Selected Reading


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Raf Phosphorylation: One Step Forward and Two Steps Back

We understand Raf-1 activation relatively well but know less about how it is inactivated. An exciting study in this issue of Molecular Cell (Dougherty et al., 2005) now describes the molecular basis underlying the transient nature of Raf-1 signaling.

Ras is a small, membrane bound G protein that activates the Raf-MEK-ERK three-tiered protein kinase cascade (Figure 1). As this pathway controls cell fate, its activity is carefully controlled with feedback loops that play an important role in its regulation. ERK stimulates transcription of protein phosphatases that mediate its own deactivation and it also deactivates the proteins that activate Ras, thus providing at least two feedback mechanisms (Figure 1). A third, previously uncharacterized feedback loop from ERK to Raf-1 has also been proposed (Asseli et al., 1995), and it is the molecular basis of this loop that is now described by Morrison and colleagues (Dougherty et al., 2005 [this issue of Molecular Cell]). Mammals possess three Raf proteins, A-RAF, B-RAF, and Raf-1 (also called C-RAF). Although they have distinct modes of regulation (Wellbrock et al., 2004), in essence they are activated by phosphorylation which occurs when they are recruited to the plasma membrane by Ras. For activation, Raf-1 requires phosphorylation on five sites within its kinase domain, one of which is serine 338 (S338) (Figure 2), but there is duplicity in its regulation by phosphorylation. For example, protein kinase A (PKA) phosphorylates three sites in Raf-1 to block its activation when cyclic AMP levels are elevated (Dumaz and Marais, 2003).

Mitogenic stimulation of cells triggers Raf-1 hyperphosphorylation, which can be seen as an electrophoretic mobility shift in SDS-gels. Initially this was used as an indicator of Raf-1 activation, but it is unreliable as such, because it can occur when Raf-1 is not activated (Samuels et al., 1993). Dougherty et al. (2005) show that in addition to previously identified sites, Raf-1 becomes phosphorylated on S29, S43, S289, S296, S301, and S642 after mitogen stimulation. Five of these sites (excluding S43) are newly identified and are directly phosphorylated by ERK; these are the sites that cause the hyperphosphorylation phenomenon. Inhibiting hyperphosphorylation does not have a significant impact on the magnitude of Raf-1 activation by mitogens, but it does prevent Raf-1 from being deactivated after its initial transient activation phase. Only S296 and S642 are conserved in A-RAF and B-RAF, respectively, and ERK does phosphorylate the C terminus of B-RAF, but the consequences of this to activity are untested (Brunner et al.,
(Dougherty et al., 2005), implying that similar numbers of molecules are activated. If hyperphosphorylation ensures that most of the Raf-1 in cells is not activated, then 6A Raf-1 should be superactivated as seen with endogenous Raf-1 when MEK-ERK signaling is inhibited (Alessi et al., 1995). Perhaps 6A Raf-1 is not superactivated because cells have a limited capacity to activate it when it is overexpressed.

Hyperphosphorylated Raf-1 does not bind to Ras, presumably partly due to steric hindrance mediated by S43, which probably explains why it cannot be activated. The other sites may also contribute to the suppression of Ras binding. They may recruit other factors that directly interfere with Ras binding or may induce a conformation change that masks the Ras binding domain. Here, there is an interesting parallel to Raf-1 regulation by PKA, which also phosphorylates several N-terminal sites, thereby blocking Ras binding. Thus, in two different situations the Raf-1 N terminus is targeted by inhibitory kinases, confirming that this domain is largely regulatory and responsible for determining the protein’s subcellular location. Indeed, a general pattern emerges with N-terminal phosphorylation generally inhibiting Raf-1, whereas C terminus phosphorylation generally activates it (Figure 2). As S642 is the only inhibitory site in the C terminus, its function is particularly interesting and will no doubt be subjected to future scrutiny.

It is unclear whether hyperphosphorylation can also inhibit fully activated Raf-1, as the sustained activity observed with 6A Raf-1 could either be because it cannot be inhibited or deactivated properly, or because although it is inhibited/deactivated normally, it continues to be activated for a longer time period. The fact that S338 is dephosphorylated more rapidly in wt Raf-1 than in 6A Raf-1 (Dougherty et al., 2005) does suggest that hyperphosphorylation makes the active protein more susceptible to deactivation. However, it may also interfere with Raf-1 binding to other proteins such as MEK or to scaffold proteins such as KSR, and it will be important to dissect the functions of the individual sites. Whatever their mode of action, as the authors comment, this feedback is likely to ensure that Raf-1 signals are carefully regulated, preventing overamplification and possibly allowing Ras to bind to other effectors.

This study raises several other interesting questions. In particular, how does Raf escape this feedback loop in the approximately 15% of cancer cells that harbor mutated Ras and so have constitutively activated ERK? A simple answer may be that A-RAF and B-RAF are not inhibited by ERK and so they couple Ras to MEK in cancer. This may explain why C-RAF is not mutated in cancer, because even if it were, the feedback loop would not
still block its signaling. It will be interesting to determine whether mutant B-RAF, present in approximately 7% of human cancers (Garnett and Marais, 2004) is inhibited by ERK. If all Raf isoforms are inhibited by the loop, then how does mutant Ras signal in cancer? Note that Pin1 is overexpressed in several human cancers, so perhaps it recycles Raf and permits Ras signaling. It will be interesting to correlate Ras and B-RAF mutations in human cancers (Garnett and Marais, 2004) with Pin1 and PP2A expression levels. Remember though that ERK activity must be carefully regulated, because too much can induce cell cycle arrest and senescence (See Wellbrock et al., 2004), so perhaps the loop produces the trickle of signaling that favors cancer cell growth. It would be cruel irony if the loop that evolved to carefully regulate Raf-1 signaling is responsible for optimizing its activity in cancer. With the molecular characterization of this loop, we can now answer many such questions and also, as the authors comment, investigate the potential of Pin1 as a therapeutic target.

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