Membrane Localization of Raf Assists Engagement of Downstream Effectors*

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We have previously described a small molecule-directed protein dimerization strategy, using coumeyycin to juxtapose Raf fusion proteins containing the coumeyycin-binding domain GyrB. Oligomerization of cytoplasmically localized Raf-GyrB fusion proteins leads to an increase in the kinase activity of both Raf and its substrate Mek. Surprisingly, more distal targets, such as Erk1 and Erk2, are not activated using this approach. Here we report that coumeyycin-induced oligomerization of a membrane-localized Raf-GyrB fusion protein potently activated Erk1 and Erk2, up-regulated Fos protein levels, and induced expression of many immediate-early response genes. Thus, both membrane localization and oligomerization of Raf-GyrB are required to target Raf signals to downstream effectors. The ability to activate the entire Raf signal transduction cascade conditionally, using coumeyycin-induced oligomerization, should prove useful for dissecting Raf-mediated effects on gene expression and cellular differentiation.

All cells sense and respond to changes in the external environment. They accomplish this, in part, by expressing a diverse set of transmembrane receptors on their surfaces that are capable of recognizing and responding to a large number of distinct ligands. Stimulation of a specific receptor typically leads to the activation of several signal transduction pathways; activation of these distinct pathways effects changes in gene transcription, thereby shaping the response to a particular stimulus. One such pathway, the ubiquitous Ras signal transduction pathway, regulates proliferative responses emanating from a variety of receptors (reviewed in Ref. 1). Ras itself was initially identified based on its potent ability to induce neoplastic transformation of a variety of cell types (2–4). This oncogenic potential is underscored by the finding that over 25% of all human tumors bear activated alleles of ras (reviewed in Ref. 5). More recently, the Ras signaling pathway has been shown to play an important role in regulating many developmental processes, e.g. vulval development in Caenorhabditis elegans, ommatidial development in Drosophila, and lymphocyte development in mice (reviewed in Refs. 6–9). Thus, understanding the molecular mechanisms by which Ras transduces signals will provide important insights into both the process of tumorigenesis and the regulation of cellular differentiation.

Ras activates a number of distinct effector molecules including the serine/threonine kinase Raf, the lipid kinase phosphatidylinositol 3-OH-kinase, the guanine nucleotide exchange factor for Raf, Raf GDP dissociation stimulator, and the Ras GTPase-activating protein (10). A key question concerns the role that these specific effectors play in directly modulating the expression of genes entrained by Ras. To evaluate the effects of stimulating a specific pathway, a method capable of activating discrete downstream effector molecules is required. We sought to engineer such an approach through coumeyycin-mediated intracellular multimerization of target proteins, focusing in particular on Raf.

Raf is normally activated through recruitment to the plasma membrane mediated by an interaction with GTP-bound Ras (11–15). This membrane localization leads to Raf activation via a process that is not yet well understood. To study Raf-specific signal transduction events, we developed an inducible dimerization strategy that allows for the conditional activation of Raf (16). Our approach uses the symmetrically dimeric antibiotic coumeyycin to promote either membrane translocation or dimerization of Raf fusion proteins that bear the coumeyycin-binding domain GyrB. During the course of these initial studies, we found that Raf kinase activity can be induced by Raf dimerization, or more likely oligomerization, even in the absence of membrane localization. This activation of Raf by oligomerization is not unique to coumeyycin-based strategies, as a similar result was obtained using FK1012-induced dimerization of Raf-FKBP chimeras (17). We have now examined more distal effects of Raf activation following coumeyycin-induced oligomerization, and here report that, although coumeycin-induced clustering of cytoplasmic forms of Raf-GyrB does lead to robust activation of Mek, it does not activate downstream signaling effectors such as Erk1 or Erk2, nor does it modulate Raf-regulated gene transcription. In contrast, coumeycin-mediated oligomerization of membrane-localized forms of Raf-GyrB, which are not by themselves constitutively active, leads to dramatic increases in downstream MAP1 kinase activation, Fos protein levels, and Raf-dependent gene transcription. These results show that membrane localization is not necessarily required for Raf activation, but is crucial for targeting Raf signals to distal effectors. In addition, our results demonstrate that coumeycin-induced activation of membrane-localized Raf-GyrB can be a useful tool for identifying both protein effectors and gene targets regulated by Raf.

