The requirement of specific membrane domains for Raf-1 phosphorylation and activation

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ABSTRACT

Activation of Raf-1 by Ras requires recruitment to the membrane as well as additional phosphorylations, including phosphorylation at serine 338 (S338) and tyrosine 341 (Y341). In this study we show that Y341 participates in the recruitment of Raf-1 to specialized membrane domains called “rafts” which are required for Raf-1 to be phosphorylated on S338. Raf-1 is also thought to be recruited to the small G protein Rap1 upon GTP loading of Rap1. However, this does not result in Raf-1 activation. We propose that this is because Raf-1 is not phosphorylated on Y341 upon recruitment to Rap1. Redirecting Rap1 to Ras-containing membranes or mimicking Y341 phosphorylation of Raf-1 by mutation converts Rap1 into an activator of Raf-1. In contrast to Raf-1, B-Raf is activated by Rap1. We suggest that this is because B-Raf activation is independent of tyrosine phosphorylation. Moreover, mutants that render B-Raf dependent of tyrosine phosphorylation are no longer activated by Rap1.
INTRODUCTION

The mitogen-activated protein (MAP) kinase family regulates diverse physiological processes including cell growth, differentiation, and death. Activation of one of these MAP kinases (the extracellular signal-regulated kinase, or ERK) is initiated by the recruitment of the MAP kinase kinase kinase Raf-1 to the small G protein Ras, a resident plasma membrane protein. The Ras family consists of three members (Ha-Ras, Ki-Ras, and N-Ras) (1) that display overlapping but distinct patterns of expression and function (2). Ras is tethered to the membrane via a carboxyl CAAX motif containing a cysteine (C) followed by two aliphatic amino acids (A) and a carboxyl-terminal amino acid (X), that directs the attachment of a farnesyl moiety (3,4). In addition to farnesylation, a second signal assists in correct membrane targeting. For Ha-Ras and N-Ras, this second signal is a palmitoyl moiety that is introduced on a neighboring cysteine. In Ki-Ras, this second site is a polybasic domain. The requirement of Ras’ membrane localization for Raf-1 activation has been confirmed by mutating the terminal cysteine in a constitutively active Ras mutant, RasV12, resulting in a mutant that can not activate Raf-1 (5).

Membrane regions rich in cholesterol and sphingolipids, termed “rafts” or detergent-insoluble glycolipid-enriched complexes have been proposed to participate in signaling events by organizing additional molecules, such as c-Src, G protein subunits, and phospholipases, into discrete membrane domains (6). Recent attention has focused on the role of these specialized microdomains in Ras signaling (7-10). A number of groups have shown that both Ras isoforms Ki-Ras (11) and Ha-Ras are targeted to rafts (7-9,12-14). Others have suggested that Ha-Ras, but not Ki-Ras, can be targeted to raft microdomains (10,15). Targeting of Ras isoforms to specific membrane domains may be determined by the characteristics of the lipid modifications on Ras, as well as other sequences found within the hypervariable region (10). Localization of Ras isoforms to distinct membrane microdomains may influence selectivity of signaling among the Ras isoforms (2). For example, Ki-Ras is thought to couple well to Raf-1 but, unlike Ha-
Ras, couples poorly to PI3-K (16,17). Differences between Ha-Ras and Ki-Ras in their promotion of cell survival have also been noted (18), suggesting that distinct localization of Ras isoforms dictate signaling pathways, as recently proposed (19). Ras isoforms have been reported to display distinct dependencies on caveolin in their coupling to Raf-1 (9,15).

Raf-1 recruitment to the membrane can be achieved independently of Ras by the addition of Ras carboxyl-terminal sequences to the carboxyl-terminus of Raf-1. The addition of twenty amino acids from the carboxyl-terminus of Ki-Ras onto Raf-1 (Raf-KiCAAX) is sufficient to redirect Raf-1 to the membrane where it is constitutively active (20,21). Maximal activity of this chimera, however, requires additional phosphorylation events (22,23), consistent with the requirement of specific kinases for full activation for wild type Raf-1 (24-26). In particular, two phosphorylations on S338 and Y341 have been shown to be required for full activity (27). Recently, two additional sites within the kinase activation loop have also been shown to be required (28). Phosphorylation of serine 338 may be mediated by the serine/threonine kinase PAK (p21-associated protein) (29) and phosphorylation of tyrosine 341 can be carried out by Src family tyrosine kinases (23,27,30). The participation of specific membrane microdomains in these modifications is not known.

In this study, we examined the membrane requirements for the post-translational modification of Raf-1. In addition, we took advantage of chimeric Raf-1 molecules that are targeted to specific membrane domains to determine the specificity of these domains for raft localization, phosphorylation, and constitutive activation of Raf-1. Understanding the molecular basis for Raf-1 activation by Ras may also help explain the actions of the related small G protein Rap1, which recruits Raf-1, but unlike Ras, cannot activate it. In the present study we address whether membrane localization also plays a role in Rap1-mediated inhibition of Raf-1 activation by Ras. We found that Raf-1 phosphorylation was intimately linked to proper membrane targeting and that the
ability of Ras and Rap1 to support Raf-1 phosphorylation dictated the biochemical actions of both Ras and Rap1 on Raf-1.
EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and Stimulations--Cos-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. Cells were transfected using LipofectAMINE 2000 according to the manufacturer’s recommendations. Unless otherwise noted, cells were transfected with a total of 10 µg of plasmid DNA, with pcDNA3.1 (vector) used to adjust DNA amounts where necessary. After 24 hrs, transfected cells were switched to low serum containing medium and incubated for a further 12 hrs. Cells were stimulated with 50ng/ml epidermal growth factor (EGF) for the indicated times. For cholesterol depletion experiments, serum starved cells were preincubated for 1 hour with 2% methyl-β-cyclodextrin (Sigma) in DMEM prior to stimulation with EGF.

