Role of Group A p21-activated Kinases in Activation of Extracellular-regulated Kinase by Growth Factors*

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The canonical extracellular-regulated kinase (ERK) signaling cascade, consisting of the Ras-Raf-Mek-ERK module, is critically important to many cellular functions. Although the general mechanism of activation of the ERK cascade is well established, additional noncanonical components greatly influence the activity of this pathway. Here, we focus on the group A p21-activated kinases (Paks), which have previously been implicated in regulating both c-Raf and Mek1 activity, by phosphorylating these proteins at Ser338 and Ser298, respectively. In NIH-3T3 cells, expression of an inhibitor of all three group A Paks reduced activation of ERK in response to platelet-derived growth factor (PDGF) but not to epidermal growth factor (EGF). Similar results were obtained in HeLa cells using small interference RNA-mediated simultaneous knockdown of both Pak1 and Pak2 to reduce group A Pak function. Inhibition of Pak kinase activity dramatically decreased phosphorylation of Mek1 at Ser298 in response to either PDGF or EGF, but this inhibition did not prevent Mek1 activation by EGF, suggesting that although Pak can phosphorylate Mek1 at Ser298, this event is not required for Mek1 activation by growth factors. Inhibition of Pak reduced the Ser338 phosphorylation of c-Raf in response to both PDGF and EGF; however, in the case of EGF, the reduction in Ser338 phosphorylation was not accompanied by a significant decrease in c-Raf activity. These findings suggest that Paks are required for the phosphorylation of c-Raf at Ser338 in response to either growth factor, but that the mechanisms by which EGF and PDGF activate c-Raf are fundamentally different.

The p21-activated kinases (Paks)2 are effectors of the small GTPases Cdc42 and Rac that have been implicated in regulating cell morphology and motility by promoting the formation of localized membrane protrusions (1, 2). The Paks are divided into two distinct subgroups: the well studied group A, comprising Pak1, Pak2, and Pak3, and the more recently identified group B, comprising Pak4, Pak5, and Pak6. In addition to regulating morphological pathways, Paks contribute to a variety of transcriptional signaling cascades, such as the p38, Jun kinase (3, 4), and ERK pathways (5, 6). The ERK pathway is of particular interest because group A Paks are known to be required for ERK activation and transformation by Ras (6), and two of the three components of this pathway, Mek1 and c-Raf (also known as Raf-1), have been proposed to be direct Pak substrates (7–9).

The canonical ERK activation pathway from receptor protein-tyrosine kinases is relatively well understood. Receptor protein-tyrosine kinases such as the epidermal growth factor (EGF) receptor or the platelet-derived growth factor (PDGF) receptor autophosphorylate upon ligand stimulation, thereby recruiting a variety of SH2-containing proteins, including Grb2, which binds SOS, an activator of Ras. Ras in turn recruits Raf to the plasma membrane where this kinase becomes activated, thereafter phosphorylating and activating Mek, which then phosphorylates and activates ERK. However, additional components play an important role in regulating ERK activation. For example, Mek1 is phosphorylated by group A Paks at position Ser298, and it is thought that this phosphorylation is essential for transmitting mitogenic signals (10). The phosphorylation of Mek1 at this site is not in itself sufficient for activation; rather, it is thought to increase the affinity of Mek1 for ERK and/or c-Raf (11). In the case of c-Raf, several groups have reported that Paks target a critical regulatory residue, Ser338 (7, 8, 12), rendering this kinase competent for activation by Ras. However, other groups have suggested that either phosphorylation of Ser338 is dispensable for c-Raf activity (13) or that Ser338 phosphorylation is required for maximal activity but that Paks are not the relevant Ser338 kinases (14).

