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Isolation of intrinsically active mutants of MAP kinases via genetic screens in yeast

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Abstract

Intrinsically active variants of a protein are powerful tools for deciphering the specific functions of that protein. Since the catalytic activity of such variants is spontaneously active *in vivo*, they can disclose very accurately biochemical and biological functions of the parental protein. It is particularly important to obtain intrinsically active variants of individual MAP kinases. This is because in response to extracellular signals, more than one MAPK is typically concomitantly activated making it difficult to reveal their individual functions and downstream targets. Until very recently intrinsically active variants were not available for MAP kinases because of their unusual mechanism of activation that requires dual phosphorylation on neighboring Thr and Tyr residues. It is not known how to mimic the phospho-Thr-Xaa-phospho-Tyr motif by mutagenesis. We describe here a genetic screen in yeast that we successfully used to isolate bona fide intrinsically active variants of the yeast MAP kinase Hog1 and all isoforms of the human p38 family. We further established a screen for isolation of intrinsically active ERKs. The rationale of our screening approach is to search for MAPK molecules that are active in the absence of their activators. The method could be applied to the discovery of intrinsically active variants of any MAP kinase of any organism. We describe in detail the rationale, the steps that should be taken for establishment of such a screen and a step-by-step protocol for carrying out the screen.

Keywords: Yeast; Constitutively active variants; Hog1; Mpk1; Fus3; Kss1; Pbs2; Ste7; Mkk1; Mkk2; p38; ERK; JNK; SAPK

1. Introduction

1.1. Intrinsically active variants of an enzyme are accurate and specific tools for studying the enzyme's biological functions

Deciphering the precise specific functions of a given protein *in vivo* can be very difficult. A powerful way to study the specific functions of a given protein is to downregulate its expression via siRNA or knockout approaches. In some cases this approach may not disclose all the functions of a protein, due to some compensatory activity of other proteins. Also, knockdown approaches are not very useful for elucidating the roles of particular splicing products, or for the identification of downstream targets of the protein of interest. A useful accurate way to address protein function, that is complementary to the downregulation approach, is to controllably elevate the activity of the enzyme in question and to follow the biochemical, molecular and physiological (or sometimes pathological) consequences. In many cases overexpression of a protein is sufficient to increase its total activity in the cell and to impose biological phenotypes. Although useful for many purposes the overexpression approach could be misleading because overexpression of a protein could impose indirect effects by sequestering proteins to which it binds or by targeting proteins that are not its natural targets. In any case the overexpression approach is useless for proteins whose activities are tightly regulated and consequently manifest no basal activity. The activity of these enzymes could be induced *in vivo* by exposing the cells to the natural stimuli or to the conditions

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known to evoke the desired activity. However, in the vast majority of cases external stimuli induce a battery of cellular responses, activating several enzymes in parallel. Under these conditions it is very difficult to relate an apparent effect of the stimulus to the enzyme in question. An alternative way, that bypasses the need of external stimulation, is to use intrinsically active variants of the enzyme. When expressed in cells or organisms (constitutively or via inducible vectors), such variants, whose activity is intrinsic and therefore does not require any induction, will be spontaneously active. Importantly, at the time that the intrinsically active molecule is active, other enzymes, that are usually co-induced and form a complex network of reactions, are not active. Because the intrinsically active proteins are active *in vivo* in such insulation it is legitimate to relate any changes in the cell to the intrinsic activity of the protein. It would thus be possible to accurately reveal the relative contribution of the enzyme to biochemical and biological outputs. Indeed, mutated, intrinsically active molecules that were identified in disease (mainly in cancer) have been very useful in basic research. For example, ectopic expression of intrinsically active small GTPases in cells revealed their oncogenic effects, their direct effectors and their downstream impact on gene expression [1–3]. Also, expression of intrinsically active transcriptional activators disclosed their target genes [4,5]. In addition to intrinsically active proteins identified in diseases, other proteins were made intrinsically active by inserting mutations that impose structural changes similar to those obtained through natural activation. It is usually most difficult to predict which mutation would render an enzyme intrinsically active. Several small G proteins (e.g., Cdc42) and some other enzymes were made constitutively active, following insertion of mutations similar to those found in active homologs (e.g., *ras*; [6]). Other mutations were designed to mimic the mechanism of activation of the enzyme. This latter approach was useful for enzymes, in particular kinases, that are activated by phosphorylation. In these cases replacement of posphoacceptors (Ser of Thr) with acidic amino acids (Asp of Glu) resulted in partial emulation of the active phosphorylated conformation that was sufficient to render the enzyme catalytically and biologically active [7,8].

