Differential Regulation of Raf-1, A-Raf, and B-Raf by Oncogenic Ras and Tyrosine Kinases*

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It has previously been shown that maximal activation of Raf-1 is produced by synergistic signals from oncogenic Ras and activated tyrosine kinases. This synergy arises because Ras-GTP translocates Raf-1 to the plasma membrane where it becomes phosphorylated on tyrosine residues 340 and 341 by membrane-bound tyrosine kinases (Marais, R., Light, Y., Paterson, H. F., and Marshall, C. J. (1995) EMBO J. 14, 3136–3145). We have examined whether the other two members of the Raf family, A-Raf and B-Raf, are regulated in a similar way to Raf-1. A-Raf behaves like Raf-1, being weakly activated by oncogenic Ras more strongly activated by oncogenic Src, and these signals synergize to give maximal activation. B-Raf by contrast is strongly activated by oncogenic Ras alone and is not activated by oncogenic Src. These results show that maximal activation of B-Raf merely requires signals that generate Ras-GTP, whereas activation of Raf-1 and A-Raf requires Ras-GTP together with signals that lead to their tyrosine phosphorylation. B-Raf may therefore be the primary target of oncogenic Ras.

Biochemical studies in vertebrate cells together with genetic analysis in Caenorhabditis elegans and Drosophila melanogaster have defined a conserved signal transduction pathway consisting of receptor tyrosine kinases, p21ras, Raf serine/threonine protein kinases, Mek (ERK activator or MAPKK) dual specificity kinases, and ERK (MAPK) serine/threonine protein kinases. One important target of this signal transduction pathway is the phosphorylation of transcription factors of the Elk and Ets families by ERKs (2). Signaling through this pathway can mediate differentiation, proliferation, or oncogenic transformation, depending on cellular context (3). While the overall organization of the pathway in all cell types appears to be similar, there may be important differences in detail that could contribute to the specificity, magnitude, or duration of signal output. For example, while C. elegans and D. melanogaster appear to have only one Ras homologue (4), mammalian cells contain Ha-, N-, and Ki-Ras, as well as TC21 and R-Ras (5–7), all of which are potentially capable of interacting with Raf protein kinases. Furthermore, C. elegans and D. melanogaster appear to have only one Raf kinase (lin45 and D Raf, respectively), whereas mammalian cells contain 3 Raf genes encoding Raf-1 (otherwise known as c-Raf) A-Raf, and B-Raf (8, 9). To add further complexity, B-Raf has been shown to exist in multiple spliced forms (10, 11). A notable difference between B-Raf and Raf-1 is the absence of two tyrosine phosphorylation sites (equivalent to Tyr-340 and -341 in Raf-1) that are involved in the Ras-dependent activation of Raf-1 by tyrosine kinases (1, 12). The presence of aspartic acid residues at the equivalent positions in B-Raf suggests that its regulation may differ from Raf-1. Tyrosines equivalent to 340/341 of Raf-1 are also absent in D Raf and lin45, and, as in B-Raf, at least one of these residues is an acidic amino acid.

Most work on the regulation of Raf protein kinases in vertebrate cells has studied Raf-1 (13) suggesting a need to determine whether A-Raf and B-Raf are similarly regulated. In cardiac myocytes, phorbol ester or endothelin treatment activates Raf-1 and A-Raf, but fibroblast growth factor only activates Raf-1 (14). B-Raf is preferentially activated in NIH3T3 cells following serum treatment (15) and in PC12 cells following nerve growth factor treatment (16). These results demonstrate that there may be differential regulation of different members of the Raf family.

Both biochemical studies and genetic analysis show that Ras proteins play a central role in the activation of Raf protein kinases. We and others have argued that Ras-GTP recruits Raf-1 to the plasma membrane for activation by membrane-mediated events that include phosphorylation by membrane-bound tyrosine kinases (1, 17–19). It is not known how A-Raf and B-Raf respond to such signals. Previous work has shown that when expressed in mammalian or insect cells Raf-1 is activated by expression with oncogenic Ras or an oncogenic tyrosine kinase, but that the strongest activation is seen when co-expressed with both oncogenic Ras and an oncogenic tyrosine kinase (20). We have therefore used mammalian cell expression to compare the activation of Raf-1, A-Raf, and B-Raf by oncogenic Ras, an oncogenic tyrosine kinase (SrcY527F), or the combination of oncogenic Ras and SrcY527F.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The cDNAs for the Myc-tagged human Raf-1, SrcY527F, and oncogenic Ras (Ha-Ras Gly-12 → Arg) were cloned into the vector pEFpLink.2 and have been described previously (1). The cDNAs for A-Raf and B-Raf were also cloned into pEFpLink.2 incorporating a N-terminal Myc epitope tag (EQKLISEEDL) recognized by the 9E10 monoclonal antibody (21). COS7 cells were transiently transfected with the LipofectAMINE™ reagent (Life Technologies, Inc.) following a protocol previously described for NIH3T3 cells (1). The cells were allowed to recover for 40 h, serum-starved for 24 h, and harvested.