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The abbreviations used are: MAP, mitogen-activated protein; EGF, epidermal growth factor; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; PMA, phorbol 12-myristate 13-acetate.
EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Novobiocin, coomassie blue, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma and dissolved in Me₂SO before use. Epidermal growth factor (EGF) was purchased from Life Technologies, Inc.

Rabbit polyclonal antibodies used to detect either phosphorylated, active or total Mek were obtained from Transduction Laboratories (Lexington, KY). Alkaline phosphatase-conjugated goat anti-rabbit and rabbit antimouse polyclonal antibodies were obtained from Amersham Pharmacia Biotech as part of their Vistra enhanced chemifluorescence Western blotting kits.

Cells and Cell Culture—NIH3T3 cells were obtained from ATCC (Manassas, VA) and grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Calabasas, CA), 2 mM l-glutamine (Life Technologies, Inc.), 50 units/ml penicillin, and 50 µg/ml streptomycin (Life Technologies, Inc.). NIH3T3 cells were transfected with various Raf-GyrB constructs by the calcium phosphate precipitation method as described previously (19). Individual clones were obtained by limiting dilution of bulk transfected cells.

Plasmid Construction—Construction of the Raf-GyrB plasmids was described previously (16). Raf-GyrB constructs bearing distinct amino termini were generated by PCR-directed mutagenesis. In brief, specific primers (listed below) encoding either the Myc epitope tag or myristylamidation sequences derived from Lck were used in conjunction with a common 3'-oligonucleotide to amplify an approximately 500-base pair fragment encompassing the amino terminus of Raf with the desired amino-terminal extension. PCR amplification was carried out for 25 cycles under the following conditions: 30 s of annealing at 59 °C, 1 min of extension at 72 °C, and 30 s of denaturation at 94 °C. PCR fragments were digested with HindIII and subcloned into pCDNA3-Raf-GyrB. For transfection studies, the various Raf-GyrB chimeras were excised from pCDNA3 by AccI and NotI digestion, the ends were blunted with Klenow, and the resulting fragment subcloned into the bicistronic expression vector EMCV neo.

Primers—Primers are as listed below.

Common 3' primer
5'-TCT GAC AGA TGT CAC AGA AGG-3'

Myc tag primer
5'-CCC AAG CTT ATT ATG GAC CAG AAG CTC ATC GAG GAC TTC AAG CAG AGA GTC CAG TGG-3'

Lck primer
5'-CCC AAG CTT ATT ATG GSC GTC G7A TGG TCA CGA GAC CAT AGA CTA CAA GGA GCT TGG-3'

Lck G2A primer
5'-CCC AAG CTT ATT ATG GSC GTC G7A TGG TCA CGA GAC CAT AGA CTA CAA GGA GCT TGG-3'

Lck C35, C55 primer
5'-CCC AAG CTT ATT ATG GSC GTC G7A TGG TCA CGA GAC CAT AGA CTA CAA GGA GCT TGG-3'

Erk and Mek Phosphorylation Assays—Cells were plated in 100-mm tissue culture dishes at 5 x 10⁶ cells/dish and allowed to adhere overnight. The following day, cells were washed twice with saline-buffered saline (PBS) and then incubated for an additional 18 h in 5 ml of serum-free DMEM containing 2 mM l-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. Cells were subsequently stimulated by adding 5 ml of DMEM containing the appropriate amounts of Me₂SO, novobiocin, coomassie blue, or PMA. At indicated time points, stimulation was achieved by washing cells twice with ice-cold PBS and then lysing them in ice-cold buffer H (20) containing 1% Triton X-100. Lysates were subjected to 1.5-M l-lactate (Beverly, MA). TDS is a murine monoclonal antibody (IgG2b) that recognizes the 24-kDa amino-terminal fragment of the B subunit of bacterial DNA Gyrase (GyrB), and was generously provided by Dr. Martin Gellert (18) (currently available from Luecent Ltd.). Rabbit polyclonal IgG specific for Erk1 and Erk2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Raf monoclonal antibody (IgG1) was obtained from Transduction Laboratories (Lexington, KY).