Plasmids--The cDNA encoding HaRas, and HaRasV12 were tagged at their amino terminus with a Flag epitope. The cDNA encoding KiRas, and Ki-RasV12 were tagged at their amino terminus with a myc epitope. The cDNA encoding NRas, and NRasV12 were tagged at their amino terminus with an hemaglutinin (HA) epitope, and were purchased from the Guthrie Institute, Sayre, Pa. All other cDNAs were tagged at the amino terminus with a 2xFlag epitope (Kodak) by PCR and introduced into the BamH1 and XbaI sites of pcDNA3.1 vector (Invitrogen), unless otherwise indicated. Raf-1 and B-Raf mutations were introduced by PCR using the QuickChange site-directed mutagenesis kit (Stratagene). The minimal membrane targeting domain of Ha-Ras(-hvr) (31) was added to the 3’ end of FlagRaf-1 by PCR using a primer with Raf-1 sequences along with an in-frame XhoI site (adding amino acids L and E) and the last nine amino acids of Ha-Ras (CMSCKCVLS), a stop codon and a XbaI site. This construct is designated Raf-HaCAAX(-hvr). Raf-HaRasCAAX(+hvr), containing both the minimal membrane targeting domain and the hypervariable domain, was constructed using a primer with an in-frame XhoI site, the carboxy-terminal 27 amino acids of Ha-Ras (IRQHKLRKLNPPDESGPJCMSCKCVLS), a stop codon and a XbaI
site by PCR. Raf-Rap1CAAX was constructed using the same strategy with the
carboxy-terminal 24 amino acids of Rap1b (LVRQINRKTPVPGKARKKSSCQLL)
introduced into the Xho1-Xba1 site of Raf-Ha-RasCAAX(+hvr). B-Raf-HaRasCAAX,
B-Raf-Rap1CAAX chimeras were constructed using a similar strategy. RapE63-
HaRasCAAX(+hvr) was constructed by adding an in frame XhoI site by PCR into
RapE63 at base pair 480 and cloning into the EcoR1-XhoI sites of FlagRaf-Ha-
RasCAAX, thereby replacing the Rap membrane targeting motif with the Ha-Ras
hypervariable domain and CAAX motif. The full-length coding sequences of human
Ha-Ras, bovine Rap1b, RapE63-Ha-RasCAAX, Raf-Rap1CAAX, and Raf(Y341D)-
Rap1CAAX were introduced in-frame into the pEGFP-C1 cloning vector (Clontech).
Wild type (CAV/WT) and mutant caveolin-3 (CAV/DGV) plasmids were constructed
as per Watson et al (32).

Western blotting and immunoblotting--Cos-7 cells were stimulated and lysates
prepared as described. Protein concentrations were determined by using the Bio-Rad
protein assay dye reagent according to manufacturer’s recommendations. Equal
amounts of lysate were immunoprecipitated with either Flag M2 antibody coupled to
agarose (Sigma) or anti-Myc antibody (9E10) coupled to agarose (Santa Cruz
Biotechnology) where indicated and examined by western blot as previously described
(33). Samples were separated by SDS-PAGE and transferred to PVDF membrane.
Expression of Flag and Myc-tagged proteins was detected using monoclonal Flag M2
antibody (Sigma) or monoclonal anti-Myc 9E10 antibody (Santa Cruz Biotechnology).
Rabbit polyclonal anti-phospho MEK1/2 antibody (Cell Signaling Technology) was
used to detect activated GST-MEK1 (Upstate Biotechnology). Polyclonal anti-phospho
ERK1/2 (Cell Signaling Technology) was used to detect activated MycERK2
(pMycERK2). Phosphorylation of Raf-1 at Ser338 or at Tyr341 was detected using
anti-phosphoRaf-1 Ser338 or phosphotyrosine (pTyr, 4G10) (Upstate Biotechnology,
Inc.). All experiments were repeated at least three times and representative blots
shown. The expression of endogenous N-Ras, Ki-Ras, and Ha-Ras was examined using subtype-specific Ras antisera from Santa Cruz Biotechnology.

**Cell Fractionation**--Cos-7 cells were washed twice in phosphate buffered saline (PBS) before scrapping into 0.5 ml of hypotonic lysis buffer (10 mM Tris pH 7.5, 5 mM MgCl, 25 mM NaF, 25 mM β-glycerophosphate, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM sodium orthovanadate, and 1 mM PMSF). After 10 min., cells were homogenized at 4°C by 50 strokes in a tight-fitting Dounce homogenizer. Nuclei and unbroken cells were pelleted by centrifugation at 1500x g. Supernatants were then centrifuged at 100,000 x g in a Beckman TLA 45 rotor at 4°C for 30 min. The supernatant was collected and designated the cytosolic fraction (S100) and the pellet was resuspended in 250 μl hypotonic lysis buffer and designated the membrane (P100) fraction.

