

## Increasing Complexity of the Ras Signaling Pathway\*

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Ras is a key regulator of cell growth in all eukaryotic cells. Genetic, biochemical, and molecular studies in *Caenorhabditis elegans*, *Drosophila*, and mammalian cells have positioned Ras centrally in signal transduction pathways that respond to diverse extracellular stimuli, including peptide growth factors, cytokines, and hormones. The biological activity of Ras is controlled by a regulated GDP/GTP cycle. Guanine nucleotide exchange factors (GEFs<sup>1</sup>; RasGRF1/2 and Sos1/2) promote the formation of the active, GTP-bound form of Ras (1). GTPase-activating proteins (GAPs; p120 GAP and NF1) accelerate the intrinsic GTP hydrolytic activity of Ras to promote formation of the inactive, GDP-bound form of Ras (1). Mutations in Ras at amino acids 12, 13, or 61 make Ras insensitive to GAP action and, hence, constitutively active in transforming mammalian cells (2, 3). These activating mutations in Ras are prevalent in a wide spectrum of human cancers. It has been estimated that 30% of all human tumors contain an activating mutation in Ras. The frequency of Ras mutations varies depending on tumor type, with the highest frequencies seen in lung, colon, thyroid, and pancreatic carcinomas (3). The frequency of Ras mutations is likely to be an underestimation of the contribution of aberrant signaling through the Ras pathway to human malignancies because chronic up-regulation of the Ras pathway can occur in the absence of mutations in Ras itself (4–6).

### Ras Directly Binds Raf and Activates a Kinase Cascade

Ras mediates its effects on cellular proliferation in part by activation of a cascade of kinases: Raf (c-Raf-1, A-Raf, and B-Raf), MEK (MAPK/ERK kinases 1 and 2), and ERK1/2 (7). Upon activation, the ERKs phosphorylate cytoplasmic targets (such as Rsk (8) and Mnk (9, 10)) and translocate to the nucleus, where they stimulate the activity of various transcription factors that include the Elk-1 transcription factor (Fig. 1). Ras activates this kinase cascade by directly binding to Raf (11, 12). The binding of Ras to Raf requires active, GTP-bound Ras and an intact effector domain. The recent observation that Ras interacts with two distinct NH<sub>2</sub>-terminal regions of Raf-1 (RID/RBS1, spanning residues 51–131 (13, 14) and Raf-CRD (14))

suggests that Ras promotes more than just membrane translocation of Raf and instead may also facilitate the subsequent events that lead to Raf-1 activation. Other components that contribute to Raf-1 activation include 14-3-3 proteins, phospholipids, and serine/threonine and tyrosine kinases (15). Therefore, the connection between Ras and Raf alone is not simply linear and requires multicomplex formation to complete Raf activation.

### Ras Targets Multiple Effectors

Ras is likely to act through additional proteins besides Raf. The earliest observations that Ras has multiple effector proteins came from genetic studies in the budding yeast *Saccharomyces cerevisiae* and later the fission yeast *Schizosaccharomyces pombe*. Budding yeast devoid of Ras function were inviable, but yeast lacking adenylyl cyclase, an effector of Ras in this organism, were often capable of forming slow growing microcolonies (16). This result suggested that Ras proteins in *S. cerevisiae* have an essential function other than the activation of adenylyl cyclase. In *S. pombe*, Ras directly interacts with two effectors: Byr2, a MAPK kinase kinase, and Scd1, a GEF for the Rho family protein Cdc42 (17). Three additional observations in mammalian cells indicated that the events downstream of Ras are more complex than simply activating the Raf kinase. First, activated Raf induces only a subset of the events mediated by activated Ras. For example, activated Ras activates three distinct MAPK cascades (ERK, JNK, p38), whereas Raf causes direct activation only of ERK (18, 19). Second, activated Raf is not sufficient to promote all functions of Ras, such as the transformation of some epithelial cells (20). Third, studies with Ras mutants that discriminate between effectors suggest that multiple effector-mediated pathways are important for establishing and maintaining the transformed state (21, 48).