Assays for Raf Protein Kinase Activity—The cells were extracted and the Raf proteins were quantitated as described previously for NIH3T3 cells (1). The activity of each Raf protein was assessed following the procedures of Alessi et al. (22). Briefly, the Raf proteins were immunoprecipitated for 2 h at 4 °C from 3–50 μg of cell extracts (dependent on...
Raf construct and the efficiency of transfection) with ~20 µg of 9E10 monoclonal antibody immobilized on Protein G-Sepharose. The beads were subjected to 1 × 500 µl wash with wash buffer (30 mM Tri-HCl, 0.2 mM EDTA, 0.3% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.1% (w/v) Triton X-100, 5 mM NaF, 0.2 mM Na3VO4, pH 7.5) containing 1.0 mM KCl, 1 × 500 µl wash with wash buffer containing 0.1 × KCl, and 1 × 500 µl wash with wash buffer. The beads were resuspended in 20 µl of reaction buffer (1) containing 6.5 µg/ml GST-Mek1 or GST-Mek2 and 100 µg/ml GST-ERK2. The beads were incubated at 30 °C for 5–30 min, and the reaction was terminated by the addition of 20 µl of reaction buffer lacking MgCl2 and ATP, but containing 20 mM EDTA. The supernatants were collected and the activity of the GST-ERK2 in 10 µl of supernatant was determined using MIF as a substrate, as described previously (1).

Microinjection and Immunofluorescence Analysis—These were performed as described previously (18).

RESULTS

Ras-GTP Translocates All Raf Proteins to the Plasma Membrane—In order to study the activation of the Raf proteins, we constructed plasmids to express Raf-1, A-Raf, and B-Raf, each with a N-terminal Myc (9E10) monoclonal antibody tag (21). The version of B-Raf used corresponds to the protein with a mass of 86 kDa. In the yeast two-hybrid systems (23) or in vitro using immobilized Ras-GTP (24). We and others have previously shown that the interaction of Raf-1 with Ras-GTP leads to translocation of Raf-1 to the plasma membrane which is a key step in Raf-1 activation (1,17,18).

We tested whether the interaction of other members of the Raf family with Ras-GTP could translocate them to the plasma membrane. Madin-Darby canine kidney cells were microinjected with expression vectors for the Myc-tagged Raf constructs in the absence or presence of expression vectors for oncogenic Ras. The cells were stained for the recombinant Raf proteins using the 9E10 monoclonal antibody or for the Ras proteins using a mixture of the monoclonal antibodies Y13-238 and Y13-259. All three Raf proteins were found to be located in the cytoplasm when expressed alone, but when co-expressed with oncogenic Ras all were found translocated to the plasma membrane (Fig. 1). The results we observed with B-Raf are in contrast to those obtained by Jelinek et al. (19) who, using biochemical fractionation techniques, failed to observe B-Raf associated with the particulate fraction of Ras transformed cells. The reason for this discrepancy is not clear but may reflect a greater sensitivity of the immunocytochemical methods we have used. The translocation to the plasma membrane of all Raf proteins is dependent on Ras being in the active GTP-bound state, since co-injection of Raf expression vectors with an N17Ras expression vector did not lead to membrane translocation (data not shown). The replacement of Ser-17 by Asn(N17Ras) has been shown to generate a form of Ras that is mainly in the GDP-bound state and cannot undergo the conformational switch on binding GTP (25,26).

