tions and raise some new issues as well. The earlier finding that anti-p38 SAPK immunoprecipitates phosphorylated Cdc25B and Cdc25C at the previously identified Chk1/2 phosphorylation sites (Bulavin et al., 2001) can now be explained by the presence of coimmunoprecipitating MK2 in the immune complex assays. Although the results obtained in fission yeast and mammalian cells were largely congruent, a puzzling discrepancy was that UV light-induced G_2 checkpoint activation was strongly dependent on Chk1 in fission yeast, whereas UV-irradiated mammalian cells relied heavily on MK2. These results suggest that, post S phase, mammalian cells perceive UV stress primarily through an extranuclear signaling cascade, leading to the activation of p38 SAPK. Finally, it is noteworthy that MAPKAP kinases are considered promising targets for the development of novel anti-inflammatory agents. These new findings suggest that MK2 inhibitors may also sensitize cancer cells to killing by antitumor agents that activate the p38 SAPK-dependent G_2 checkpoint pathway.

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Molecular Cell, Vol. 17, January 21, 2005, DOI 10.1016/j.molcel.2005.01.001

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 (Alessi 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 mitogenic 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 (Brummer et al.,

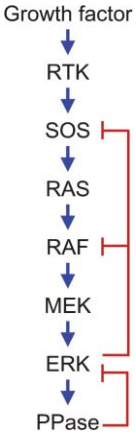

Figure 1. The Ras/RAF/MEK/ERK Pathway

Receptor tyrosine kinases (RTK) activate exchange factors such as SOS, which then activates Ras. Ras stimulates the RAF/MEK/ERK kinase cascade. ERK induces protein phosphatase (PPase) expression and consequently inhibits its own activity, and it also inhibits the activity of SOS and Raf-1 by direct phosphorylation.

2003). We do not yet know if S296 and S642 alone can inhibit Raf-1, but these observations raise the interesting possibility that all Raf isoforms are regulated by an ERK feedback loop.

Dougherty et al. (2005) also show that hyperphosphorylated Raf-1 is dephosphorylated by protein phosphatase 2A (PP2A) and that this requires Pin1, a prolyl isomerase that converts phosphoserine (and phosphothreonine)-proline from the *cis* to the *trans* conformation. Only the *trans* forms are efficient substrates for PP2A, and in Pin1$^{-/-}$ cells Raf-1 is constitutively hyperphosphorylated and refractory to activation. These results provide a molecular explanation of feedback inhibition, whereby ERK phosphorylates Raf-1, producing a form that is refractory to activation until it is recycled through Pin1 and PP2A. Thus, phosphorylation allows Raf-1 to take a step forward to become active but then forces it to take two steps back to a state where it cannot be activated any more.

The physiological role of this feedback loop is currently unclear. Only a fraction of Raf-1 is activated in mitogen-stimulated cells, but the whole population is hyperphosphorylated and the hyperphosphorylated protein cannot be activated, thus preventing sequential rounds of Raf-1 signaling. With this in mind, it is somewhat surprising that when all six sites are mutated (6A Raf-1), mitogen-stimulated Raf-1 kinase activity and S338 phosphorylation are not significantly elevated (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

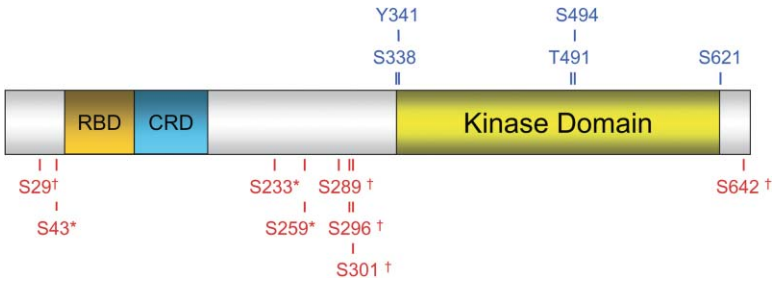

Figure 2. Schematic Representation of Raf-1

The important features of the protein are shown, including the Ras binding domain (RBD), a cystein-rich domain (CRD), and the kinase domain. Phosphorylation of residues marked in blue is required for Raf-1 activity, whereas those in red block its activation. Residues marked with an asterisk are phosphorylated by PKA, and those with a cross symbol are phosphorylated by ERK.

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 cancer 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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Acknowledgments

N.D. and R.M. are funded by Cancer Research UK (C107/A3096) and The Institute of Cancer Research.

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Domain Swapping and Retroviral Assembly

Ivanov et al. (2005) recently reported the structure of a mammalian SCAN domain. The molecule is a structural homolog of the HIV-1 capsid protein and forms a domain-swapped dimer in solution. The authors propose a similar domain-swapping event facilitates HIV-1 assembly, providing a new model for protein-protein interactions underlying viral particle formation.

The assembly of retroviruses is directed by a single protein, Gag, which is an extended molecule composed of multiple domains. Several thousand copies of Gag are arranged radially within each spherical and enveloped viral particle, with their N termini associated with the lipid bilayer and their C termini contacting the RNA genome in the virion interior. Sometime after particle formation, in a process termed maturation, the Gag protein is cleaved by the viral protease, releasing the structural proteins found in the infectious virus. These include the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins. CA reorganizes to form the shell of the mature viral core, which has a distinctive conical shape in HIV-1.

Formation of the immature particle is driven by the interactions of Gag with itself, with RNA, and with cellular membranes. The CA region of Gag is the most important locus of protein-protein interactions during immature particle assembly, but the nature of these interactions has remained unclear. While HIV-1 is highly organized, viral particles differ both in size and in the number of copies of Gag they incorporate. This precludes direct determination of the virion structure by cryo-electron microscopy or X-ray crystallography. Hence the molecular architecture of HIV-1 and other retroviruses has been slowly inferred through study of their components.

Mature CA is composed of two independently folded, predominantly helical domains. The C-terminal of these domains (the CTD) contains the most highly conserved sequence within Gag, a stretch of 20 amino acids termed the major homology region (MHR). Some residues within the MHR are invariantly conserved across all eight retroviral genera, a striking anomaly in sequences that have elsewhere widely diverged, and one that implies an essential function for the MHR in the viral life cycle. Structurally, the MHR encompasses much of the first of the CTD's four helices, as well as the β-turn and extended