The regulation of c-Raf by phosphorylation is complex. In addition to the Ser338 and the adjacent Ser339 positions, phosphorylation at Tyr340 and Tyr341 also influence c-Raf conformation and activity. The Ser338/339 and Tyr340/341 sites have been identified as being coordinately regulated and required for maximal c-Raf activity (15) or to promote the redistribution of c-Raf to different subcellular compartments (16). Recently, activation of c-Raf by angiogenic factors has demonstrated a differential phosphorylation of these two proximal regulatory sites: Ser338/339 and Tyr340/341. Basic fibroblast growth factor was reported to promote c-Raf activation through Pak-dependent phosphorylation of Ser338/339, whereas vascular endothelial growth factor was reported to promote c-Raf activation by Src-dependent phosphorylation of Tyr340/341 (16). In other systems phosphorylation of Tyr341 is dependent on the Src-like kinase Lyn (17).

In this report, we used a specific inhibitor of group A Paks, the Pak1 inhibitor domain (PID) (18), to analyze the Raf/Mek/ERK cascade in murine fibroblasts stimulated with growth factors. When expressed in NIH-3T3 cells, the Pak1 PID efficiently blocks the activation of all three group A Paks and blocks stimulation of ERK by platelet-derived growth factor (PDGF) but not by epidermal growth factor (EGF). An independent approach, siRNA, yielded similar results, but only if both Pak1 and Pak2 were simultaneously knocked down. For both EGF and PDGF, PID expression blocked Mek1 phosphorylation at Ser298, suggesting that this site is not required for Mek1 activation of ERK. Stimulation of c-Raf Ser338 phosphorylation by either growth factor was also blocked by PID expression. However, this blockade reduced c-Raf activation by PDGF but not by EGF. These results demonstrate that Ser338 phosphorylation...
of c-Raf is dependent on Pak activity in NIH-3T3 cells and that this phosphorylation is required for c-Raf activation by PDGF but not EGF. Further, these results show that alternate c-Raf activation mechanisms exist in NIH-3T3 cells and that similar growth factors use distinct signaling pathways to activate the ERK cascade.

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal anti-hemagglutinin (HA) antibodies (12CA5) were obtained from BabCo, and polyclonal anti-Myc and monoclonal anti-GST antibodies were obtained from Santa Cruz Biotechnology, Inc. Antibodies against group A Paks were from Zymed Laboratories Inc. (Pak1) and Cell Signaling Technology (Pak2 and Pak3). Antibodies against activated ERK and Mek (Ser217/221) were obtained from Promega and Cell Signaling Technologies, respectively, and antibodies against total ERK and Mek were from Cell Signaling Technologies. Monoclonal anti-c-Raf and polyclonal anti-Ser(P)328 c-Raf antisera were from BD-Pharmingen and Upstate Biotechnology, Inc., respectively. Polyclonal anti-phospho Mek Ser298 were obtained from BIOSOURCE International. Secondary horseradish peroxidase-conjugated antibodies were obtained from Jackson Laboratories. SMARTpool® siRNAs against human Pak1 and Pak2 were purchased from Dharmacon.

**Expression Plasmids**—The Pak1 PID (amino acids 83–149 in Pak1) was cloned as a BamHI/Acc65I fragment into pEBG (19). This plasmid was then mutagenized to create Sall sites upstream of the glutathione S-transferase (GST) leader and downstream of the 3′ end of the PID. The insert was then excised with Sall and cloned into pTet-Splice (20). An expression vector for activated c-Raf (c-Raf S338D/Y341D/T491E/S494D (DDDE)) was obtained from K.-L. Guan (21).

**Adenoviruses**—The AdenoEasy system was used to create recombinant viruses bearing the PID or its inactive control PID L107F (22). The PID or PID L107F was subcloned into pAdTrack cytomegalovirus, and this plasmid, along with the adenoviral backbone plasmid, pAdEasy-1, was cotransfected into B/5183 bacteria. Recombinants were identified by restriction analysis, and the adenovirus was amplified in 293 cells and purified by CsCl step gradient.