A plethora of candidate Ras effectors in addition to Raf have been reported. These include p120 Ras GAP (22), GEFs for the small GTPase Ral (RalGDS, RGL, RLF/RGL2) (23), AF6/Canoe (24, 25), RIN1 (26), and phosphatidylinositol 3-kinase (PI3K) (27). Although these candidate effectors comprise a very diverse collection of structurally and functionally distinct proteins, they all show preferential affinity for active Ras-GTP. Therefore, it is not surprising that residues corresponding to the switch I (Ras residues 30–37) and II (residues 59–76), which define the conformation differences between the GDP- and GTP-bound Ras, are involved in effector recognition. Specifically, an intact core Ras effector domain (residues 32–40) is essential for all effector interactions. Mutation of residues in sequences flanking this region (spanning residues 25–45) show differential impairment of effector interactions and provide useful mutants to decipher the contribution of specific effectors for Ras function (28). Thus, Ras residues important for effector interaction are more extensive than originally believed. The interaction of Ras with candidate effectors is often direct (interaction is observed *in vitro* using proteins purified from bacteria). For some, the interaction with Ras is observed *in vivo* upon co-immunoprecipitation, but these experiments are often done under conditions in which the Ras target is overexpressed. To date, Raf is the only Ras target protein for which genetic studies confirm its fundamental role in Ras signaling in a normal cellular context. Nonetheless, the interaction of Ras with at least some of these target proteins is likely to be critical

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<sup>1</sup> The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; MAPK, mitogen-activated protein kinase; ERK, extracellular receptor-stimulated kinase; MEK, MAPK/ERK kinase; RID, Ras-interaction domain; RBS, Ras-binding site; CRD, cysteine-rich domain; JNK, Jun NH<sub>2</sub>-terminal kinase; PI3K, phosphatidylinositol 3-kinase; PH, pleckstrin homology; CAAX, cysteine, aliphatic, aliphatic, terminal amino acid.

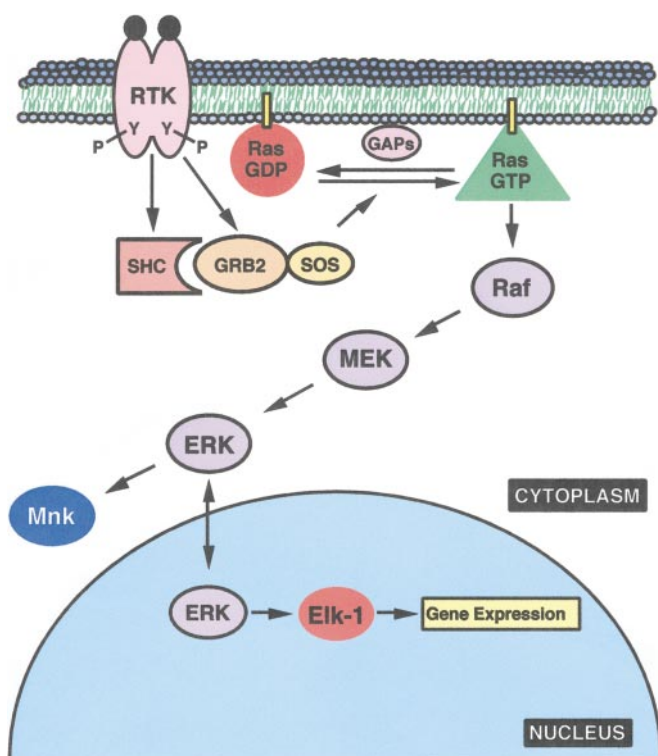


FIG. 1. **Ras regulates a cascade of kinases.** Ras is a GDP/GTP-regulated binary switch that resides at the inner surface of the plasma membrane and acts to relay extracellular ligand-stimulated signals to cytoplasmic signaling cascades. A linear pathway where Ras functions downstream of receptor tyrosine kinases (RTK) and upstream of a cascade of serine/threonine kinases (Raf > MEK > ERK) provides a complete link between the cell surface and the nucleus. Activated ERKs can translocate into the nucleus to phosphorylate and activate transcription factors, such as Elk-1. Activated ERKs also phosphorylate substrates in the cytoplasm, including the Mnk kinase, and thus contribute to translation initiation of mRNAs with structured 5'-untranslated regions.

for mediating the role of oncogenic Ras in malignant transformation.

#### Multiple Effector Pathways Contribute to Ras-mediated Transformation

What is the contribution of each of the known effector-mediated pathways to malignant transformation? The current state of affairs is depicted in Fig. 2. As described earlier, activation of the Raf/ERK pathway, with its concomitant activation of transcription factors, is essential for cell proliferation. The Ras GTPase-activating protein, p120 GAP, in addition to negatively regulating Ras function may impinge on the Rho family via its association with p190, a GAP for Rho family members (29). Activation of members of the Rho family of GTPases is likely to contribute significantly to the Ras-transformed phenotype (reviewed in Ref. 30).