Ras and Src Synergize to Activate Raf-1 and A-Raf, whereas B-Raf Is Maximally Activated by Oncogenic Ras Alone—We next examined whether A-Raf and B-Raf are activated by oncogenic Ras and oncogenic Src. Plasmids expressing each Raf protein were transected into COS7 cells alone or together with oncogenic Ras, SrcY527F, or with both oncogenes together. We have previously demonstrated that when Raf-1 is expressed in NIH3T3 cells together with oncogenic Ras and SrcY527F, it becomes phosphorylated, and consequently its mobility in SDS gels is reduced (1). In COS cells, Raf-1 migrates as a protein with a mass of ~70,000 (Fig. 2A, closed arrow), and this mobility is little affected by the presence of oncogenic Ras (Fig. 2A, lanes 1 and 2). In the presence of SrcY527F, a small portion of Raf-1 undergoes a reduction in mobility (Fig. 2A, lanes 1 and 3). By comparison, in the presence of both oncogenic Ras and SrcY527F, a greater proportion of Raf-1 undergoes a larger mobility shift than in the presence of SrcY527F alone (Fig. 2A, lanes 3 and 4). It has been shown previously that the mobility shift characteristic of Raf-1 that results from activation of ERK kinases does not directly reflect the activation status of Raf-1 (27,28).

A-Raf is also subject to a Ras- and Src-induced mobility shift, although to a lesser degree than seen for Raf-1. In this gel system, A-Raf migrates with a mass of ~68,000 (Fig. 2A, open arrow) and undergoes a weak substoichiometric mobility shift in the presence of oncogenic Ras, SrcY527F, or in the presence of both oncogenes together (Fig. 2A, lanes 5–8).

B-Raf migrates as a protein with a mass of 93 kDa on this gel system (Fig. 2A, lane 9, open arrowhead) although its calculated mass including the Myc tag (1,510) is 86 kDa. In the presence of oncogenic Ras alone, B-Raf undergoes a large, stoichiometric shift in mobility, with an apparent increase in mass of ~10,000 (Fig. 2A, lane 10, closed arrowhead). In the presence of SrcY527F, there is a minor shift in B-Raf mobility, and, in the presence of both oncogenes, the degree of shift is similar to that seen with oncogenic Ras alone (Fig. 2A, lanes 9–12).

In these experiments, the plasmid ratios have been optimized to give similar amounts of Raf protein in each sample. However, there is some variability in the accumulation of Raf protein as a consequence of transfection variability and the actions of the oncogenes on the expression of Raf proteins. Therefore, in order to ensure equivalence in the subsequent kinase assays, the amount of Raf in each sample was determined by quantitative immunoblot analysis, using the 9E10 monoclonal antibody and iodinated protein A in conjunction with a PhosphorImager (data not shown). The samples were then mixed with appropriate extracts from cells transfected.
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with oncogenic Ras, SrcY527F, or both oncogenes (but in the absence of Raf) to give samples with the same amount of cellular proteins and the same amount of exogenous Raf protein, but differing in which Raf isotype they contain.

We next examined the activation of the Raf kinases. Equivalent amounts of each kinase were immunoprecipitated with the 9E10 monoclonal antibody and subjected to a cascade kinase assay in which the Raf proteins were used to phosphorylate and activate recombinant GST-Mek1 which in turn was used to phosphorylate and activate recombinant GST-Erk2, both of which were produced in bacteria (22). We have previously shown that Raf-1 is weakly activated by oncogenic Ras and more strongly activated by SrcY527F, but both oncogenes synergize to give maximal Raf-1 activity (1). Fig. 2B shows that like Raf-1, A-Raf requires synergistic interactions from both oncogenic Raf and SrcY527F for maximal activation. B-Raf is clearly different from the other two Raf proteins. It is highly activated by oncogenic Ras and is not activated by Src, and there is no synergy between Ras and Src. To determine whether the activation of B-Raf by oncogenic Ras requires plasma membrane localization, we tested the ability of the HrasV12S186 double mutant to activate B-Raf. This double mutant is “locked” in the GTP-bound active state by the Gly-12 → Val mutation, but because Cys-186 is mutated to a serine it cannot be farnesylated and localized at the membrane (29). HRasV12S186 failed to activate B-Raf or any other of the Raf proteins (data not shown).