**Cell Culture**—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal calf serum. NIH-3T3 cells and derivatives were maintained in DMEM plus 10% calf serum and the antibiotics penicillin and streptomycin. The NIH-3T3 variant S2-6 (20), bearing a tetracycline-regulated transactivator, was used to construct cell lines that inducibly express the Pak1 PID. To prepare stable, regulated clonal cell lines, S2-6 cells were cotransfected with the pTet-Splice-PID plasmids plus a plasmid encoding a puromycin resistance gene using a calcium phosphate precipitation method (23). Forty-eight hours post-transfection, the cells were selected in medium containing 2.5 mM histidinol (to retain the tetracycline-VP16 transactivator), 2 μg/ml puromycin (to select for the tetracycline-regulated expression vector), and 1 μg/ml tetracycline (to repress transgene expression during selection. Clonal cell lines were isolated and expanded, and at least 24 lines were examined for inducible transgene expression by anti-GST immunoblot.

**Transient Transfection**—The cells were plated at 3.2 × 10⁵ cells/35-mm culture dish in DMEM plus 10% calf serum and transfected with expression plasmids using Lipofectamine (Invitrogen). siRNAs were transfected into HeLa cells plated in 24-well dishes using Oligofectamine (Invitrogen) according to the manufacturer’s recommended protocol. For double siRNA, the amounts of each SMARTpool was reduced by a factor of two.

**Immunoblot**—The cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1% glycerol, 1% Nonidet P-40, 50 mM NaF, 10 mM β-glycerol-phosphate) or radioimmune precipitation assay buffer (24) containing 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin. Equivalent amounts of cleared cell lysate extract were fractionated by 10% SDS/PAGE and transferred to polyvinyl difluoride membranes. The membranes were blocked using fat-free milk or 5% bovine serum albumin (for phospho-specific antibodies) in Tris-buffered saline containing 0.1% Tween 20, probed with antibodies, and developed using an alkaline-phosphatase-based chemiluminescent system (PerkinElmer Life Sciences).

**Kinase Assays**—Raf kinase assays were preformed as suggested by Muller and Morrison (24), using endogenous c-Raf immunoprecipitated from 100-μm plates of 25A3 cells. In-gel kinase assays were preformed as described by Ding and Badway (25) with histone H4 (Fluka) as substrate rather than p47phox.

**RESULTS**

**The PID from Pak1 Serves as a Potent Inhibitor of All Group A Paks**—Based on similarities between the group A Paks (Pak1, Pak2, and Pak3), we postulated that expression of the PID (residues 83–149 of Pak1) (18) could serve as a group-specific Pak inhibitor. This construct has previously been used to inhibit Pak1 and Pak3 kinase activity (18), but its ability to inhibit Pak2 has not been assessed. Indeed, Fackler et al. (26) have argued that the Pak1 PID should not affect Pak2 activity, because the N termini of these two kinases differ substantially. This is a particularly important issue because Pak2 is abundantly expressed in most cell types. To evaluate the ability of the Pak1 PID to inhibit group A Paks, recombinant GST-PID or an inactive control (GST-PID Phe107) (18) was added to recombinant His-Pak1, which was then activated by exposure to GTP-loaded Cdc42 and assayed for kinase activity. As expected, the Pak1 PID effectively blocked Pak1 activation, whereas the inactive inhibitor did not (Fig. 1A). The Pak1 PID also inhibited Pak2 activity to approximately the same extent as it inhibited Pak1 (Fig. 1B).

Similar behavior was observed in cell-based assays. U2OS cells were infected with recombinant adenovirus expressing GST-PID or GST-PID Phe107 (Fig. 1C). One day after adenoaviral infection, the cells were cotransfected with an expression plasmid encoding an active allele of Myc-Cdc42 and an expression plasmid encoding HA-Pak1 or -Pak2. These kinases were subsequently immunoprecipitated and tested for autokinase activity. As shown in Fig. 1D, the PID of Pak1 potently inhibited both Pak1 and Pak2. Group B Paks, such as Pak4 and Pak5, were unaffected (data not shown). Because the Pak1 PID has already been shown to act on Pak3 (18), these results show that, despite the sequence differences between Pak1 and Pak2, this protein fragment can be used as a general inhibitor of all three group A Paks in cells.