RESULTS

We have previously demonstrated that coomassie blue-mediated oligomerization of Raf-GyrB fusion proteins leads to robust activation of the direct target of Raf, the dual-specificity
kinase or cytosolic compartments (Fig. 2, of Raf-GyrB chimeric proteins that localized either to mem-
phosphorylation. To test this hypothesis, we generated a series
for coupling Raf/Mek activation to efficient Erk1 and Erk2
the plasma membrane, and this localization may be important
downstream targets Erk1 and Erk2. As shown in Fig. 1A, EGF treatment led to a
a dramatic increase in phosphorylated Erk1 and Erk2 within 5
min of stimulation (17.8-fold induction of phospho-Erk2). In
contrast, coumermycin stimulation led to a very modest (2.7-
fold induction of Erk2) and transient increase in Erk phosphory-
similarity, PMA treatment, which is known to potently
activate the MAP kinase pathway, gave a greater than 20-fold
increase in Erk1 and Erk2 phosphorylation while coumermycin
addition stimulated only weak MAP kinase activation (Fig. 1C). This result was not due to ineffective Mek activation as
coumermycin stimulation of Raf-GyrB transfectants led to Mek
phosphorylation (and hence activation) at comparable levels to
those observed for PMA stimulation of either these same cells
(Fig. 1B and Ref. 16) or non-transfected NIH3T3 cells (data not
shown). These results indicate that, despite robust activation of
Mek (Ref. 16 and Fig. 1B), coumermycin-induced Raf-GyrB
oligomerization does not lead to significant activation of the
downstream targets Erk1 and Erk2.

Activation of Raf normally occurs following its recruitment to
the plasma membrane, and this localization may be important
for coupling Raf/Mek activation to efficient Erk1 and Erk2
phosphorylation. To test this hypothesis, we generated a series
of Raf-GyrB chimeric proteins that localized either to mem-
brane or cytosolic compartments (Fig. 2, A and B). The
membrane-localized form of Raf-GyrB was created by adding an
amino-terminal myristylation and palmitylation sequence de-
lected from the Src family kinase Lck (referred to as Lck-Raf-
GyrB). Previous work has established that this sequence is
sufficient for targeting heterologous proteins to the membrane
compartment, and that both myristylation and palmitylation
are required for this effect (26, 27). We also generated three
cell lines in which either a Myc tag or mutated myri-
stylation/palmitylation sequences were added to the amino ter-
minus of Raf-GyrB. In the first mutant (C5S,C5S), the cysteine
residues required for palmitylation were changed to serines. In
the second mutant (G2A), the glycine residue at position 2 was
changed to alanine. The latter mutation prevents both myri-
stylation and palmitylation, since prior myristylation is re-
quired for palmitylation (27). Using these constructs, we gen-
erated a panel of stable NIH3T3 transfectants. The Lck-Raf-
GyrB constructs were expressed at levels equivalent to
dogenous Raf, and the other three constructs were expressed
at 2–3-fold higher levels, as judged by immunoblotting (data
not shown). Subcellular fractionation studies demonstrated
that the Lck-Raf-GyrB construct was predominate localized
to the membrane fraction, while the other three constructs
localized with endogenous Raf and were found predomin-
antly in the cytoplasm (Fig. 2B).

These cell lines were then tested for the ability of coumer-
mycin to induce Mek and Erk phosphorylation. As seen in Fig.
3 (panels A–D), coumermycin stimulation leads to an increase
in Mek phosphorylation in all cell lines tested. The level of Mek
activation seen in Myc-Raf-GyrB, C3S,C5S, and Lck-Raf-GyrB
transfectants was comparable to that observed when stimulat-
ing these cell lines with PMA. The G2A transfectant responded
to coumermycin to a lesser but still significant extent. In con-
trast, neither the Myc-Raf-GyrB construct nor the G2A or
C3S,C5S constructs exhibited notable, sustained increases in

![Fig. 1. Coumermycin-induced Raf-GyrB oligomerization does not lead to significant activation of Erk1 and Erk2.](image)
Erk activation following coumermycin stimulation (Fig. 3, panels E–G). However, stimulation of Lck-Raf-GyrB transfectants with coumermycin resulted in a rapid and dramatic increase in the level of phosphorylated Erk1 and Erk2 (Fig. 3, panel H). Appreciable activation could be seen within 1 min of stimulation, with maximal phosphorylation occurring after 5 min. Membrane localization mediated by the Lck myristylation/palmitylation sequence did not lead to constitutive activation of the Raf pathway (see Fig. 3, panel H). Moreover, none of the cell lines tested responded to novobiocin (a monomeric form of coumermycin), indicating that the response was truly dependent on coumermycin-induced oligomerization of Lck-Raf-GyrB, and not just due to nonspecific effects of drug binding to the GyrB domain (data not shown). All cell lines tested, irrespective of the construct with which they were transfected, remained responsive to PMA.

