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Pak protein kinases as mediators of Ras signaling and cell transformation. A place for Pak on the Ras MAP: More than just another JNK bond

Review Article

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Summary

Ras plays a key role in regulating cellular proliferation, differentiation, and transformation. Raf is the major effector of Ras in the Ras>Raf>Mek>Erk (MAPK) cascade. A second effector is phosphoinositide 3-OH kinase (PI 3-kinase) which synthesizes several lipid second messengers that activate small G proteins such as Rac and Cdc42. Rac also has multiple effectors, one of which is the serine threonine kinase Pak (p65Pak). Here we review studies documenting a novel Ras signal through PI 3-kinase and Rac/Cdc42 to Pak. The signal appears essential for maintaining cell transformation and Erk activation.

I. Introduction

Ras is one of the most commonly mutated oncogenes and is found activated in 20-30% of tumors (Lowy and Willumsen, 1993). Ras encodes a small G protein that binds GTP and GDP and possesses an intrinsic GTPase activity. In its oncogenic form Ras acquires a point mutation that inactivates the GTPase activity and causes it to be locked into its activated GTP-bound state. Normally, Ras is activated by growth factor receptors through its guanine nucleotide exchange factors (Egan and Weinberg, 1993).

The major oncogenic signal from Ras utilizes the serine threonine kinase, Raf, as the effector (Van Aelst et al., 1993; Vojtek et al., 1993). GTP bound Ras binds and activates Raf and simultaneously recruits it to the membrane. Upon activation Raf phosphorylates and activates another kinase Mek which in turn activates ERK (MAPK). This Ras>Raf>Mek>ERK signal is usually referred to as the MAP kinase cascade (Marshall, 1995). In recent years Ras has also been shown to bind other effectors, besides Raf, and activate other signaling pathways that cooperate with the Raf> ERK signal (White et al., 1995). The other pathways are not as well defined as the Raf cascade. The three effectors that have been most widely studied are Rin, RalGDS and PI 3-kinase (Afar et al., 1997; Peterson et al., 1996; Rodriguez-Viciana et al., 1997). Each binds RasGTP and can, in some experimental systems, cooperate with partially activated Raf mutants to transform cells (Rin cooperates with Abl). Further evidence of the importance of these effectors in Ras signaling comes from new Ras point mutants, known as effector mutants, that bind and activate only subsets of Ras effectors (White et al., 1995). Ras^{V12S35} binds and activates Raf, Ras^{V12G37} binds and activates RalGDS and Rin1 and Ras^{V12C40} binds and activates PI 3-kinase (Joneson et al., 1996; Rodriguez-Viciana et al., 1997; White et al., 1995; White et al., 1996). These mutants are deficient in signaling when tested alone, but cooperate when introduced into cells together. Signals from Ras through the alternate effectors utilize other small G proteins. Ral GDS uses Ral, and PI 3-kinase uses the small G protein Rac (Rodriguez-Viciana et al., 1997; White et al., 1996).

Rho and two related proteins, Rac and Cdc42, are members of the Rho family of small G proteins. These proteins are about 50% identical to Ras and regulate the actin cytoskeleton. Rho induces stress fibers and focal adhesions (Ridley and Hall, 1992), Rac induces accumulation of actin rich ruffles or lamellipodia at the periphery of cells (Ridley et

al., 1992) and Cdc42 induces microspikes or filopodia (Nobes and Hall, 1995). Each Rho family member also activates a kinase cascade that leads to transcriptional activation similar to the MAP kinase cascade but not as well defined. Rho activates the ternary complex factors (TCF), and Rac and Cdc42 activate the Jun N-terminal kinase cascade JNK(SAPK) (Coso et al., 1995; Frost et al., 1996; Hill et al., 1995; Minden et al., 1995). Dominant negative mutants of Rac, Rho and Cdc42 each inhibit Ras transformation and activated mutants cooperate with Raf to transform cells (Khosravi-Far et al., 1995; Qiu et al., 1995; Qiu et al., 1997; Qiu et al., 1995). These observations suggest that the signals through the Rho family of small G proteins play essential roles in Ras transformation.

