Signal transduction: hanging on a scaffold

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Recent data concerning scaffolding proteins profoundly challenge our conceptions of multicomponent signal transduction systems. Recent studies of the phototransduction system in *Drosophila* suggest two points. First, scaffolding markedly limits the possibilities for signal amplification. Second, the methods generally available to study signal transduction may be too crude to assess the *in vivo* roles of scaffolds. Studies of the mitogen-activated protein kinase pathway scaffold, Ste5, indicate functions beyond that of a passive structural element. Finally, the identification of new mitogen-activated protein kinase pathway scaffolds suggests the existence of multiple 'signalosomes' or 'transducisomes.'

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Abbreviations

ERK	extracellular-signal-regulated protein kinase
Ina-D	inactivation no-after potential
JIP	JNK interacting protein
JNK	c-jun N-terminal kinase
KSR	kinase suppressor of ras
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
PLC	phospholipase C
RKIP	Raf kinase inhibitor protein
SAPK	stress-activated protein kinase

Introduction

Rather than re-review an already well-reviewed field, we will focus on the implications of scaffold proteins on the function of signal transduction pathways. Thinking about the function of scaffold proteins raises new questions about the nature of signaling pathways. We will also discuss how technical limitations may impede a greater understanding of scaffolds. Recent findings suggest that our understanding of scaffolds is likely to take unexpected and surprising turns. For scaffolding novices, any one of the referenced reviews can provide excellent introductory overviews of the field [1–3,4•,5,6•].

Most of our ideas about scaffolds are influenced by work on the prototype mitogen-activated protein kinase (MAPK) scaffold, Ste5, a yeast protein involved in the Fus3 MAPK pathway [7,8]. Given the remarkable conservation of the MAPK pathways in eukaryotic cells, many investigators believe that Ste5 equivalents must exist in mammalian cells. To date, no protein homologs of Ste5 have been identified, however. Rather, working on the premise that Ste5 binds to all three kinase components of the MAPK cascade, the use of modern protein interaction methods have identified a growing list of molecules that can operationally fit this description of an Ste5-like scaffold (e.g. [9•]). None of these MAPK pathway scaffolds shows significant homology to Ste5 or to each other (with the exceptions of kinase domains in Pbs2p and the MAPK kinase kinase MEKK1).

Most investigators ascribe two related functions to scaffolds. First, scaffolds are said to maintain the specificity of the signaling pathway — a function variously described as 'isolating' or as 'stabilizing' the otherwise weak interactions between the kinases of a single cascade. Second, scaffolds are said to catalyze the activation of the pathway components. MAPK scaffolds may hold the kinases in a manner that directly enhances their mutual interactions, theoretically enhancing the rate of the phosphate transfer. Examples of proteins that could be considered as 'catalytic scaffolds' include Ste5, MAPK kinase (MEK) partner 1 (MP1), c-jun N-terminal kinase (JNK) interacting protein (JIP1), JNK/SAPK (stress-activated protein kinase) activating protein 1 (JSAP1) and kinase suppressor of Ras (KSR). However, the premises that scaffolds function to catalyse activation and to ensure specificity are largely untested.

Another perspective on scaffold function in non-MAPK-based signal transduction figures larger in the literature. In this model, the scaffold functions to co-localize a group of molecules that participate in the same signaling process to a specific area of a cell. In contrast to the MAPK pathways, the co-localized proteins do not necessarily directly act on each other, but rather they are all involved in the same signaling pathway. Recent work (described below) suggest that scaffolds enhance the efficiency of signal propagation. Examples of such 'anchoring' scaffolds include the A-kinase anchoring proteins (AKAPs) [3], Ina-D (inactivation no-after potential) [10], and Yotiao (an NMDA-receptor associated protein) [11•]. Also included in this category are the tyrosine phosphorylated scaffolds such as the platelet-derived growth factor (PDGF) receptor cytoplasmic domain, insulin response substrate-1 (IRS1), and the T cell proteins LAT (linker for activation of T cells) and SLP76 (Src homology 2 domain containing leukocyte protein of 76 kDa) [12].