The family of GEFs for Ral have also been implicated as target proteins for Ras (31, 32). A role for Ral in regulation of phospholipase D and in actin cytoskeletal rearrangements (via interaction with RalBP1) has been suggested (33, 34). In one report, RalA has been reported to cooperate with Ras for transformation (35), but others have not seen this cooperativity (36). Perhaps the RalGDS targets other proteins in addition to Ral that can influence the transformed phenotype. There is precedence for multiple functions residing in GEFs; SOS facilitates the exchange of nucleotides on Ras and couples Ras to Rac through its Dbl and pleckstrin homology (PH) domains in a PI3K-dependent manner (37).

RIN1 was identified in a genetic selection for mammalian

cDNAs that were capable of suppressing the phenotypes associated with constitutive activation of the Ras pathway in *S. cerevisiae* (38). RIN1 interacts directly with Ras in a GTP- and effector domain-dependent fashion and localizes to the plasma membrane (26). Subsequently, RIN1 was shown to interact with Abl and Bcr/Abl *in vitro* and *in vivo* through a domain distinct from the Ras binding domain (39, 40). Moreover, RIN1 can enhance the transforming activity of Bcr/Abl and rescue several transformation-defective mutants of Bcr/Abl (40). The aspects of Ras function mediated by RIN1 are still the subject of investigation, but one possibility is that RIN1 coordinates signals from Ras and Abl.

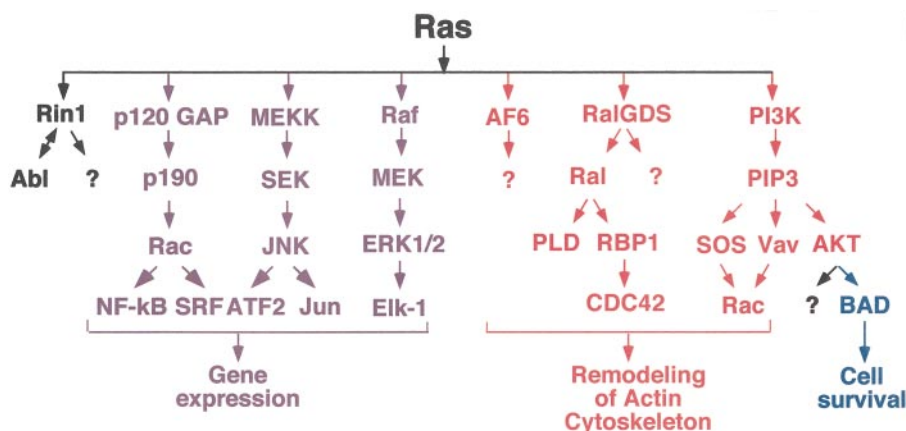
Biochemically, AF6/Canoe are candidate Ras effectors (25). In addition, genetic studies in *Drosophila* have linked Canoe to Ras in eye development (41). Canoe/AF6 have a GLG(F/D)HR motif, a conserved sequence found in proteins that associate with cellular junctions, so perhaps Canoe/AF6 coordinate signaling events at the plasma membrane to remodeling of the actin cytoskeleton.

Finally, activation of PI3K, via a direct interaction between Ras and the catalytic subunit of the protein, is necessary for actin cytoskeletal rearrangements associated with the transformed phenotype (36). PI3K is a lipid kinase with specificity for the 3-position of the inositol ring. Activation of PI3K by a variety of extracellular stimuli leads to the accumulation of the second messenger phosphatidylinositol 3,4,5-trisphosphate. What are the downstream targets of this second messenger? One target is the serine/threonine kinase Akt/PKB. Binding of Akt/PKB via its PH domain to phosphatidylinositol 3,4,5-trisphosphate localizes Akt/PKB to the plasma membrane and leads to a partial activation of its kinase activity (42). Akt/PKB activity is further increased by phosphorylation on 2 residues by two different kinases, one of which, PDK1, is itself a lipid-regulated kinase (43). The events downstream of Akt/PKB are the subject of intense investigation in many laboratories. Akt/PKB phosphorylates and inactivates the pro-apoptotic protein BAD (44, 45) but is likely to have additional substrates. Other targets of the products of PI3K include the PH domains of Vav (46), SOS (37), and GRP1 (47).