Overall the response of A-Raf to oncogenic Ras or Src and the synergy between Ras and Src for activation is similar to Raf-1. However, it is clear that the level of A-Raf activity obtained under maximal activation conditions is only ~20% of that for the same amount of Raf-1 and even less compared to an equivalent amount of B-Raf (Fig. 2B). We considered a possible explanation for this difference to be that A-Raf was unable to phosphorylate and activate GST-Mek1 as efficiently as Raf-1 and B-Raf. We therefore examined whether A-Raf was able to activate GST-Mek2 and how this compared to the abilities of Raf-1 and B-Raf to do likewise. We find that there is no difference in the ability of any of the Raf kinases to phosphorylate and activate either GST-Mek1 (Fig. 2C). Importantly, the activity of A-Raf from cells transfected together with oncogenic Ras and SrcY527F is also only ~20% that seen with Raf-1 under these conditions. We conclude that with at least two substrates the activity of A-Raf is considerably lower than Raf-1 under comparable conditions. These data differ from the recent data of Wu et al. (30) who have shown that A-Raf is a better activator of GST-Mek1 than of GST-Mek2 and we are unable to explain the differences between their data and ours.

B-Raf Has an Elevated Basal Kinase Activity That Is Independent of the Ras-Raf Interaction—When equivalent amounts of each Raf protein are compared, it is clear that not only is B-Raf maximally activated by oncogenic Ras alone, but that it also has a much higher basal activity than Raf-1 or A-Raf. Fig. 2B shows that the basal activity of B-Raf when expressed in serum-starved cells is 15–20 times that of Raf-1 expressed.

Fig. 2. Activation of Raf-1, A-Raf, and B-Raf by oncogenic Ras and Src. A, immunoprotein blot analysis. COS cells were transfected with expression vectors for N-terminally Myc-tagged Raf-1, A-Raf, or B-Raf together with either empty expression vector (−), with oncogenic Ras (Ras), with SrcY527F (Src), or with both oncogenes (Ras/Src). The cells were serum-starved for the last 24 h of incubation, and detergent extracts were prepared. 40 μg of protein were analyzed by immunoprotein blot analysis with the 9E10 monoclonal antibody, using iodinated protein A to detect the Raf proteins. An autoradiogram of the blot is shown. The position of migration of Raf-1 (closed arrow), A-Raf (open arrow), B-Raf (open arrowhead), and shifted B-Raf (closed arrowhead) are indicated as are the positions of migration of marker proteins (to the right of the figure). B, activation of Raf-1, A-Raf, and B-Raf by Ras and Src using Mek1 as a substrate. The amount of Raf kinase in each of the samples from panel A was determined by PhosphorImager analysis. Each sample was mixed with control extracts from cells transfected with appropriate oncogene combinations but in the absence of exogenous Raf to generate samples with the same amount of cellular proteins, the same amount of exogenous Raf protein, but differed from each other in which oncogenes and which Raf isotype they contained. The exogenous Raf from equivalent amounts (16 μg) of these extracts was immunoprecipitated with the 9E10 monoclonal antibody, and a cascade kinase assay was performed using GST-Mek1 as a Raf substrate. The results are expressed as fold activation where one unit of activity is defined as the amount of activity seen with Raf-1 in the absence of any oncogenes and in this experiment represents ~259 cpm of 32P (Cerenkov channel) incorporated into MBP. The assay shown represents samples from a particular transfection in which the samples were assayed in triplicate. Standard deviations are indicated as error bars; where no error bars are shown, the standard error is less than 2% of the x-axis co-ordinate scale. Similar results were obtained in 3 independent experiments.
under the same conditions. Since B-Raf is highly responsive to Ras-GTP, we considered it possible that the high basal activity of B-Raf was a consequence of its interaction with the low level of Ras-GTP that exists even in serum-starved cells. Depending on cell type and method of analysis, the basal level of Ras-GTP in unstimulated cells is between 0.5 and 10% (31, 32). Using radioactive methods we routinely see about 5% Ras-GTP in serum-starved COS cells (data not shown). To determine whether the basal kinase activity of B-Raf depends on interaction with Ras-GTP, we constructed an Arg-187 → LeuB-Raf (R187LB-Raf) mutant. This mutation mimics the Arg-89 → Leu mutation of Raf-1 which cripples the interaction of the Ras binding domain and Ras-GTP (33, 34) and prevents B-Raf from being recruited to the plasma membrane when microinjected with oncogenic Ras (data not shown). We therefore determined the basal level of kinase activity of R187LB-Raf and found it to be indistinguishable from the activity of wt B-Raf (R187LB-Raf activity = 88% ± 12% of that of wt B-Raf). Thus the basal kinase activity of B-Raf is not a consequence of activation through interaction with basal Ras-GTP.