**Sucrose gradients**--Membrane microdomains were isolated based on their buoyant density using isopycnic equilibrium sucrose density gradient centrifugation. A non-detergent method for lipid raft isolation was used based on the method of Smart and colleagues (34), but modified for equilibrium centrifugation and sucrose was used instead of Optiprep. Briefly, transfected Cos-7 cells (2x10⁶ cells) were rinsed twice in PBS and scrapped into 0.5 ml MES buffer (25 mM MES pH 6.5, 10 mM NaCl, 5 mM MgCl, 10 μg/ml aprotinin, 10 mg/ml leupeptin, 25 mM NaF, 25 mM β-glycerophosphate, 5 mM sodium orthovanadate). Cells were homogenized at 4°C by 30 passes through a 23 gauge syringe and sonicated on ice for 30 seconds at setting 2, 30 seconds at setting 3, and 30 seconds at setting 4 (Sonic Dismembrater, Fisher). The lysate was mixed with 0.5 ml of 90% sucrose in MES buffer and placed at the bottom of a 2.4 ml Beckman ultracentrifuge tube. The gradient was constructed by overlaying the 45% sucrose/lysate mixture with 1.2 ml of 35 % sucrose, 1 ml of 30% sucrose, 1 ml of 25% sucrose, and ultimately 1 ml of 5% sucrose. The tubes were centrifuged at 4°C
in a Beckman SW 55 rotor for 16 hrs at 48,000 rpm. A visible band 3-4mm from the top was observed after centrifugation and corresponded to the lipid raft/caveolae fraction. Twelve 0.43 ml fractions were collected from the top of the gradient. From each fraction, 40 µl was removed for refractometry and protein determination using the Bradford method (BioRad). The remainder of each fraction was diluted with 1 ml of MES buffer to dilute out the sucrose and membranes and proteins were pelleted at 100,000 x g for 45 min. in a TLA 45 rotor. Pellets were resuspended in Laemmli buffer, separated by SDS-PAGE, and transferred to a PVDF membrane for analysis by immunoblotting. Only the first 10 fractions are shown for each gradient. Similar results were obtained using the sodium carbonate method of raft preparation (10).

**Immunofluorescence**—Cos-7 cells were grown on glass coverslips and transfected with 100 ng of FlagRaf cDNAs using LipofectAMINE 2000. Cells were fixed in PBS containing 4% formaldehyde, permeabilized in PBS containing 0.1% Triton X-100, and blocked with PBS/5% horse serum. Localization of transfected proteins was detected with a 1:2000 dilution of Flag M2 antibody (Sigma) in 5% horse serum/0.01% Tween followed by a 1:10,000 dilution of an anti-mouse-FITC conjugate in 5% horse serum/0.01%Tween. Cells were visualized using a Zeiss Axioplan 2 microscope. To examine the expression patterns of the myc-tagged wildtype caveolin (CAV/WT) or DGV mutant (CAV/DGV) constructs, cells were transfected with 5 µg of plasmid cDNAs as described above. The expressed caveolin constructs were detected by mouse anti-myc monoclonal antibody (Santa Cruz) at 1:100 dilution, followed by Texas-Red anti-mouse secondary antibody at 1:100 (Jackson Immunoresearch Labs). Endogenous caveolin was detected by rabbit anti-caveolin polyclonal antibody (Transduction Labs) at 1:200 dilution, followed by Alexa-488 anti-rabbit secondary at 1:100 dilution (Molecular Probes). Cells were imaged with a Zeiss 510 scanning laser confocal microscope.
**Raf-1 kinase assays**--Cos-7 cells were transfected, cells lysed and lysates prepared as described (28). FlagRaf proteins were immunoprecipitated from 500 µg of cell lysate with 30 µl of Flag M2 agarose (Sigma) at 4°C for 6 hrs. Immune complexes were washed twice with lysis buffer and once with kinase assay buffer (20 mM MOPS pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 5 µg/ml aprotinin). Pellets were resuspended in 40 µl of kinase assay buffer with 1.5 mM MgCl₂ and 7.5 µM ATP along with 0.4 µg GST-MEK1 (Upstate Biotechnology) and the reaction was incubated for 30 min. at 30°C. The kinase reaction was terminated by adding 45 µl of 2x Laemmli buffer, boiled for 5 min., resolved by SDS-PAGE and transferred to PVDF membrane. Raf-1 activity was evaluated by immunoblotting with anti-phospho MEK1/2 antibody (Cell Signaling Technology).

**PhosphoMycERK2 Assay**--For MycERK2 assays, treated and untreated cells were lysed in ERK assay buffer and activation of MycErk2 was detected as described previously (33).
RESULTS

*Raf-1 is relocalized to raft microdomains upon EGF stimulation.* Activation of Raf-1 by Ras-dependent signals induced by growth factors is associated with a redistribution of Raf-1 from the cytoplasm to the cell membrane, where it associates with Ras (35). Recent studies suggest that Ras may be localized to specific cholesterol-rich membrane microdomains called rafts (11), and upon Ras activation, Raf-1 may also be recruited to rafts (12).