One of the more elegant approaches to understanding the contribution of each of the effector pathways to Ras-mediated transformation has been the use of Ras effector mutants that are impaired in binding a specific target (21, 36, 48, 49). For example, studies with effector domain mutants have revealed a bifurcation of the signaling pathways downstream of Ras leading to remodeling of the actin cytoskeleton and DNA synthesis. RasV12C40, an activated mutant of Ras with an alteration of tyrosine to cysteine at position 40 in the effector domain, is unable to bind Raf. This mutant fails to activate the ERK cascade and cannot activate a Ras-responsive reporter construct, but it is capable of inducing membrane ruffling to the same extent as an activated Ras with an intact effector domain (49). These results suggest that stimulation of membrane ruffling and activation of the ERK cascade are mediated by distinct Ras effector proteins. Subsequently, RasV12C40 was shown to bind to and activate PI3K, suggesting that Ras-induced morphological alterations may be mediated in part through activation of PI3K (36). In addition to binding PI3K, RasV12C40 will also interact with AF6 (48), and a role for AF6 in modulation of the actin cytoskeleton by RasV12C40 cannot be excluded. Finally, the ability of this mutant to cause tumorigenic transformation demonstrates that Raf-independent pathways alone are sufficient to promote Ras transformation.

Two additional approaches to dissect the contributions of this surfeit of candidate effector proteins to Ras function have been to overexpress or membrane-target a specific effector to

FIG. 2. A surfeit of candidate Ras effectors. Multiple effector pathways contribute to Ras function. Our current understanding of the downstream targets of each of the Ras effectors is shown in the figure (see text for details). *PLD*, phospholipase D; *PIP3*, phosphatidylinositol triphosphate; *MEKK*, MEK kinase; *SEK*, SAPK/JNK kinase; *SRF*, serum response factor.



see if this mimics any aspect of Ras function. Targeting to the plasma membrane is often achieved by adding the sequence containing the CAAX box of Ras to the effector of interest. Membrane targeting has been shown to cause constitutive activation of Raf and PI3K. If membrane targeting of the candidate effector does not reproduce any aspect of Ras function, it may be that the target protein under study is already constitutively localized in this cellular compartment. Although Raf and PI3K reside in the cytoplasm and become associated with the plasma membrane upon receipt of stimulatory signal(s), other Ras target proteins, such as adenyl cyclase in yeast and the RalGDS, are constitutively membrane localized. Membrane-targeted RalGDS did not exhibit any transforming potential.<sup>2</sup> Finally, a powerful (but as yet not common) approach to decipher the role of a Ras target protein is to determine whether fibroblasts derived from mice deficient in a target protein are impaired in transformation (for example, see Ref. 50).

### Ras Mediates Life and Death Decisions by Distinct Effector Pathways

One perplexing aspect of the Ras signaling pathway is that Ras can promote both cell death and cell survival through interactions with distinct effector proteins. Using Ras mutants, Kauffmann-Zeh *et al.* (51) demonstrated that activation of Raf by Ras promotes apoptosis in fibroblasts containing an inducible c-Myc oncoprotein, whereas activation of PI3K by Ras promotes cell survival. In this assay, oncogenic Ras enhanced apoptosis. This result suggests that Ras has the potential to trigger two seemingly contradictory biological outcomes: cell death by activation of Raf and cell survival by activation of PI3K. At least in this assay system, cell death (the Raf pathway) is dominant over cell survival (the PI3K pathway). How this seemingly discordant choice of cell death *versus* survival is achieved is not known.

What is the contribution of each of these pathways to tumor initiation and/or progression? Promotion of cell death by activation of Raf may be an important factor in limiting the expansion of cells harboring Ras mutations, whereas promotion of cell survival by activation of PI3K may contribute to tumor expansion and metastases. It will be interesting to see if the relative contributions of these antagonistic pathways can be modulated by other signaling pathways and whether such modulation plays a role in tumor formation.

Activated Ras also promotes cell survival in epithelial cells upon detachment from an extracellular matrix (52). This action of Ras is mediated through activation of PI3K and Akt/PKB. Given that the majority of human tumors are of epithelial cell

origin, a pharmacological intervention that could switch a Ras-dependent survival signal into an apoptotic signal might be of considerable value in the treatment of human malignancies.

### Summary

The last 5 years have seen an impressive expansion in the number of candidate Ras effectors. Much progress has been made toward deciphering the aspects of Ras function mediated by each of these proteins, and many studies, in particular those with effector domain mutants, have convincingly demonstrated that Ras must target at least three different pathways for transformation. The corruption of the signaling pathways that lie downstream of Ras is a recurring theme in the initiation and/or progression of human malignancies. Pharmacological interventions have directly impeded Ras function by interfering with its farnesylation and membrane targeting or have blocked activation of components of the kinase cascade downstream of Ras (53). However, the function of Ras and its downstream kinase cascade is central to many cellular processes, and this may limit the usefulness of these approaches. The diversity of Ras target proteins and the necessity for activation of multiple effector pathways for malignant transformation by Ras open new directions for the design of additional therapeutic interventions that may negate Ras transformation without abolishing all of Ras function.

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