Recognizing the different roles of catalytic and anchoring scaffolds, reviews of MAPK scaffolds (the presumed 'catalytic-type') rarely mention the existence of the other 'anchoring' class. The two perspectives on scaffold function are not mutually exclusive: catalytic scaffolds also co-localize components and co-localization is a means to enhance mutual interactions and thus signaling efficiency. Given that the idea that scaffolds perform a catalytic function is largely untested, it seems very possible that the distinction between these two perspectives will continue to blur.

Signals and signal amplification

It is widely believed that multi-component signaling pathways function to amplify signals. In a multicomponent kinase cascade, if each kinase can phosphorylate and activate many downstream kinases, the net result of a three-step kinase pathway is to geometrically amplify the initial signal. The strict utilization of scaffolds in a multikinase cascade, however, is likely to severely limit the amount of signal amplification. Furthermore, the aggregate of components binding to a particular scaffold suggests the possibility of a discrete cellular complex that mediates a specific signal. Zuker and co-workers, in recent studies of the *Drosophila* phototransduction pathway, addressed these issues [10,13••].

Ina-D scaffolds this system via its five PDZ (post-synaptic densitiy/disc-large/ZO1 domains. The known Ina-D ligands include phospholipase C (PLC), protein kinase C and the channel protein, TRP (transient receptor potential). Acting as an anchoring scaffold, Ina-D functions by assembling the relevant signaling molecules at a specific subcellular location in the *Drosophila* photoreceptor cell. Photoreceptor signal transduction begins with photon activation of rhodopsin, which in turn activates a G-protein-coupled PLC leading to the opening of an ion channel. Ina-D mutants that cannot bind all components demonstrate defective signaling. These findings lead Zuker and co-workers to suggest that Ina-D functions to assemble a signaling complex, a 'transducisome', as a quantal unit of signal transduction.

Exploiting mutants that express various amounts of the α subunit of G protein (Gα) and PLC, Scott and Zuker [13••] have shown that signal amplification is not a feature of this scaffolded signal transduction process. In a mutant in which signaling inactivation is defective, the activation of a single rhodopsin molecule by a single photon molecule results in a continuous signal. Single photon responses were measured by whole cell patch clamp techniques. Each molecule of rhodopsin repeatedly activated the same cluster of channels. If amplification was the primary function of the multicomponent system, varying the number of intermediate molecules should affect the amplitude of the response. However, mutants with decreased expression of the 'cascade's' intermediate components (i.e. Gα or PLC) have the same amplitude of response to single ligands as wild-type photoreceptors. Therefore, the number of channels activated was not determined by the availability of intermediates. Rather, the number of channels localized in a particular transducisome solely determines the 'amplification'. Thus, one photon activates one rhodopsin molecule, which activates one Gα, activating one entire transducisome. While, the activation of a single transducisome appears to be a highly co-operative, all-or-nothing process, Scott and Zuker argue that the multicomponent nature of the pathway apparently does not provide a means of amplification — violating a central tenet of signal transduction dogma.

Scaffolds and ideas about 'switch-like' signaling

If not for amplification, why have multicomponent signal transduction systems? Ferrell and co-workers [14••,15] have suggested that the multistep nature of MAPK pathways allows for 'switch-like' responses that effectively eliminate 'noise'. The model that represents switch-like signaling (known as the 'ultrasensitive' model) originating within the MAPK pathway is based on the multicomponent nature of the pathway and the requirement for two distinct phosphorylation events to activate both MAPK kinase (MAPKK or MEK) and MAPK. The model requires two related assumptions: first, that all the enzymes (MAPKK kinase [MAPKKK], MAPKK, and MAPK) freely diffuse with respect to each other (i.e. there is no transducisome); second, that the activating dual phosphorylations of MAPKK and MAPK occur nonprocessively. (Nonprocessive describes a reaction in which the kinase dissociates from its substrate protein after the first phosphate is transferred. The second phosphorylation event requires a second binding of kinase to its substrate protein.)

Contrary to the expectation that the more efficient processive mechanism would be used, *in vitro* studies using purified enzymes show that MAPK (or ERK [extracellular-signal-regulated kinase] activation by MEK is nonprocessive [16,17], which seems to validate one of the necessary assumptions underlying this model of 'switch-like' signaling. However, current models of scaffold function are incompatible with the quantitative model of 'switch-like' signaling because scaffold functions violate critical assumptions necessary for the model [15]. First, scaffolds will limit mutual diffusion of the pathway components. Second, by holding the kinase and its substrate in proximity to each other, the scaffold would convert a nonprocessive reaction to a processive one. Therefore, in addition to limiting amplification, scaffold function may also preclude the recently proposed model in which the MAPK pathways generate 'switch-like' responses.