Hence, the lack of response to coumermycin did not reflect a generalized defect in the Ras pathway in these transfectants (Fig. 3).

Typically, activation of Erk1 and Erk2 results in the phosphorylation of additional effector proteins including transcription factors such as the Ets family member Elk-1. Phosphorylated Elk-1, in turn, can bind to the promoters of a number of genes including those for c-fos. This leads to an increase in fos mRNA transcription and ultimately an increase in Fos protein abundance (28, 29). Therefore, we examined whether coumermycin stimulation of Raf-GyrB or Lck-Raf-GyrB transfectants could lead to up-regulation of Fos protein levels. As shown in Fig. 4, coumermycin stimulation of Lck-Raf-GyrB transfectants resulted in a significant increase in Fos protein accumulation (3.6-fold induction). This result compares well with that obtained when stimulating cells with PMA. In contrast, Raf-GyrB transfecteds showed no significant increase in Fos protein levels following coumermycin stimulation, although they still remained responsive to PMA (Fig. 4).

To examine this issue more broadly, we compared wholesale changes in mRNA abundance using gene macroarrays. Lck-

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**Fig. 2.** Membrane localization of Raf-GyrB is required for coumermycin-dependent activation of Erk1 and Erk2. A, unique amino-terminal extensions were added to Raf-GyrB consisting of a Myc tag, an 8-amino acid Lck-derived myristylation/palmitylation sequence, or two distinct mutants of the Lck myristylation/palmitylation sequence. These constructs were transfected into NIH3T3 cells, and stably expressing cell lines were generated. B, subcellular localization of Raf-GyrB constructs. Subcellular fractionation was carried out by differential centrifugation as described under “Experimental Procedures.” The ratio of Raf-GyrB to Raf was determined in membrane (M) versus cytosolic (C) fractions. -Fold enrichment of Raf-GyrB constructs in the membrane fraction was determined relative to endogenous Raf and was calculated as the ratio of Raf-GyrB to Raf in the membrane fraction divided by the ratio of Raf-GyrB to Raf in the cytoplasmic fraction. Results for Lck-Raf-GyrB, Raf-GyrB, and Myc-Raf-GyrB are representative of four or more experiments, while the results for the G2A and C3S,C5S constructs are representative examples of two experiments.

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**Fig. 3.** Coumermycin-induced activation of membrane-localized Raf-GyrB leads to increases in Erk1 and Erk2 phosphorylation. NIH3T3 cells were stably transfected with the Myc-Raf-GyrB, G2A, C3S,C5S, or Lck-Raf-GyrB constructs. Cells were serum-starved for 20 h and then stimulated with either coumermycin (Cou., 900 nM) or PMA (10 ng/ml). Phospho-Mek (panels A–D) and phospho-Erk1 and -Erk2 levels (panels E–H) were determined as in Fig. 1. The y axis represents -fold induction relative to cells stimulated with vehicle (DMSO). Experiments for G2A and C3S,C5S constructs are representative examples of two or more experiments, while experiments for Myc-Raf-GyrB and Lck-Raf-GyrB are representative examples of five or more experiments; error bars represent the range of duplicate measurements.
Raf-GyrB transfectants were stimulated with novobiocin (as a negative control), coumermycin, or PMA (as a positive control). Transcript abundance in cells treated with novobiocin was equivalent to that seen in cells treated with carrier alone (Me2SO). In contrast, stimulation of Lck-Raf-GyrB cells with either coumermycin or PMA led to very similar changes in the pattern of gene expression. Specifically, stimulating Lck-Raf-GyrB transfectants with coumermycin for 40 min led to a greater than 2.5-fold change in mRNA levels for 71 out of 588 genes examined. These included a number of genes known to be induced downstream of the Raf pathway, most notably egr-1 (20-fold induction), c-fos (4.4-fold induction), and c-myc (5.5-fold induction) (29–31) (Fig. 5 (A and B) and Table I). In contrast, no changes in gene expression were observed when stimulating Raf-GyrB-transfected cells with coumermycin (Fig. 5A and data not shown). Taken together, these results suggest that both oligomerization and appropriate membrane localization of Raf are critical for connecting Raf activation to changes in downstream gene transcription.