The signals through Rac are directly connected to Ras (Bar-Sagi and Feramisco, 1986; Ridley et al., 1992). This is because Ras and Rac both cause membrane ruffling when microinjected into cells and a dominant negative Rac mutant inhibits Ras induced ruffling. The signal from Ras to Rac is likely to be mediated by PI 3-kinase since Ras^{V12C40}, which activates PI 3-kinase, and activated mutants of PI 3-kinase both induce ruffles (Joneson et al., 1996; Rodriguez-Viciana et al., 1997). The mechanism that PI 3-kinase uses to activate Rac probably involves stimulation of Rac GEFs by PI 3-kinase products such as phosphatidylinositol-3,4,5-triphosphate (Han et al., 1998; Nimnual et al., 1998). The immediate effector downstream of Rac in Ras signal transduction to both the JNK and actin pathways has remained elusive. The first candidate to be isolated was the serine threonine kinase p65Pak (Manser et al., 1994). Pak was isolated because it binds both Rac and Cdc42 in their GTP bound forms. Pak is homologous to Ste20, a protein kinase in the yeast *S. cerevisiae* regulated by Cdc42 (Lim et al., 1996; Sells and Chernoff, 1997). Some of the activities of Pak resemble those of Ras and Rac. For example, microinjection of Pak into some cells causes ruffling and breaks up stress fibers and membrane targeting of Pak in PC12 cells induces extension of neurites (Daniels et al., 1998; Manser et al., 1997; Sells et al., 1997). Although microinjection of Pak can cause membrane ruffling, ruffling does not require Pak kinase activity (Sells et al., 1997). In addition, Rac^{V12H40}, an effector mutant that does not bind to Pak, still cooperates with Raf to transform cells and causes membrane ruffling when it is microinjected (Joneson et al., 1996; Lamarche et al., 1996; Westwick et al., 1997). These studies suggest that there may not be a role for Pak in Ras transformation or signaling and even question the existence of any direct signals from Ras to Pak.

This report reviews work linking Ras directly to Pak through a PI3-kinase dependent pathway and discusses the role of this link in cell transformation (Tang et al., 1997; Tang et al., 1998; Tang et al., 1999).

II. Evidence linking Pak to cell transformation

Signal transduction studies routinely use reagents to activate or inhibit specific candidate components of the Ras signaling pathway. Such studies suffer from the limitation that high levels of expression may lead to non-physiological signals. To circumvent this problem, it is necessary to use multiple strategies to link signaling molecules to Ras. A general strategy has emerged in the field that is based on three types of observations. First, Ras must signal to the candidate. Second, Ras signals must be potentiated by activating or overexpressing the candidates. Third, Ras signals must be blocked by inhibiting the candidate molecule. Finally, the biological assays must be backed up with biochemical assays to known Ras signals. We shall now discuss how this has been applied to place Pak on the Ras signaling pathway. The observations are presented in reverse order for historical reasons.

III. Kinase deficient Pak inhibits Ras transformation

The first observation linking Ras to Pak was the demonstration that expression of kinase deficient mutants of Pak inhibit Ras transformation in Rat-1 fibroblasts (Tang et al., 1997). This type of mutant is routinely used to study protein kinases because they behave as dominant negative mutants. The assumption is that the kinase defective mutants specifically bind to key substrates but fail to phosphorylate them while preventing the endogenous kinase from phosphorylating them. For Pak, a single amino acid substitution at amino acid number 299 (K299R), a key residue in the catalytic domain, inactivates the kinase activity (Sells et al., 1997; Zhang et al., 1995). Since Rac is required for Ras transformation, the trivial explanation that the mutants were sequestering Rac was addressed by mutating the Rac/Cdc42 binding site in the kinase deficient Pak. The mutant, $Pak1^{L83,L86,R299}$, has substitutions of leucines for conserved histidines at positions 83 and 86, along with the original R299 in the kinase domain. This mutation is as effective at inhibiting Ras transformation as the original mutant, which rules out the sequestering model. Hence, kinase deficient Pak mutants are potent inhibitors of Ras signaling, even when they cannot bind Rac and Cdc42. Inhibition was specific for Ras since the Pak mutants did not inhibit v-Raf. Interestingly, almost no Ras inhibition was observed in NIH 3T3 cells, but was observed in other cells (Tang et al., 1998).

IV. Pak can be uncoupled from JNK but not MAPK signaling

The major signaling pathway downstream of Ras is to the MAP kinase Erk, while the major signal downstream of