To evaluate the physiological effect of ablation of group A Pak activity, we engineered a cell line that inducibly expresses a GST-tagged Pak1 PID construct. When grown in the presence of tetracycline, these cells showed no detectable expression of the PID; in the absence of tetracycline, the PID was strongly induced (Fig. 2A). This inducibility was not affected by the presence of growth factors or calf serum; inducible expression of the GST-PID construct was seen when cells were maintained in serum-free DMEM or in DMEM supplemented with calf serum as long as tetracycline was absent (data not shown).

We determined whether this construct could inhibit endogenous Paks in NIH-3T3 cells. To evaluate the activity of endogenous Paks, we relied on an in-gel kinase assay with histone H4 as substrate, because this assay allows simultaneous readouts for Pak1 and Pak2 (Pak3 is not resolvable from Pak1 on standard SDS/PAGE, but is, in any case,
and EGF (Fig. 2C). When the PID was expressed, these renaturable kinases, which migrated at the same molecular mass as Pak1 and Pak2 (Fig. 2B), were inhibited irrespective of the stimulus used. It is important to note that there are several renaturable, H4 kinases of unknown origin that were unaffected by expression of the PID, suggesting that the inhibition is highly selective for group A Paks. These results indicate that the PID serves as a potent and specific inhibitor of group A kinase activity in vitro and in vivo.

Group A Paks Are Required for PDGF, but Not EGF or Lysophosphatic Acid. Stimulation of ERK—The ERK cascade is critical to many of the cellular responses to growth and motility signals. Two components of this cascade, c-Raf and Mek1, are thought to be targets of group A Pak kinase activity, suggesting that the loss of Pak activity might affect ERK activation. The cells were grown to ~75% confluence in the presence of tetracycline and then transferred to serum-free DMEM ± tetracycline for 19–22 h prior to stimulation with growth factors. ERK activity was measured 5 min after growth factor stimulation, because maximal ERK activity was seen at this time point for most growth factors that we tested, and this property was not affected by PID expression (data not shown). As demonstrated in Fig. 3A, inhibition of group A kinase activity inhibited PDGF but not EGF or lysophosphatic acid-mediated stimulation of ERK. Expression of the PID had no effect on the activation of Ras by either PDGF or EGF, indicating that this inhibitor does not interfere with proximal signaling from these receptors (Fig. 3B). The inability of PID expression to inhibit EGF activation of ERK was not due to overstimulating cells with excess growth factor, because similar effects were seen even at the lowest concentrations of EGF (Fig. 3C). These results, combined with those in Fig. 2, show that group A Pak activity is required for ERK activation downstream of the PDGF, but not the EGF receptor, and suggest that these two receptors employ different strategies to activate ERK.

As an independent check on the PID approach, we also performed gene knockdown experiments, using siRNA pools against Pak1, Pak2, or both together. For these experiments, we used HeLa in place of NIH-3T3 cells, because of their ease of transfection. Transfection with siRNA pools directed against human Pak1 gave potent and selective knockdown of this protein (Fig. 4A). Similarly, Pak2 siRNAs yielded knockdown of Pak2. When transfected together, the Pak1 and Pak2 siRNA pools caused a marked decrease in the expression levels of both these proteins. Having established that these siRNA pools are potent and selective, we tested their effect on ERK activation by EGF and PDGF. As
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FIGURE 3. Effects of PID on ERK activation by PDGF and EGF. A, S2-6 cells expressing tetracycline-regulated PID were grown under repressive (Tet−) or permissive (Tet+) conditions as indicated. Quiescent cells were stimulated with the indicated growth factors and assayed for PID expression, ERK activity (pERK), and total ERK. B, cell lysates were incubated with Ras RBD beads, and the bound proteins were eluted and separated by SDS/PAGE. An anti-Ras immunoblot is shown. C, quiescent S2-6 cells induced for PID expression were treated with increasing concentrations of growth factor (PDGF: 0, 0.82, 1.25, 2.5, 5.0, and 10 ng/ml; EGF: 0, 0.031, 0.062, 0.125, 0.25, and 0.5 ng/ml) for 5 min and assayed for ERK activity (pERK), and total ERK. The data were quantified by densitometry. LPA, lysophosphatidic acid.