Proteins that localize to cholesterol-rich microdomains (rafts) can be detected within low-density fractions of sucrose density gradients (10,14,36). The method is illustrated in Fig. 1A. The density gradient achieved following equilibrium centrifugation is shown in Fig. 1B with the corresponding protein concentrations. One of these raft proteins, caveolin-1, was used to identify the density of these cholesterol-rich raft domains (Fig. 1C, top panel) (6). Using this technique, we show that endogenous Raf-1 was excluded from raft microdomains in untreated cells (Fig. 1C, second panel) and EGF treatment induced the redistribution of endogenous Raf-1 into raft domains (Fig. 1C, third panel). A significant fraction of Raf-1 protein was also detected at higher densities within the gradient.

Similar results were seen using transfected cDNAs encoding wild type Raf-1, tagged with the Flag epitope. We show that wild type Raf-1 was excluded from raft microdomains in untreated cells (Fig. 1D, top panel) and EGF treatment induced the redistribution of wild type Raf-1 into raft domains (Fig. 1D, middle panel). Raft domains can be disrupted by the cholesterol-depleting agent methyl-β-cyclodextrin (CD) (11). In the presence of CD, EGF recruitment of Raf-1 to low density fractions was inhibited (Fig. 1D, bottom panel), confirming that these low density fractions represented cholesterol-rich membrane microdomains. This provides strong evidence that the buoyant fractions containing Raf-1 represented cholesterol-rich membrane
microdomains consistent with rafts. Raf-1 could also be redistributed to rafts in cells transfected with constitutively active mutants of Ha-Ras, Ki-Ras and N-Ras (Fig. 1E).

The localization of Ras proteins to rafts is not completely understood and some controversies remain (37), with some groups showing that Ha-Ras, but not Ki-Ras, requires raft localization for full activity (9,15). Cos-7 cells express detectable levels of Ki-Ras and N-Ras (Fig. 2A; middle and right panels, untr.). The expression of Ha-Ras was not detected (Fig. 2A; left panel, untr.). As a control, cells were transfected with each Ras isoform and lysates probed with isoform-specific antisera. Endogenous Ki-Ras and N-Ras were localized to lipid rafts in resting cells (Fig. 2B). Similar results were seen in cells transfected with wild type (WT) as well as constitutively active (V12) Ha-Ras, Ki-Ras, and N-Ras mutants, although transfected N-Ras WT appeared in higher density fractions as well (Fig. 2C, D).

*Raf-1 activation and S338 phosphorylation require intact raft microdomains.* It has long been appreciated that Raf-1 recruitment to Ras is insufficient by itself to trigger Raf-1 activation and that additional post-translational modifications are required (27). The two best-studied modifications are serine phosphorylations on serine 338 and tyrosine 341. Fig. 3A demonstrates this requirement. Constitutively active Ras (RasV12) activated wild type Raf-1 (Raf-1 WT), as measured by *in vitro* Raf-1 assay. However, Raf-1 that was mutated at either S338 to alanine (RafS338A) or Y341 to alanine (RafY341A) could no longer be activated by HaRasV12 (Fig. 3A).

Raft microdomains were required for phosphorylation on S338, since CD inhibited EGF-induced phosphorylation of FlagRaf-1 on S338 (p338) and Raf-1 activation (Fig. 3B). These data suggest that targeting to raft domains was required for full Raf-1 activation and phosphorylation at S338. In contrast, tyrosine phosphorylation of FlagRaf-1 was not affected by CD (Fig. 3C, upper panel, lanes 1-4). The absence of
phosphorylation of Y341 in FlagRaf-Y341A is provided as a negative control (Fig. 3C), upper panel, lanes 5-8).

Recent studies have shown that Ras proteins are localized to specialized raft domains called caveolae (11). These microdomains are enriched for caveolin, and a requirement for caveolin can be assessed using interfering mutants such as CAV/DGV, a truncated form of caveolin-3 (9,10,32). In Cos-7 cells, expression of CAV/DGV was detected within cytoplasmic vesicles, as previously reported (9,10) and interferes with the localization of caveolin-1 (Fig. 4A). CAV/DGV did not interfere with the ability of either EGF (Fig. 4B) or constitutively active Ras mutants (Figs. 4C) to activate Raf-1 or stimulate S338 phosphorylation.

**Y341 is required for targeting to raft microdomains and phosphorylation of S338.** We next examined the requirement of S338 and Y341 in Raf-1 localization. EGF was able to direct RafS338A into rafts (Fig. 5A, upper panel), but was not able to direct RafY341A into rafts (Fig. 5A, lower panel). Neither Raf mutant RafS338A nor RafY341A entered rafts in the absence of EGF stimulation (data not shown). This suggests that phosphorylation of Y341 participates in localization of Raf-1 to lipid rafts, while S338 phosphorylation occurs once proper localization has been achieved. In Fig. 5B, we show that the tyrosine at residue 341 was essential for EGF and HaRasV12 to phosphorylate S338 (Fig. 5B, upper panel) and activate Raf-1 (Fig. 5B, middle panel). Replacing tyrosine with aspartate in the mutant Raf Y341D did not effect EGF’s actions (Fig. 5B).