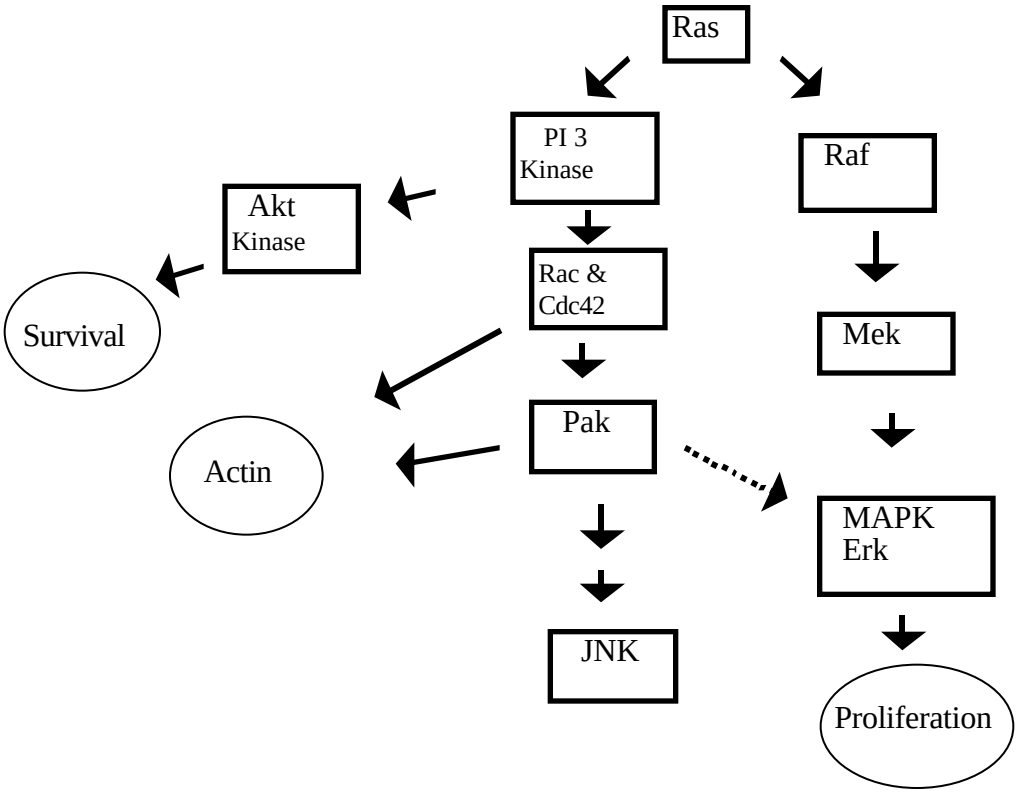

Figure 1 Model of Ras signaling through Pak to ERK. Although Pak can activate JNK, Ras does not appear to utilize Pak for this signal.

Rac is to the related kinase JNK. As expected, kinase deficient Pak, $Pak1^{R299}$, inhibits JNK activation by Ras and by Rac (~75%). However, no inhibition was observed with the $Pak1^{L83,L86,R299}$ construct which cannot sequester Rac or Cdc42. Since both of these mutants inhibit Ras transformation, JNK inhibition is not obligatory for Pak mutants to inhibit Ras transformation. However, when the analogous experiment was performed to measure Erk signaling, both of the kinase deficient Pak mutants inhibited Ras signals to Erk. These observations suggest that the dominant negative Pak1 mutants may inhibit Ras transformation by interfering with the MAP/ERK kinase cascade and not the JNK cascade. Thus, the third criteria is met--in both biochemical and biological assays, kinase deficient Pak mutants inhibit Ras.

V. Pak potentiates signals to Erk

The studies described above suggest that Pak mediates signals to ERK to sustain cell transformation. Several laboratories have independently observed that Pak can stimulate Erk (Frost et al., 1997; King et al., 1998; Lu et al., 1997; Tang et al., 1999). The first was the study from Mayer and his colleagues who found that targeting Pak to the membrane by fusion to a localization signal stimulated Erk (Lu et al., 1997). Two mechanisms have since emerged to trace the signals from Pak to Erk. (1) Pak was shown to phosphorylate Mek in vitro and in cell culture studies. In this study a kinase deficient Pak mutant that failed to bind Rac/Cdc42 also inhibited signals to Erk from several growth factors (Frost et al., 1997). (2) Pak was also shown to phosphorylate Raf at a novel residue, 338. Moreover, mutation of residue 338 prevented Raf from signaling to Erk and transforming cells (King et al., 1998). All four of the studies have in common the observation that Pak alone is not sufficient for Erk activation but can potentiate signals to Erk from components of the Ras signaling pathways. These data demonstrate that Pak may be necessary for Ras signaling. However, Pak is clearly not sufficient for Ras signaling since $Pak1^{L83,\ L86}$ an hyperactive kinase mutant does not transform most cells nor does it cooperate with Ras or Raf to transform cells (Tang et al., 1997). The one exception is a cell line that is hypersensitive to Ras transformation because it has mutations that activate MEK. In this cell line, Pak cooperates with Raf to transform the cells (Tang et al., 1999). However, as discussed above evidence from many labs demonstrate that Pak can promote signals to Erk. This constitutes the second

arm of the case for Pak as a key modulator of Ras signaling--potentiation signals to Ras targets.