in NIH-3T3 cells. Erk was strongly activated by EGF and by PDGF in HeLa cells (Fig. 4B). Knockdown of Pak1 or Pak2 alone had little effect; however, simultaneous knockdown of both proteins selectively reduced ERK activation by PDGF but not by EGF. These results are consistent with those obtained in NIH-3T3 cells using the PID fragment to simultaneously reduce the function of all group A Paks. Further, these data show that Pak1 and Pak2 are likely to share overlapping function in ERK activation, and that, in cells expressing these two forms of Pak, both must be inhibited to block ERK activation by PDGF, but that neither are required for ERK activation by EGF.

Group A Paks Are Selectively Required for PDGF-mediated Activation of Mek. Unrelated to Its Effects on Mek Ser298 Phosphorylation—The results above indicate that Pak1 is required for PDGF, but not EGF stimulation of ERK. We next examined the upstream kinase required for ERK activation, Mek. Pak1 is particularly interesting, because this protein has been reported to be a direct substrate for Pak1, and the phosphorylation of Mek1 by Pak1 at Ser298 is thought to be required for Mek1 activation (10, 11). As with ERK, we found that expression of the PID resulted in a PDGF-specific ~70% reduction in Mek1 activation, as assessed by phosphorylation of Mek1 at Ser298 (Fig. 5A). We also confirmed these results using an siRNA approach. Combined knockdown of Pak1 and Pak2 in HeLa cells prevented Mek activation by PDGF but not by EGF (Fig. 5B). We then examined the effect of PID expression on Mek1 Ser298 phosphorylation. Unexpectedly, we found that Mek1 activity and Mek1 Ser298 phosphorylation were not coupled. In control cells, Mek1 was heavily phosphorylated at Ser298 under basal and growth factor-stimulated conditions, and expression of the PID blocked this phosphorylation (Fig. 5C). These results are consistent with the idea that Paks act as Mek Ser298 kinases in vivo but cast doubt on the relevance of this phosphorylation to growth factor-mediated activation of Mek and ERK.

Group A Paks Are Required for Ser338 Phosphorylation of c-Raf—To investigate whether the reduced Mek1 activity in cells expressing the PID is due to defects in c-Raf activation, we examined the phosphorylation status and activity of c-Raf. Both PDGF and EGF promoted phosphorylation of c-Raf at Ser338, as determined by immunoblotting with phospho-specific sera (Fig. 6A). Expression of the PID strongly inhibited this phosphorylation by either growth factor, consistent with the notion that group A Paks are required for the phosphorylation of c-Raf at Ser338. Interestingly, in the case of EGF, the phosphorylation of c-Raf at this site did not strictly correlate with enzymatic activity, as assessed by immune complex protein kinase assay. PDGF activated c-Raf ~8-fold; this activation was inhibited about 40% by the PID (Fig. 6B). In contrast, EGF had a more modest stimulatory effect on c-Raf; this effect was not significantly inhibited by the PID.
If Paks are required for efficient phosphorylation and activation of c-Raf by PDGF, then expression of an appropriate phosphomimic c-Raf mutant should bypass this requirement. Pid-expressing cells were transfected with a constitutively activated form of c-Raf (21) and assayed for ERK activity under basal and growth factor-stimulated conditions. This activated form of Raf stimulated ERK activity, and the stimulation was unaffected by Pid expression (Fig. 6). These results show that the Pid acts primarily at the level of Raf, either by interfering with its catalytic activity and/or its ability to couple to downstream effectors.