Are we largely misunderstanding the roles of scaffolds?

Recent data show that the prototype MAPK pathway scaffold, Ste5p, does a lot more than just co-localize the components of a signal transduction system. Ste5 was the first recognized MAPK pathway scaffold and it binds three sequentially activated protein kinases in the yeast MAPK pathway [7,8]. In response to mating pheromone, Gβ recruits Ste5 to the plasma membrane. The molecule forms homo-oligomers [18,19] to which many pathway constituents apparently remain bound regardless of the presence of pheromone. Using a combination of nuclear import mutants, and synthetic Ste5 molecules that have varying degrees of nuclear import efficiency, Elion and co-workers [20••] identified an unexpected role of Ste5; specifically, shuttling of the Ste5 complex through the nucleus is required for maintenance of the pathway constituents in a state competent to participate in signal transduction. This study suggests that

scaffolds may have functions in addition to their role as a binding site for three separate kinases.

Recent lessons about kinase suppressor of Ras

A flurry of recent papers suggest that KSR deserves the epithet of scaffold as much as any other protein discussed in recent reviews on this subject [21•,22,23•,24•,25]. KSR was originally identified as acting downstream of Ras and in parallel, or upstream of, Raf [26]. It also binds Raf, MEK and ERK, forming a high molecular weight aggregate that includes 14-3-3 and hsp90, constituents previously shown to be part of the aggregate that contains the cytosolic fraction of Raf [23•]. Overexpression studies have led to several different conclusions about whether KSR is a positive or negative regulator of the MAPK pathway [21•,22,23•,24•]. For example, overexpression studies in (human kidney epithelial) 293 cells indicated that KSR negatively regulates epidermal growth factor receptor signal transduction [24•]. In functional assays based on germinal vesicle breakdown (GVBD), ectopic expression of KSR resulted in enhancement of GVBD while overexpression resulted in inhibition [21•]. Although confusing, these findings are entirely consistent with KSR functioning as a scaffold (discussed below).

Are molecules that have been described as inhibitors really scaffolds?

Given that the function of a scaffold is to bring together a variety of different proteins, the concentration of a scaffold needs to be titred closely to the concentration of the components to which it binds. Too much scaffold protein will dilute the pathway components, working to defeat the purpose of the scaffold by sequestering an activating kinase from its substrate (a finding used to identify a scaffold for the I-kappa-B kinase complex [27•]). JIP-1 and -2 were originally termed 'JNK inhibitor proteins'; the inhibition is now thought to be an overexpression artifact [28•]. The inhibitory function attributed to KSR may also arise at least partially from these kinds of trivial effects.

This kind of 'informative artifact' was demonstrated with MP1, where the enhancement of signal transduction was only apparent when ERK1 was also overexpressed [29•]. Considering this, the recent identification of a small protein that binds raf, MEK and ERK as a Raf kinase inhibitor protein (RKIP) might be viewed with caution. While the data reported are consistent with an inhibitory function of RKIP, history suggests that RKIP may one day be known as 'Raf kinase interacting protein'. If a 'scaffold' plays a strictly catalytic role and it is already present in the cell at near stoichiometric amounts, then overexpression will have little effect on transduction. This suggests that the standard 'proof' for a scaffold, that overexpression enhances signaling, may be difficult to achieve and may be achievable only when the scaffold's ligands are also overexpressed. Therefore, inhibition of signaling associated with overexpression of a candidate scaffold may be a criterion for defining a scaffold protein (Figure 1).

Amplification and regulation

Scaffolds complicate our understanding of the utility of multistep signal transduction pathways. The first assumption is that scaffolds lock the various interacting components in place with respect to each other, thereby simplifying signal transduction. But if the role of scaffolds is to reduce complexity, why has evolution conserved this complex system of interacting kinases? Why not just use a single kinase? Signal amplification and the opportunity for fine regulation are often offered as responses to these questions.