Role of the Tyrosine Residues at Amino Acids 301/302 in A-Raf and Aspartate Residues at Amino Acids 447/448 in B-Raf in Determining Response to Oncogenic Tyrosine Kinases, Basal Kinase Activity, and Ras Responsiveness—The similarity to Raf-1 in the pattern of response of A-Raf to oncogenic Src or Ras suggested that Ras-dependent tyrosine phosphorylation is required for activation of A-Raf. In A-Raf amino acids 301/302, which are equivalent to Tyr-340/341 of Raf-1, are also tyrosines suggesting that these amino acids play a role in the activation of A-Raf. We substituted Tyr-301 and -302 in A-Raf with aspartic acid residues (A-RafDD) or phenylalanine residues (A-RafFF) and compared the activation of these mutants with oncogenic Ras or Src.

Fig. 3A shows that substitution of Tyr-301 and -302 in A-Raf with phenylalanine eliminates the ability of A-Raf to be activated by SrcY527F, either with or without oncogenic Ras. Substitution with aspartic acid generates a protein that has an elevated basal level of activity of about 2.5 times that of wt A-Raf (p ≤ 0.01). A-RafDD is strongly activated by oncogenic Ras and is not activated by SrcY527F, and there is no synergy by these oncogenes in activating A-RafDD. These results essentially parallel those we previously obtained with Raf-1 although, unlike Raf-1, the levels of activation of A-RafDD are lower than is seen with wt A-Raf together with oncogenic Ras and SrcY527F (1). These data reinforce the conclusion that the regulation of A-Raf parallels that of Raf-1 and that tyrosine phosphorylation plays a key role in regulating the activity of these two Raf proteins by oncogenes.

Amino acids 447 and 448 in B-Raf, equivalent to Tyr-340/341 in Raf-1, are aspartic acid residues. We have previously shown that substituting Tyr-340/341 in Raf-1 with aspartic acid residues generates (Raf-1DD) a kinase which like B-Raf is very responsive to oncogenic Ras and has a high basal activity (1). We therefore investigated whether Asp-447/448 are responsible for the Ras responsiveness and high basal activity of B-Raf. Substitution of aspartic acid residues 447 and 448 in B-Raf with phenylalanine markedly reduces B-Raf basal kinase activity (Fig. 3, compare lanes J and 9) and reduced its responsiveness to oncogenic Ras to ~30% of wild type levels. This is in contrast to what is seen with Raf-1Y340F/Y341F (Raf-1FF) and A-RafFF which cannot be activated by oncogenes (Ref. 1 and Fig. 3A). We next examined how substitution of tyrosines at positions 447/448 in B-Raf (B-RafYY) affected activation by oncogenes. The basal kinase activity of B-RafYY is similar to wild type B-Raf, but surprisingly, B-RafYY still responds strongly to oncogenic Ras and is not significantly activated by SrcY527F (Fig. 3B). Furthermore, there is no synergy between oncogenic Ras and SrcY527F in activating B-RafYY (Fig. 3B).

This observation suggests that while aspartic acid residues at 447 and 448 control B-Raf basal kinase activity additional factors control the response to Ras-GTP. The proposal that additional factors to the residues at 447/448 in B-Raf control Ras responsiveness is reinforced by the observation that whereas Raf-1FF is unresponsive to oncogenic Ras (1), B-RafFF retains ~30% of the response of wild type B-Raf to oncogenic Ras (Fig. 3B).

**DISCUSSION**

We have compared the abilities of oncogenic Ras or the activated tyrosine kinase, Y527Fsrc, to activate Raf-1, A-Raf, or B-Raf. B-Raf is shown to be maximally activated by oncogenic Ras unlike Raf-1 or A-Raf. In contrast, oncogenic Src is a...
better activator than Ras of Raf-1 and A-Raf and does not appear to activate B-Raf. Activation of Raf-1 and A-Raf by Src requires tyrosine phosphorylation at residues 340 and 341 in Raf-1 and 301 and 302 in A-Raf. However, as we have demonstrated previously, activation of Raf-1 by tyrosine phosphorylation at amino acids 340/341 is a Ras-dependent event as p21ras-GTP is required to bring Raf-1 to the plasma membrane for tyrosine phosphorylation (1). This requirement for the Ras-Raf-1 interaction for tyrosine phosphorylation of Raf-1 results in the synergy between Ras and Src in Raf-1 activation. We propose that a similar mechanism results in the synergy between Ras and Src for the activation of A-Raf. B-Raf lacks the tyrosines equivalent to 340/341 in Raf-1 and thus cannot be regulated in the same way.