**DISCUSSION**

The Ras signal transduction pathway plays a key role in regulating both cell proliferation and differentiation. A large number of specific effectors of Ras have been identified that propagate Ras signals (reviewed in Ref. 10). However, the exact role that these different effectors play in regulating downstream protein function remains to be elucidated. For example, it has recently been shown that Ras exists as a dimer and that driving Ras dimerization also activates the Raf pathway (34). Furthermore, in yeast the scaffolding protein Ste5 has been shown to organize signaling of the yeast homologs of Raf (Ste11), Mek (Ste7), and MAP kinase (Fus3) (35–37). Dimerization of Ste5 is both necessary and sufficient to activate Ste11 and its downstream targets.

### TABLE I

| Gene       | Coumermycin | PMA |
|------------|-------------|-----|
| Egr-1      | 20.2        | 17.4|
| APC        | 3.9         | 2.9 |
| EB1        | 4.7         | 4.0 |
| Mdm2       | 3.9         | 3.3 |
| NF2        | 2.9         | 4.1 |
| P130       | 2.5         | 2.6 |
| Rb         | NC*         | NC  |
| ZO-1       | NC          | NC  |
| c-Fos      | 4.4         | 4.3 |
| c-Myc      | 5.5         | 5.5 |

* NC, not calculated since expression levels were undetectable in novobiocin-stimulated cells.
Ste7 and Fus3 (38, 39). Our initial results suggested that oligomerization of Raf not only leads to its activation but may, in fact, obviate the membrane localization step that typically occurs during this process (16). To investigate this in greater detail, we examined more distal events such as the activation of Erk1 and Erk2 and the accumulation of fos protein levels. Following coumeycin-induced oligomerization of cytoplasmic Raf, we found that Erk1 and Erk2 were only minimally activated and that no accumulation of fos protein could be detected. Furthermore, using gene macroarrays that included a number of genes thought to be regulated by the Raf pathway (i.e. fos, myc, and egr-1), we saw no changes in gene transcription. Thus, although cytoplasmic clustering of Raf was sufficient to activate Mek, this did not lead to efficient coupling of activated Mek to more distal effectors.

One possible explanation for this result, especially in light of what is known about Ste5 functioning in yeast, is that membrane localization of Raf is required to target Raf/Mek signals to appropriate substrates. To test this possibility, we generated Raf-GyrB fusion proteins that contained an amino-terminal myristylation and palmitylation signal sequence derived from the Sre family kinase Lck. This resulted in a membrane-localized form of Raf-GyrB (Lck-Raf-GyrB). Previous studies have demonstrated that transiently expressing farnesylated versions of Raf (Raf-CAAX) in Cos cells results in membrane localization of Raf and constitutive activation of the Raf pathway (32, 33). In contrast, we found that membrane localization of Raf, achieved by amino-terminal acylation, does not lead to constitutive activation of Raf in stably transfected NIH3T3 cells. It is unclear why membrane targeting of Raf via farnesylation should result in constitutive activation of Raf, while targeting via myristylation/palmitylation does not. This discrepancy may reflect differences in the expression level achieved in our stable NIH3T3 transfecants, in which Lck-Raf-GyrB is expressed at the same level as endogenous Raf, versus transient Cos cell transfecants, in which Raf-CAAX is most likely expressed at supraphysiological concentrations. Overexpression of Raf-CAAX could lead to fortuitous association, mimicking oligomerization, and hence activation, such as has been seen following the overexpression of other kinases (40) or of caspases (41). Supporting this hypothesis, Minio et al. (42) have found that stable expression of Raf-CAAX in NIH3T3 cells also does not lead to constitutive activation of the Raf pathway. In addition, it is possible that farnesylation of Raf directly promotes interactions with effector substrates. For example, farnesylation of Ras2 (in yeast) leads to a 100-fold increase in its affinity for adenyl cyclase (43). This result reflected a change in binding affinity, and was not merely an effect of enhanced proximity due to membrane association, as a similar change was observed using solubilized Ras2 and adenyl cyclase. Regardless of the actual mechanism, our results suggest that the effect achieved by transient expression of Raf-CAAX in Cos cells most likely involves more than simply targeting Raf to the plasma membrane.