1.2. MAPKs are activated via dual phosphorylation that cannot be mimicked by mutagenesis

MAP kinases consist of a large family of enzymes that are expressed in all eukaryotic cells. The mammalian MAP kinases are divided into subgroups that include the ERKs, BMKs, p38s and JNKs [the p38s and JNKs are also known as stress-activated protein kinases (SAPK)]. The two approaches described above for producing intrinsically active enzymes are not useful for obtaining active mutants of MAP kinases. First, mutated, intrinsically active variants of MAPKs were not found so far in any disease. Second, although many aspects of the mechanism of activation of MAP kinases have been revealed it is not known how to bypass or to emulate this mechanism. MAPK activation is obtained through an unusual mechanism that is unique to MAPKs (i.e., dual phosphorylation; see below). Ways to bypass or to mimic this activation mechanism were developed, but were only partially successful [7–10]. Thus, the unique mode of activation hinders the production of intrinsically active variants.

When isolated from un-induced cells, or produced as recombinant proteins in *Escherichia coli*, MAPKs manifest very low, sometimes undetectable catalytic activity. They are activated following dual phosphorylation on neighboring Thr and Tyr residues residing in a unique domain of the MAPK proteins called the phosphorylation lip [11–18]. This phosphorylation is catalyzed by enzymes of the MAPK kinase (MEKs, also known as MAPKKs, MAP2K, or MKKs) family, which are the only enzymes in nature possessing such capability of dual Thr and Tyr phosphorylation. Recent studies suggested that dual phosphorylation of MAPKs may also be achieved by autophosphorylation induced either by interaction of the MAPK with another protein [19], or following phosphorylation of the MAPK on another site [20]. In any case, dual phosphorylation is critical for MAPK activity. It is not known how to mimic the phospho-Thr-Xaa-phospho-Tyr motif by mutagenesis. Replacing the Thr phosphoacceptor with Glu was not effective and in fact even reduced the potency of the MAPK to become active [21,22]. Detailed structural data that explains the conformational changes required for MAPK activation have been obtained for some MAPKs, mainly for ERK2 [11,12,14]. It was shown that the major effect of dual phosphorylation on ERK2 occurs at the phosphorylation lip that upon phosphorylation forms several interactions with the L16 domain. These interactions impose structural changes on the L16 itself and facilitate the formation of active dimers [11]. Unfortunately, this detailed information does not provide useful ideas for how to enforce similar conformational changes by mutations.

As rationale approaches, based on structural and biochemical studies, have not been useful for developing intrinsically active MAPKs, the employment of genetic unbiased screens may be more appropriate for this purpose. Several screens were previously performed in yeast and *Drosophila* and provided some interesting gain of function mutants. These mutants were not intrinsically active, or manifested very low activity [23–27] [reviewed in [28]. In this paper we provide a general scheme and a particular protocol for the establishment of screens specifically planned for isolation intrinsically active MAPKs. The screen we describe was already applied for the isolation of intrinsically active MAPKs of the Hog1/p38 family [29–31] and of the Mpk1/ERK family [Levin, V., and D.E., In preparation]. Molecules of the Hog1/p38 family isolated via this screen were shown to be valuable tools for studying Hog1 in yeast and p38 in mammalian cells [Askari, N., et al. Submitted]. The same screening strategy could be applied for obtaining intrinsically active variants of any MAPK of any organism. The screens take advantage of the MAP kinase