The requirement of Y341 phosphorylation for Raf-1 activation can be overcome by targeting to raft domains--Raf-1 can be constitutively targeted to the membrane following the attachment of a Kirsten Ras carboxy-terminal domain (Raf-KiCAAX) (20,21). Here, we examined the localization of a related chimera created by fusing the Raf-1 protein to the carboxyl 27 amino acids of Ha-Ras including both the CAAX
domain and the hypervariable domain (hvr); Raf-HaCAAX(+hvr) (16). As expected, wild type Raf-1 was located within the cytoplasm of resting cells (Fig. 5C, left panel), and the chimera Raf-HaCAAX(+hvr) was present on the plasma membrane (Fig. 5C, middle panel). The chimera Raf-HaCAAX(-hvr) that lacked hvr sequences was also present on the plasma membrane (Fig. 5C, right panel).

Raf-HaCAAX(+hvr) is constitutively localized within raft domains (Fig. 5D, upper panel). Unlike Raf Y341A, the introduction of Y341A into Raf-HaCAAX(+hvr) [RafY341A-HaCAAX(+hvr)] did not prevent raft localization (Fig. 5D, middle panel), constitutive phosphorylation of S338 or activation of Raf-1 (Fig. 5E). These data suggest that phosphorylation of Y341 is required for raft localization, S338 phosphorylation and activation of wild type Raf-1, but that Y341 phosphorylation is not required if Raf-1 is constitutively targeted to rafts. In contrast, mutating S338 to alanine (A), in the chimera RafS338A-HaCAAX(+hvr), completely abolished activation of Raf-1 (Fig. 5E), without affecting raft localization (Fig. 5D, lower panel), demonstrating the requirement of pS338 for kinase activation, but not raft localization.

In addition to the CAAX domain, Ha-Ras contains a hypervariable (hvr) region that influences specific membrane localization (10). Here we examined chimeras either containing the Ha-Ras hypervariable region (+hvr) or lacking these sequences (-hvr). Like Raf-HaCAAX(+hvr), Raf-HaCAAX(-hvr) was present within the particulate (P100) fraction (Fig. 6A), and was detected on the plasma membrane (Fig. 5C, right panel). Unlike Raf-HaCAAX(+hvr), Raf-HaCAAX(-hvr) was excluded from low-density gradient fractions (Fig. 6B, upper panel), suggesting that despite its membrane localization, the chimera was targeted differently than Raf-HaCAAX(+hvr). Raf-HaCAAX(+hvr) gradients were included as a control (Fig. 6B, lower panel).

Only Raf-HaCAAX(+hvr) (lane 4), but not Raf-HaCAAX(-hvr) (lane 3) displayed constitutive phosphorylation of S338 (Fig. 6C, p338, first panel). Raf-
HaCAAX(+hvr), but not Raf-HaCAAX(-hvr), was active in Raf-1 kinase assays in vitro (Fig. 3C, pMEK, second panel) and ERK activation assays in vivo (Fig. 6C, pMycERK2, fourth panel). As a control, EGF stimulation of wild type Raf-1 was included, which resulted in phosphorylation of S338 and activation of both Raf-1 and ERK (lanes 1, 2). High levels of activation of Raf-HaCAAX(+hvr), but not Raf-HaCAAX(-hvr), were also seen in coupled in vitro kinase assays (data not shown). Therefore, both S338 phosphorylation and biochemical activity of these chimeras paralleled raft localization.

Y341D restored raft localization and activity to Raf-HaCAAX(-hvr)—For Raf-HaCAAX(-hvr), membrane targeting was not sufficient to trigger S338 phosphorylation and Raf-1 activity. Since Y341 phosphorylation potentiates the localization of Raf-1 within rafts, which may be required for subsequent S338 phosphorylation, we tested whether the Y341D mutation could restore S338 phosphorylation in the HaCAAX(-hvr) chimera. Indeed, this mutant, RafY341D-HaCAAX(-hvr), but not RafS338D-HaCAAX(-hvr), was capable of entering raft domains (Fig. 7A). Moreover, RafY341D-HaCAAX(-hvr) was phosphorylated on S338 and showed constitutive activity to levels similar to those seen with Raf-HaCAAX(+hvr) (Fig. 7B). Furthermore, the introduction of Y341D into Raf-HaCAAX(+hvr) did not significantly increase Raf-1 activity (Fig. 7B, C). These data suggest that one of the functions of Y341 is to localize Raf-1 to specific membrane microdomains permitting efficient phosphorylation of S338 and coupling to downstream effectors.

Rap1 is unable to activate Raf-1 because it can not induce Y341 phosphorylation—The ability of small G proteins to recruit Raf-1 to the membrane is not sufficient for full activation of Raf-1 (22). For example, Rap1 is a small G protein within the Ras family that can associate with Raf-1 but cannot activate it (38). Unlike Ras, which is located at the plasma membrane, Rap1 is located in vesicular membranes (39-41). This localization is directed by carboxy-terminal sequences of Rap1 that
contain a distinct CAAX motif that regulate the attachment of geranyl modifications that direct Rap1 to vesicular membranes (42,43). This could be shown using green fluorescent protein (GFP) fusions to the RapE63 protein (GFP-Rap) which, unlike GFP alone (Fig. 8A, a), was localized to perinuclear vesicles within the cytoplasm (Fig. 8A, b). In contrast, GFP-HaRasV12 was detected at the plasma membrane, consistent with recent reports (19) (Fig. 8A, c). The chimera GFP-RapE63-HaRasCAAX was also present on the plasma membrane, confirming that the carboxyl terminal sequences of HaRas could redirect ectopic proteins (Fig. 8A, d).