DISCUSSION

A number of groups have shown that group A Paks can phosphorylate Mek1 at Ser298 and c-Raf at Ser338. In the case of Mek1, the Ser298 phosphorylation is thought to increase the affinity of Mek for ERK and/or c-Raf (11), thus assisting in activation of the cascade. In fibroblasts, such phosphorylation has been reported to be essential for activation of ERK in response to growth factor and integrin signals. In the case of c-Raf, phosphorylation at Ser338 is thought to relieve autoinhibition (8, 12). However, there is substantial controversy regarding the role of Paks in the ERK cascade. For this reason, we reassessed the function of endogenous group A Paks in this signaling pathway using a loss of function technique.

Using two independent approaches, a peptide inhibitor and siRNA, we found that group A Pak activity is required for ERK activation by PDGF but not by EGF. This result is surprising, because the receptors for PDGF and EGF are thought to use similar mechanisms to activate the ERK cascade. However, recent studies have indicated that the signaling mechanisms activated by these receptors are distinct. For example, Mann’s group has recently shown that the phosphorylation profile elicited by PDGF is substantially different from that elicited by EGF (27).

In the present study, we found that PDGF-dependent ERK inhibition is mirrored by reduced Mek Ser217/221 phosphorylation, which is known to correlate with Mek activity (28). However, this inhibition is not due to the loss of Mek1 phosphorylation on Ser298. Under all of the conditions tested, expression of the PID strongly suppressed Mek1 Ser298 phosphorylation, but this suppression did not correlate with Mek activity. Thus, our data confirm that Pak activity is required for Mek1 Ser298 phosphorylation but also show that phosphorylation at this site is not required for ERK activation by EGF. These results are seemingly at odds with those of Slack-Davis et al. (10) and Edin and Juliano (29), who have reported that Pak-mediated phosphorylation of Mek1 Ser298 and c-Raf Ser338 is essential for adhesion signal propagation to ERK in NIH-3T3 cells. It is possible that the requirement for Pak-mediated Mek1 Ser298 phosphorylation applies to integrin-generated signals but not to growth factor-generated signals.

Reduced Mek activation in PID expressing cells is likely to be the result of the reduced ability of c-Raf to phosphorylate Mek. c-Raf kinase activity and Ser338 phosphorylation in response to PDGF are both reduced by reduction of group A kinase activity; however, the reduced c-Raf activity is still nearly equal to that seen with EGF stimulation (Fig. 6B). Why then, are PDGF stimulation or Mek and Erk so severely affected? It is known that Raf activity in vitro is often not strictly correlated with its effects on signaling. For example, some transforming mutants of v-Raf are less active than the wild-type kinase (30). In such cases, it is thought that changes in Raf complexes (e.g. with c-Raf) mediate the effects on signaling. It is possible that, rather than its mild inhibitory effects on c-Raf kinase activity, the loss of Pak function has a greater effect on the ability of PDGF-stimulated c-Raf to efficiently couple to Mek, perhaps by affecting its interactions with other Raf members or with specific scaffolding proteins. This latter possibility is strongly supported by the recent report that the Mek scaffolding protein MP1 regulates Pak-dependent ERK activation by adhesion but is not required for ERK activation by PDGF (31).

