in a dominant gain-of-function allele that activates the pheromone response signaling pathway even in the absence of mating pheromone. This allele can bypass the need for all upstream components of the pathway (the pheromone receptor and its associated G protein). In contrast, loss-of-function point mutations in the CRIB domain that reduce binding to Cdc42 cause a defect in pheromone signaling because these point mutations prevent release of inhibition [4]. Thus, depending on the nature of the mutation, mutations in the N-terminal noncatalytic domain of Ste20 can have opposite effects. This is because this domain has two types of binding sites with opposite functions: one interaction is an autoinhibitory one in which the noncatalytic domain binds to the kinase domain while the other is an activating interaction with Cdc42.

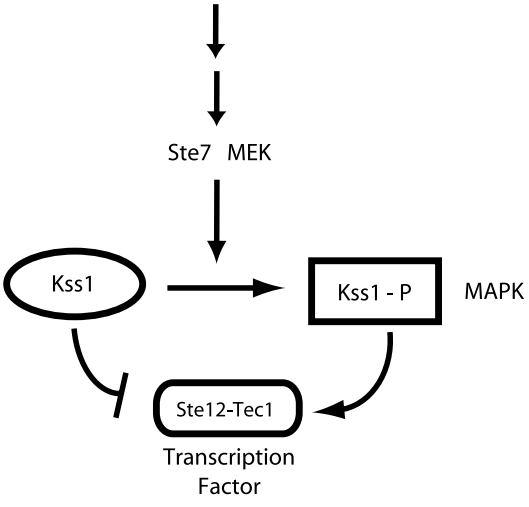

Fig. 2. The yeast Kss1 MAPK has kinase-dependent and kinase-independent functions.

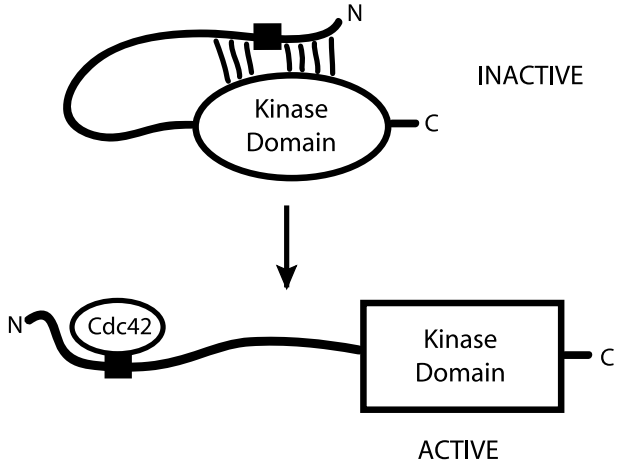

Fig. 3. Activation of the Ste20 PAK by inhibition of autoinhibition.

4.3. Ste7 (MEK)

Ste7 is the MAPK/ERK kinase (MEK) of both the pheromone response and filamentous growth MAPK cascades in yeast. Like mammalian MEK enzymes, its enzymatic kinase activity requires it to be phosphorylated by a MAP3K on two residues in its activation loop. The MAP3K that activates Ste7 is Ste11, a MAP/ERK kinase or MEKK enzyme. A gain-of-function mutation in Ste7 has been described [5,6]. It is a substitution in the activation loop of the kinase. The mutation allows Ste7 to be recognized and phosphorylated on its activating residues by kinases that do not normally recognize it as a substrate. Thus, this allele represents a gain-of-abnormal-function allele or a neomorph. In this case, the abnormal function corresponds to the ability to be recognized by a noncognate upstream kinase. Despite its neomorphic nature, this mutant version of Ste7 is useful for activating the pheromone response and filamentous growth pathways since other aspects of its activity such as substrate preference is likely to be unaltered.

4.4. Ste11 (MEKK)

A common task in the analysis of kinase signaling networks is the placement if a newly discovered component into a pathway. In a linear pathway such as a MAPK cascade, it is useful to know if a component acts prior to ("upstream") or after ("downstream") of another component. This can be accomplished through the analysis of double mutants (also termed "epistasis analysis"). In the case where the factors of interest function positively in a pathway, one can determine which factor is downstream by analyzing cells that contain a hypermorphic mutation in one component and a null mutation in another. If the double mutant displays precisely the phenotype of the null mutant, then the component corresponding to the null mutant functions downstream. An example is provided by Ste11, the MEKK/MAP3K that functions in the pheromone response pathway as well as the filamentous growth and HOG pathways. Expression of a version of Ste11 in which the noncatalytic autoinhibitory N-terminal domain is deleted results in induction of a pheromone pathway-specific gene expression and other mating responses [7]. Combining this mutation with a null mutation in the gene encoding downstream kinase Ste7 results in a strain that is no longer responsive to expression of this version of Ste11. On the other hand, expression of this version of Ste11 in cells lacking the β subunit of the upstream G protein still results in induction of mating responses. This double mutant analysis therefore places Ste11 between the G protein and Ste7.

5. Implications for systematic biology

Above I emphasize how the sophistication of protein kinases as enzymes means that genetic analysis that is restricted to reducing the levels of a protein kinase provides only initial clues about the function of a kinase. Mutations that eliminate one specific function or interaction of a protein kinase provide far more specific information. To analyze all kinase networks using general methods, one envisions the necessity of moving beyond the realm of systematic depletion of individual gene products and into the realm of systematic analysis of individual amino acid residues. Ideally one would like to assay for mutations that specifically affect each of the activities/states described above. Given that one does not know beforehand, every residue in a kinase would need to be mutated. Systematic methods for quantifying genetic interactions have been developed in yeast [8–10]. These include Systematic Genetic Array (SGA) analysis and a modification called Epistatic Mini-Array Profiling (EMAP). By combining libraries of protein kinase mutations with these genetic techniques, it may be possible to identify regulators and targets for a given protein kinase. Because the affinities of the interactions between kinases and other proteins may be low (due to the requirement for transient interactions during signaling), genetic approaches could offer substantial technical advantages over purely biochemical approaches.

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