VI. Ras activates Pak

The third arm linking Ras to Pak comes from a study showing that Ras activates Pak (Tang et al., 1999). Ras activation was observed in transfection assays of Rat-1 fibroblasts. Activation was comparable to the levels observed by Rac. Activated PI 3-kinase, but not Raf also activated Pak suggesting that the signal was mediated by PI 3-kinase. Furthermore, the PI 3-kinase specific inhibitor LY294002 inhibited both Ras and PI3-kinase activation of Pak with similar dose responses. Dominant negative Rac and dominant negative Cdc42 both inhibited Ras and PI3-kinase activation. Thus a Ras>PI3-kinase>Rac/Cdc42 signal was traced. The mechanism of action of Rac and Cdc42 dominant negative mutants both involve the sequestering of exchange factors. Many of these exchange factors can activate both Rac and Cdc42, so the dominant negative mutant results do not necessarily distinguish between them. Interestingly, in a remarkable synergy between the biological responses and biochemical signaling, Ras could not activate Pak in NIH 3T3 cells. The differences between the two cell lines and their signals from Ras to Pak lie downstream of PI3-kinase since neither Ras nor PI 3-kinase could activate Pak in NIH 3T3 cells.

VII. A convergence of signals from small G proteins on Pak

The studies with Ras demonstrated that it can activate Pak directly. Further studies suggested that Pak mediates signals from other G proteins through indirect mechanisms (Tang et al., 1999). First, transformation by Rac, Rho, Ras and several Rac and Ras effector mutants were all inhibited by dominant negative Pak mutants. Second, cooperative ERK activation by all three GTPases was inhibited by the Pak dominant negative mutants. Third, all combinations of Ras, Rho and Rac mutants that yielded high efficiency transformations also activated Pak. Finally, in the presence of a partially activated Raf all small G proteins, including Rho, could activate Pak. It should be noted that these correlations suggest that Pak activation may be necessary for high efficiency transformation but clearly Pak activation is not sufficient for transformation since Ras^{V12C40}, Rac^{V12}, Rac^{V12L37} and $Pak1^{L83,L86}$, all of which activate the kinase activity of Pak, transform poorly when tested individually. This study suggested that multiple signals from several small G proteins utilize Pak even when they do not signal to Pak directly (Tang et al., 1999). A similar conclusion was reached by the study of Frost et. al. which found that Rho and Rac were signaling through Pak to Erk (Frost et al., 1997).

VIII. A Role for Pak in Ras signaling beyond Rat-1 fibroblasts

While the studies with Rat-1 fibroblasts provide a useful experimental system to study the role of Pak in Ras signaling, it is not appropriate for tumor cells. To develop cell systems for studying Pak more appropriate for a specific tumor, Tang et. al. developed several cell systems relevant to Neurofibromatosis. Neurofibromatosis type 1 (NF1), a common autosomal dominant disorder caused by loss of the NF1 gene, is characterized clinically by neurofibromas, and more rarely by neurofibrosarcomas (Gutmann et al., 1997; Viskochil et al., 1990; Wallace et al., 1990). Abnormalities in Schwann cells are thought to be responsible for both types of tumors (Kim et al., 1997; Sheela et al., 1990). While the basis of the Schwann cell as the affected cell is not well understood, the underlying mechanism of transformation for both tumors is through the Ras signaling pathway. Neurofibromin, the protein encoded by NF1, possesses an intrinsic GTPase accelerating activity for the Ras proto-oncogene. Through this activity neurofibromin acts as a negative regulator of Ras. Hence, loss of neurofibromin causes elevated levels of activated Ras which leads to hyperactivation of downstream signals (Ballester et al., 1990; Basu et al., 1992; DeClue et al., 1992; Martin et al., 1990; Xu et al., 1990). First, transfection assays demonstrated the central role of Pak in Ras transformation and signaling to Erk in Rat Schwann cells. Then, kinase deficient Pak mutants were found to revert a transformed cell from a patient with NF1. Thus, in at least one tumor cell, Pak plays a central role in Ras signaling and transformation (Tang et al., 1998).

The observation that Rho, Rac and Cdc42 are all required for Ras transformation has prompted several searches for key downstream effectors. Pak emerged as one candidate because it was the first protein kinase found to bind Rac/Cdc42 and it was homologous to Ste20, a Cdc42 effector in yeast (Manser et al., 1994). Subsequent studies with effector mutants suggested that Pak does not play a role in transformation. However, by the three criteria discussed earlier, Pak has been linked to Ras. First, Ras signals directly to Pak through PI-3 kinase. Second, several laboratories have found that Pak is required to sustain signals to Erk. Third, Ras transformation and Erk signaling can be inhibited by dominant negative Pak mutants. Hence, Pak becomes part of a growing list of Ras downstream signaling proteins, such as Raf, PI 3-kinase and Mek that may be targets for novel anti-neoplastic drugs.

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Note added in proof

We have recently determined that the Akt proto-oncogene is a key intermediate between Ras and Pak suggesting that Pak may transduce cell survival signals from Ras [Tang Y, Zhou H, Chen A, Pittman RN, and Field J (**2000**) The Akt ptoto-oncogene links Ras to Pak and cell survival signals. **J Biol Chem** 275, 9106].

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