There are no data to date, however, showing that the reason for conserving the multiple steps is amplification. In fact, the Ina-D-scaffolded photoreceptor system suggests that amplification does not occur. Although generating switch-like responses is an attractive reason for the existence of multi-step signaling pathways, we have argued above that the current model is incompatible with the use of scaffolds. Scaffolds could play some role in switch-like responses but in a completely distinct manner.

Another possible advantage to multiple steps is to allow multiple opportunities for fine regulation. This appears relevant for mechanisms of Raf activation. But there is no data that fine regulation occurs in the distal, and purportedly scaffolded, steps (i.e. activation of MAPKKs and MAPKs) of the cascade. Even in the Ina-D system, there is no definitive evidence that regulation of the individual components within the 'transducisome' is used to modulate signaling.

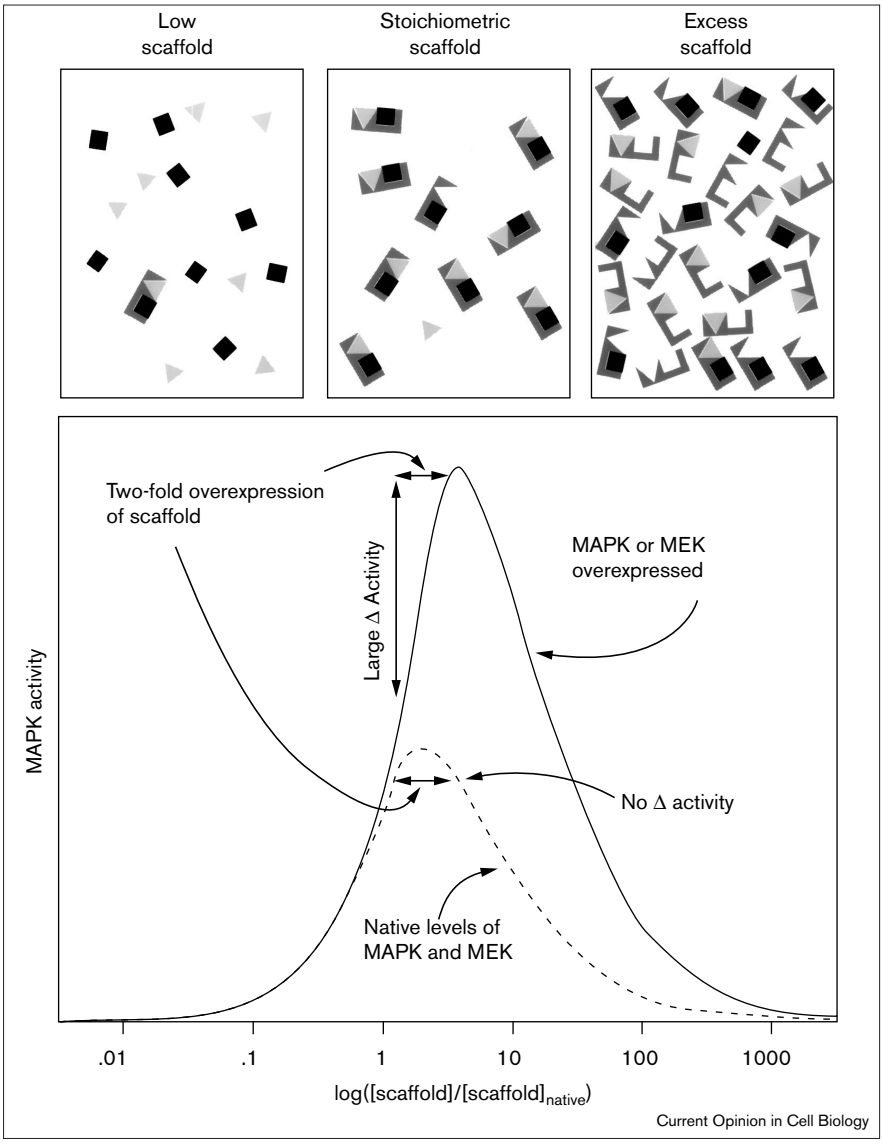
Some specific questions for 'scaffoldologists' in the future

How do the MAPK scaffolds affect the kinetics of kinase cascades *in vitro*? An analysis of *in vitro* Michaelis–Menten kinetics for the activation of the MAPK pathway components in the presence or absence of a particular scaffold would be very informative. These kinds of measurements would clinch the issue of whether the 'catalytic scaffolds' are really catalytic or not. Furthermore, these experiments would answer the question whether scaffolds convert non-processive pathways to processive ones.