At the positions equivalent to Tyr-340/341 in Raf-1, B-Raf has aspartic acid residues (amino acids 447/448), and these acidic amino acids control at least in part responsiveness to oncogenic Ras. Substitution of Asp-447/448 in B-Raf with phenylalanine markedly reduces the activation by Ras, and substitution of Tyr-340/341 with aspartic acid in Raf-1 or Tyr-301/302 in A-Raf increases responsiveness to Ras. However, since B-Raf/Ff is still more responsive to Ras than Raf-1 or Raf-1FF, additional differences from Raf-1 must also control the response of B-Raf to oncogenic Ras. The nature of the amino acids at these sites also seems to affect the basal rate of kinase activity; B-Raf and Raf-1DD have a 10–20-fold greater basal kinase activity than Raf-1, and B-Raf/Ff has a very much reduced basal rate compared to wild type B-Raf. The kinase activity of A-Raf/Ff is also elevated relative to wild type A-Raf. Since C. elegans lin-45 and D. melanogaster DrAraf have either aspartic acid or glutamic acid at the positions equivalent to Tyr-340/341 in Raf-1 (35, 36), the regulation of these Raf proteins is likely to resemble B-Raf rather than Raf-1 or A-Raf. Thus for these Raf protein kinases the signal from the receptor tyrosine kinase to activate Ras may be the only signal needed to activate the MAPK cascade, whereas receptor tyrosine kinase activation of Raf-1 and A-Raf may require Ras activation and tyrosine phosphorylation of Raf.

Surprisingly the levels of activity of A-Raf in these experiments never reached those achieved by activation of Raf-1 or B-Raf. The reason for this discrepancy is not clear. Careful titration of input DNA together with equalization of protein in the samples, using the same epitope tag for each Raf family member, ensured that the same amount of each Raf protein was assayed. Low levels of A-Raf/Mek kinase activity have also been reported by McMahon and collaborators (37) who compared oncogenic versions of the three Raf proteins and found that A-Raf had the least ability to activate Mek in vitro. The lower level of A-Raf activity relative to Raf-1 was reflected in the proportion of protein with a retarded electrophoretic mobility in co-transfection with the activators. The slower migrating forms of A-Raf constituted less than 30% of total A-Raf, whereas at least 50% of the Raf-1 co-transfected with oncogenic Ras and Src had a retarded mobility. Interestingly co-transfection of B-Raf with oncogenic Ras resulted in most of the B-Raf having a retarded electrophoretic ability; co-transfection of B-Raf with activated Src had a much smaller effect. Another observation we have made suggests that there may be some differences in the activation of Raf-1 compared to A-Raf. While increasing the input of Raf-1 expression vector in the transfections had little effect on the activation of Raf-1, increasing the input of A-Raf DNA eventually resulted in A-Raf no longer becoming activated, suggesting that A-Raf activation required some limiting cellular component.

These results point to important differences in the regulation of different Raf family members. Since B-Raf exists in multiple isoforms (10, 11), it is possible that some of the other isoforms of B-Raf may be regulated differently from the isoform studied here. Oncogenic Ras maximally activates B-Raf, unlike Raf-1 and A-Raf and suggesting that B-Raf rather than Raf-1 or A-Raf may be the primary target of oncogenic Ras in cell transformation. Such a hypothesis would be consistent with observations from Takai and collaborators (38) who have shown that a purified complex of B-Raf and 14-3-3 can be directly activated by recombinant Ras in vitro. Jelinek et al. (19) failed to find increased B-Raf activity in Ras transformed cells; however, our own results with Ras transformed cells show that both Raf-1 and B-Raf are activated.2 Our data (1) and that of Jelinek et al. (19) show that activation of Raf-1 by oncogenic Ras requires tyrosine phosphorylation. This tyrosine kinase activity may arise from the basal levels of tyrosine kinase activity in cells or could result from the stimulation of tyrosine kinase activity by autocrine growth factors which are known to be elaborated by Ras transformed cells (39). Thus, cells containing oncogenic Ras could activate B-Raf by direct interaction with Ras-GTP and Raf-1 and A-Raf through Ras-dependent tyrosine phosphorylation.

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