Activated Rap1 does not activate Raf1 (44). Rap1’s inability to permit S338 phosphorylation and activation of Raf-1 could be partially overcome by swapping Rap1’s CAAX domain with that of Ras (RapE63/HaRasCAAX) (Fig. 8B). Although RapE63 could not activate wild type Raf-1, it could activate RafY341D, as measured by S338 phosphorylation and kinase activation (Fig. 8C). These data suggest that the inability of RapE63 to activate Raf-1 was due to the inability of Raf-1 to be correctly phosphorylated when recruited by Rap1, since Rap1 was capable of supporting S338 phosphorylation in the Y341D mutation. These data also suggest that the inability of Rap1 to activate Raf-1 is not just a consequence of the interaction between Rap1 and Raf-1, as has been proposed (44), but may also be dictated by the localization of Rap1. However, HaRasV12 was better than RapE63-HaCAAX(+hvr) in activating both Raf-1 and RafY341D (Fig. 8B,C), suggesting that sequences within Ras distinct from the carboxy-terminal membrane-targeting domain are critical for maximal activation of Raf-1.

To examine the effect of relocalizing Raf-1 to Rap1-containing membranes, we generated chimeras of Raf-1 fused to the Rap1 carboxy-terminal CAAX motif (Raf-Rap1CAAX) (Fig. 8D). Raf-Rap1CAAX was not constitutively active, and could not be activated (Fig. 8D, middle panel) or phosphorylated on S338 by EGF (Fig. 8D, upper panel). These data suggest that Raf-1 needs to be targeted to specific membranes
in order to be activated and that Raf-1 targeting to Rap1-specific membrane domains
does not support S338 phosphorylation or activation. The inability of Rap1 to direct the
proper phosphorylation of Raf-1/Rap chimeras could be overcome by introducing
negative charges into Raf-1 at Y341. Mutation of Y341 to aspartate to generate
RafY341D-RapCAAX increased the basal levels of both phosphorylation of S338 and
Raf-1 activation compared to Raf-Rap1CAAX (Fig. 8D), which were not further
increased by EGF. In Fig. 8E, we show the subcellular localization of GFP-fusion
proteins, GFP-Raf-Rap1CAAX (Fig. 8E, a), GFP-RafY341-Rap1CAAX (Fig. 8E, b).
Both chimeras are largely localized to perinuclear regions, with little or no staining
detected at the cell surface.

*Rap1 activation of B-Raf requires aspartic acid at residues D447/D448--*The
Y341D mutation in Raf-1 resembles the naturally occurring sequence within the Raf
isoform, B-Raf. B-Raf lacks tyrosines at the site corresponding to Y341 in Raf-1 (448
in B-Raf). Instead, it contains aspartic acids at residues 447 and 448, that appear to
mimic phosphorylation at these sites (27). Moreover, B-Raf was phosphorylated at the
serine corresponding to S338 in Raf-1 (S445 in B-Raf) in resting cells (Fig. 9A, lane 1,
upper panel), and remained phosphorylated at this residue in the presence of the raft-
disrupting agent, CD (data not shown).

Unlike Raf-1, B-Raf can be activated by the small G protein Rap1 (44). Because
of this, Rap1 can activate MEK and ERK in B-Raf-expressing cells (45-47). The
ability of Rap1 to activate B-Raf is shown in Fig. 9A (lanes 1-3). Both constitutively
active mutants of Ras (RasV12) and Rap1 (RapE63) could activate wild type B-Raf (B-
RafWT) (Fig. 9A, lanes 2, 3). In contrast to B-RafWT, expression of a mutant B-Raf in
which the aspartic acid residues were mutated to the corresponding tyrosines residues in
Raf-1 (B-RafYY) showed no basal phosphorylation on S445 (Fig. 9A, lane 4). B-
RafYY was no longer activated by constitutively active Rap1 (RapE63), as measured
by both S445 phosphorylation and kinase activity (lane 6). HaRasV12 stimulated S445
phosphorylation and activity of the B-RafYY mutant (lane 5). When Rap1-CAAX sequences were coupled to B-Raf, the resulting chimera, B-Raf-Rap1CAAX, was phosphorylated on p445 and activated ERKs to a similar degree as B-Raf-HaCAAX(+hvr) (Fig. 9B). Although wild type B-Raf was constitutively phosphorylated on S445 and displayed detectable constitutive kinase activity against MEK in vitro (Fig. 9A, lane 1), it could not activate ERKs unless it was targeted to Ras or Rap1 (Fig. 9B), reflecting the requirement of specific membrane targeting for B-Raf’s activation of MEK/ERK in vivo. Therefore, we propose that B-Raf’s ability to mimic phosphorylation at residues 447 and 448 is critical for its ability to be activated by Rap1.
DISCUSSION