In the case of PDGF, our results suggest that PDGF activates Pak and that, in this setting, Pak-dependent phosphorylation of c-Raf at Ser338 is required for activation of the ERK cascade in fibroblasts. In the case of EGF, loss of group A Pak activity inhibits the phosphorylation of c-Raf at Ser338, but, in contrast to the results with PDGF, c-Raf activity is nearly unaffected. These findings suggest that c-Raf activation in response to EGF circumvents the requirement for Ser338 phosphorylation. As discussed above, these results may indicate that different scaffold proteins engage c-Raf when cells are activated by PDGF versus EGF.
Role of Pak in ERK Activation

To date, the contribution of Paks to c-Raf activation have relied predominantly on the use of inhibitors of Pak activators or dominant negative alleles of Pak or Rac/Cdc42 (32). Wortmannin and LY294002, established PI3K inhibitors, have been used as means to inhibit Pak (14), because Pak activation is thought to rely on a Ras to PI3K to Cdc42 or Rac pathway (33, 34). In cells treated with such inhibitors, Ser338 phosphorylation of c-Raf was retained even under conditions in which PI3K and downstream signaling molecules such as Pak were strongly inhibited, prompting the conclusion that Ser338 phosphorylation of c-Raf is independent of Pak (14). However, because PI3K inhibitors inhibit a protein several steps upstream of Pak and also because Pak can be activated by a variety of different mechanisms, the use of such inhibitors is not ideal for this purpose. On the other hand, dominant negative (kinase-dead) alleles of Pak have been used to support the notion that Paks are required for c-Raf Ser338 phosphorylation, but these results are difficult to interpret because of the propensity of overexpressed, dominant negative molecules to sequester limiting signaling components. This is likely to be true for Paks, which bind SH3 proteins such as Nck, Grb2, and PIX; GTPases such as Cdc42 and Rac; and a host of other signaling proteins. Because the PID is not known to bind any proteins besides Pak itself, we believe the results obtained by this approach are less subject to misinterpretation. In addition, our siRNA data show that Pak1 and Pak2 are likely to share a common function in ERK activation and that both must be inhibited to elicit this signaling defect.

The regulation of c-Raf kinase activity is complex and involves a variety of different regulatory mechanisms (32, 35). The Ser338 and Tyr340/341 sites that are thought to be targeted by Pak and Src, respectively, are critical to the activation of c-Raf. The proximity of these sites and the results of a variety of different mutational studies suggest that these two sites operate, either in isolation or cooperatively, to relieve Raf autoinhibition (36, 37). This notion is supported by early observations that truncation of the C terminus leads to constitutively activation of c-Raf. The interdependence of these sites is not entirely clear, because early results suggested that mutationally inactivating Ser338 prevented c-Raf activation by Src, but more recent results have suggested that c-Raf activation by either site can occur independently of the other site. For example, Alavi et al. (16) demonstrated that two pro-angiogenic factors both activate Raf but do so by different mechanisms; basic fibroblast growth factor stimulates c-Raf by a Pak and Ser338-dependent mechanism, whereas vascular endothelial growth factor stimulates c-Raf in a Src and Tyr340/341-dependent mechanism. In each case the specificity of the phosphorylations was nearly absolute; very little vascular endothelial growth factor-induced c-Raf Ser338 phosphorylation or basic fibroblast growth factor-induced Tyr340/341 phosphorylation was observed. However, another recent report showed that permeabilized cells loaded with GTP activate c-Raf by a mechanism that does not require Ser338 phosphorylation (13). In light of the work by Alavi et al. (16), a possible explanation for these seemingly contradictory results is that GTP loading of permeabilized cells promotes the Src-dependent activation of c-Raf. These results suggest that, although Ser338 phosphorylation correlates well with Pak-dependent c-Raf activation, Ser338 phosphorylation is clearly dispensable for c-Raf activation by certain stimuli. If so, these results also mean that phospho-specific Ser338 antibodies might not be generally useful as a surrogate marker for Raf activation.

Despite these reservations, whether Ser338 phosphorylation is required for maximal activation of c-Raf still remains to be determined. In the works cited above, Src-mediated activation of Raf was shown occur without Ser338 phosphorylation, but whether c-Raf would be further activated by Ser338 phosphorylation remains an unanswered ques-

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