The ability to target Raf to the membrane fraction, without concomitantly activating it, allowed us to examine the effects of oligomerization of Raf in this compartment. In striking contrast to what we observed with cytoplasmic forms of Raf, coumeycin-induced oligomerization of membrane-localized Raf led to dramatic increases in Erk1 and Erk2 phosphorylation, a significant increase in Fos protein accumulation, and the up-regulation of mRNAs for many genes. These changes paralleled those seen when cells were stimulated with PMA. Such overlap in response is not surprising since protein kinase C is thought to act in part via activation of the Ras/Raf pathway (44). Interestingly, we observed that mRNA levels for at least one gene, egr-1, are particularly sensitive to signals entrained by Raf alone. In addition, several of the genes induced by coumeycin-mediated activation of Raf-GyrB are involved in regulating cell proliferation (e.g. myc, Rb, fos; see Table I), thereby underscoring the role of Raf in this process. Finally, these findings allow us to separate Raf activation from the activation of Erk1 and Erk2. Neither oligomerization nor membrane localization of Raf is sufficient to potently engage the entire Raf signal transduction cascade; only the combination of these events leads to a robust activation of distal Raf effectors and an increase in Raf-dependent gene expression.

Why does oligomerization of membrane-localized Raf (Lck-Raf-GyrB) lead to complete activation of the Raf pathway, while oligomerization of cytoplasmic Raf (Raf-GyrB) does not? One potential explanation is that the strength of the signal delivered by clustering Raf in the cytoplasm could be insufficient to activate the pathway fully. This possibility seems unlikely since coumeycin-induced oligomerization of Raf-GyrB or Myc-Raf-GyrB activated Mek to the same degree as that seen when stimulating cells with PMA (Figs. 1 and 3) or fetal bovine serum (16). Since both PMA and fetal bovine serum potently activate Erk1 and Erk2 (Figs. 1 and 3 and Ref. 16), we cannot attribute the failure of coumeycin stimulation to activate Erk1 and Erk2 to inadequate activation of Raf-GyrB and its downstream substrate Mek. An alternative explanation is that the simple addition of acylan groups to the amino terminus of Lck-Raf-GyrB might facilitate interaction with substrates, much in the same way that farnesylation of Ras promotes interactions with adenyl cyclase and other targets (43, 45). However, the finding that the myristylated C3S,C5S mutant cannot activate downstream effectors following coumeycin-induced oligomerization demonstrates that simple amino-terminal acylation does not account for the effect we see using Lck-Raf-GyrB.

A more likely explanation is that membrane-localization is required to couple Raf effectively to downstream targets. The myristylation/palmitylation signal sequence used to target Lck-Raf-GyrB to the membrane compartment has been previously shown to target heterologous proteins to caveolae or glycosphingolipid-enriched membrane compartments (26). Separate studies demonstrated that in quiescent fibroblasts these membrane compartments contain all components of the Ras signaling pathway including inactive Erk1 and Erk2, and suggested that this may promote efficient activation of the MAP kinase pathway following stimulation with growth factors (46). In examining Lck-Raf-GyrB subcellular distribution, we have found that a substantial fraction can be detected in caveolae (data not shown). Thus, activating Lck-Raf-GyrB within caveolae could facilitate interactions of the activated Raf/Mek complex with the inactive MAP kinases Erk1 and Erk2 sequestered there. Additional membrane-localized activators of Raf (such as Src family or p21-activated kinases (47, 48)), or potential membrane-proximal scaffolding proteins that could coordinate Erk signaling (such as those described for Jun kinase signaling (Ref. 49)), could contribute to activation of this pathway as well. Interestingly, such a process bears striking similarities to the activation of protein kinase A, wherein distinct protein kinase A-anchoring proteins target protein kinase A to different subcellular compartments and thereby determine accessibility to both upstream activators and downstream substrates (reviewed in Ref. 50).

The ability to regulate Raf activation in a dose-dependent and temporal manner should prove to be very useful for studying Raf’s role in development. In particular, the magnitude of the induced Raf signal has been postulated to play a key role in determining the biological outcome of signaling via the MAP...
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kinase pathway (51). Using the Lck-Raf-GyrB construct, Raf-derived signals can be titrated, permitting direct correlation between the extent of kinase activation and changes in gene expression or protein function. Such information will prove invaluable in furthering our understanding of the molecular mechanisms that underlie cell fate decisions.

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