Is there amplification in the MAPK pathway? If components are bound to scaffolds with sequential elements present at near 1:1 molar ratios, then little or no amplification will occur. As this predicted function is at odds with a central tenet of signal transduction dogma, it will be important to answer this question.

Does the MAPK pathway signaling in general display 'switch-like' behavior and how are scaffolds involved in this process? The existence of scaffolds, at least if they function by co-localizing the various kinases so that they efficiently act on each other, is inconsistent with the proposed mechanism by which 'switch-like' cellular responses are generated in the MAPK cascade.

Figure 1



Overexpression of a simple passive scaffold may give many different results depending on the native concentration of scaffold and the relative concentrations of it ligands. The figure shows a numerical simulation of the simple model of scaffold function in which two molecules, MAPK and MEK for example, are bound to each molecule of scaffold and assumes that formation of this trimolecular complex is essential for MAPK activation. When scaffolds and ligands are present initially at near-stoichiometric levels (dashed line), overexpression of a scaffold may result in little change or even a decrease in the level of signaling. Enhanced signaling is predicted only when the ligand molecules are also overexpressed (solid line). Depending on the exact initial conditions and degree of scaffold overexpression, this very simple model of scaffold function predicts many seemingly contradictory results. Both positive and negative regulatory roles could be ascribed to a scaffold depending on the exact experimental conditions.

Do MAPK pathway scaffolds really function to maintain pathway fidelity (i.e. prevent cross-talk)? To our knowledge, no one has yet demonstrated that a specific scaffold actually maintains pathway fidelity. Many of the enzymes in the MAPK pathway appear to display specific substrate specificity suggesting that scaffolds are not necessary to ensure fidelity. In support of this, Brunet and Pouyssegur [30] used p44 ERK/p38 chimeric molecules to demonstrate that a specific region of these MAPK conferred pathway specificity. Analogous experiments could determine whether scaffolds determine specificity.

Is there a unitary 'transducisome' in the MAPK pathways (analogous to the Ina-D transducisome)? The oligomeric states of Ste5p, KSR, and Raf suggest that this may be the case. In fact, many signal transduction molecules occur as components of high molecular weight aggregates. The Ina-D transducisome can be identified partially because the system lends itself to electrophysiology. Therefore, a single transducisome can be studied *in situ* and monitored with millisecond accuracy. Importantly, phototransduction still functioned in Ina-D null cells, although the kinetics and amplitude were clearly altered and the signaling components mislocalized. This kind of impairment might not be apparent if one had to rely on the 'grind and blot' techniques that constitute the only available approaches to most other signal transduction systems. The photoreceptor studies leave us wondering if the roles of scaffolds in the organization of MAPK signal transduction might be indiscernible without the development of new techniques.

Conclusions

If one only learns two new things about scaffolds this year, we suggest that they are the following: first, amplification doesn't happen in what is considered the best characterized multicomponent signal transduction pathway to date (Ina-

D); second, the MAPK-pathway 'scaffold' prototype may not be simply a passive docking site for multiple kinases. We have learned this year that we do not know very much about the roles of scaffolds in MAPK signal transduction. What we assumed we knew was based on prejudices that have been undermined by provocative data presented in the past year.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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Using a yeast two-hybrid approach, the authors identify a 145 kDa protein that associates with JNKs 1,2, and 3, as well as SEK1 (a JNK kinase) and MEKK1, suggesting that it acts as a scaffold for the JNK/SAPK pathway. There is no homology between this molecule and the previously described JIPs.

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The authors exploit an array of yeast mutants to probe the trafficking of sterile 5 (Ste5). In one striking experiment using temperature-sensitive export mutants, they show that only Ste5 that has recently passed through the nucleus is competent to support transduction. The results suggest that Ste5 undergoes some modification within the nucleus and the authors propose that phosphorylation may be the modification.

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