Multiple Ras isoforms localize Raf-1 to raft microdomains--Both endogenous Ki-Ras and N-Ras were readily detected in Cos-7 cells and localized to raft domains. In contrast, Ha-Ras was not detected in Cos-7 cells, using isoform-specific antisera. This is consistent with the results of Kranenburg et al. (11), who also detected little or no Ha-Ras in Cos-7 cells. In that study, Ki-Ras, largely, and N-Ras, partially, colocalized with caveolin. Colocalization with caveolin did not appear essential for activation as EGF activated N-Ras but not Ki-Ras in Cos-7 cells (11). We show that the cholesterol-depleting agent CD blocked EGF activation of Raf-1 kinase activity and S338 phosphorylation, consistent with the CD’s ability to block EGF’s activation of MEK/ERK in these cells (11). Although, it has been shown that CD potentiated activation of Ki-Ras (but not N-Ras), CD completely blocked the coupling of Ras to Raf-1/MEK/ERK (11).

S338 phosphorylation of Raf-1 requires targeting to raft microdomains--Recent studies suggest that the ability of small G proteins to regulate signaling cascades is dictated not only by the specificity of effector utilization, but also by their subcellular localization (2). Differences in the localization of specific Ras isoforms within rafts has been reported by some (2), but not others (11). Paradoxically, disruption of rafts by CD could completely inhibit coupling to downstream effectors, while actually increasing the GTP loading of selected Ras isoforms (11). This may reflect the need for selected Ras isoforms to shuttle in and out of the raft (10,48). In this study, we focused our attention on the requirement of raft localization not on Ras activation but activation of the proximal downstream effector Raf-1. We show that one of the functions of raft localization is that it permits phosphorylation of Raf-1 on S338.

Localization of Raf-1 to rafts appears to be required for full activation of ERKs (12). Using the cholesterol-depleting agent CD, we and others (11,13) have shown that
disruption of raft microdomains interferes with signaling of Raf-1 to ERKs. Raf-1 activation also requires phosphorylation at serine 338. This activating phosphorylation occurs within the plasma membrane for Raf-1, subsequent to Raf-1’s recruitment to Ras (27). Using CD to disrupt rafts, we show that intact rafts are required for proper phosphorylation at 338. Therefore, the requirement of raft localization for full Raf-1 activity is coupled to S338 phosphorylation, extending previous studies showing that the membrane-localized S338 kinase was required for Raf-1 activation by oncogenic Ras (29). A candidate kinase, PAK, has been proposed (49-51), however, its role has been challenged (52).

**CAV/DRG does not disrupt Ras activation of Raf-1 in Cos-7 cells**—A number of studies have demonstrated that at least two types of rafts exist; those that contain caveolin and those that do not (6,11,32). Caveolins are integral components of caveolae, 50-100 nm vesicular invaginations of the plasma membrane involved in vesicular trafficking and cell signaling (53). The role of caveolin in ERK signaling has received much recent attention, and both positive and negative affects on ERKs have been reported (9,11,54-57). Ras isoforms appear heterogeneous in their ability to couple to caveolins and to localize to caveolin-containing membranes. For example, in one study, Ki-Ras largely colocalized with caveolin in Cos-7 cells, whereas N-Ras only partially colocalized with caveolin (11). In BHK cells, Ki-Ras was largely excluded from caveolin-containing membrane fractions (10).

The caveolin-3 mutant DGV (CAV/DGV) acts as an interfering mutant of caveolin function (10,32). In BHK cells, this mutant inhibits Raf-1 activation by Ha-RasV12 but not by Ki-RasV12 (N-Ras was not examined) (9,15). In these cells, CAV/DGV expression increased the buoyant density of Ras-containing membranes. The effect of this shift was reflected in the redistribution of caveolin and to a lesser extent of Ha-Ras, with no redistribution of Ki-Ras detected (9). This suggests that Ki-Ras, and to a lesser extent H-Ras, remains localized to low density raft domains even in
the presence of CAV/DGV. This is likely true for N-Ras as well, since N-Ras only partially colocalizes with caveolin-containing membranes.

We show data that disrupting caveolin function by overexpressing CAV3/DGV did not block the activation of Raf-1 by EGF, Ha-RasV12, Ki-RasV12, or N-RasV12 in these cells. Our finding that CAV3/DGV did not block EGF activation of Raf-1 supports the results of Kranenburg et al. (11), and may reflect the prominent role of endogenous Ki-Ras and N-Ras in EGF’s actions in Cos-7 cells. However, the inability of CAV/DGV to block the action of Ha-RasV12 on Raf-1 appears to conflict with the results of Roy, et al. (9). This may reflect differences in the membrane compositions of the BHK cells (9,10) and the Cos-7 cells used in this study. For example, Ki-Ras, which we and others identified in raft microdomains in Cos-7 cells (11), has been localized to non-raft domains in BHK cells (10).

In this study we compared the ability of distinct CAAX motifs to potentiate the phosphorylation and activation of a variety of chimeric Raf-1/CAAX proteins whose carboxyl-terminal domains were derived from Ha-Ras or Rap1. We show that a Raf-1 chimera that included the complete carboxyl-terminal membrane targeting domains from Ha-Ras was localized to rafts, showed both constitutive activity and phosphorylation of S338, and activated ERKs. Chimeras containing only the minimal membrane-targeting motif [Raf-HaCAAX(-hvr)], however, had no basal activity. We suggest that the lack of activity of this chimera was a direct consequence of its inability to be phosphorylated on serine 338. The ability of Y341D to restore the raft localization and S338 phosphorylation of Raf-1-HaCAAX(-hvr) and kinase activity argues that localization to specific raft microdomains may be necessary and sufficient for S338 phosphorylation and activation of Raf-1. Recent studies have proposed that hvr sequences help shuttle HaRas out of the rafts in a GTP-dependent fashion (10) and cooperate in effector utilization (16). Differences in the localization of mutant Ha-Ras proteins and Raf/Ras chimeras may be due to the influence of sequences in Ras mutants.
that are absent from the Raf/Ras chimeras. It is also possible that activated Ras shuttles 
Raf-1 into the raft where it is phosphorylated on S338 and subsequently exits the raft, 
as suggested by recent studies (10,48).

Phosphorylation of Y341 is required for proper raft localization and subsequent 
phosphorylation of S338—Upon EGF stimulation, RafS338A was localized to a raft 
microdomain. Moreover, RafS338A-HaCAAX(+hvr) was constitutively localized to a 
raft domain. These data demonstrate that S338 phosphorylation was not required for 
raft localization, but likely occurs subsequently. This is consistent with a model that 
Raf-1 activation by Ha-Ras requires post-translational modifications, including S338 
phosphorylation that occur within specialized microdomains.

We show here that Y341 phosphorylation of Raf-1 was a prerequisite for S338 
phosphorylation, consistent with previous results (27). Marais and colleagues also 
showed that tyrosine phosphorylation by Src enhanced S338 phosphorylation of Raf-1 
(27). The data presented here suggest that one of the consequences of Y341 
phosphorylation may be the repositioning of Raf-1 near potential S338 kinases. The 
requirement of Y341 in Raf-1 activation and S338 phosphorylation, however, could be 
overcome by membrane targeting, suggesting that one of the functions of Y341 
phosphorylation is to facilitate proper membrane localization. Indeed, RafY341A 
mutants were unable to enter rafts upon EGF stimulation, unless linked to ectopic raft-
targeting domains.

One explanation for the increased phosphorylation on S338 seen in Y341D 
mutants is that this reflects the strong cooperativity between the phosphorylations of 
these sites (50). However, in studies examining the ability of PAK to phosphorylate 
Raf-1 in vitro, this was not the case (58). Another explanation is that Y341D mutants 
relocalize Raf-1 to sites of S338 phosphorylation. Phosphorylation of Y341 has been 
proposed to function in concert with pS338 to provide a negatively charged surface on
the Raf-1 protein (27). We suggest that one additional function of phosphorylation of Y341 that is distinct from that of S338, is to target Raf-1 to specific membrane sites that participate in subsequent phosphorylations.

*Rap1 association with Raf-1 is not sufficient for the phosphorylation of Y341*--The inability of some small G proteins to activate Raf-1 despite recruiting Raf-1 to the membrane also suggests that recruitment to the membrane is not sufficient for Raf-1 activation. One small G protein that binds Raf-1 without activating it is Rap1 (44). Chimeric Ras/Rap1 proteins that replace membrane targeting domains of Ras with those of Rap1 are growth inhibitory (42), but this inhibition can be relieved by constitutively active Raf-1, suggesting that the inhibitory effects of this chimera were due to impaired Raf-1 activation.

One proposed function for Ras is the displacement of the 14-3-3 protein from its binding site on residue 259 within Raf-1 (35). The inability to displace 14-3-3 from Raf-1 may explain the inability of selected G proteins to activate Raf-1 (59). However, for Rap1, such a model has been ruled out (59). This suggests that other mechanisms account for the inability of Rap1 to activate Raf-1. Studies have demonstrated that activation of endogenous Rap1 limits Ras activation of Raf-1 (33,38,44). It has been proposed that Rap1 interferes with Ras by trapping the Ras/Raf-1 complex in an inactive conformation (60,61). However, recent studies have demonstrated that Ras and Rap1 occupy distinct subcellular regions (39,41,43), even following Rap1 activation (40).

In part because of its distinct location, Rap1 has been proposed to inhibit Ras activation of Raf-1 by sequestering Raf-1 from Ras. This is consistent with studies showing a loss of Ras/Raf-1 association (and a parallel increase in Rap1/Raf-1 association) upon Rap1 activation (38). Data presented here suggest a possible explanation for the inability of Rap1 recruitment of Raf-1 to activate Raf-1--the
inability of Rap1 to support Raf-1 phosphorylations. First, Raf-1 chimeras that were targeted to Rap1-containing membranes via Rap1CAAX motifs were neither activated nor phosphorylated on S338. Second, retargeting Rap1 by swapping in HaRasCAAX sequences allowed Rap1 to activate Raf-1 and to phosphorylate S388. Mutation of Raf-1 to mimic Y341 phosphorylation (Y341D) resulted in a Raf-1 protein that could be activated and phosphorylated on S338 following Rap1 activation, suggesting that the phosphorylation on Y341 can partially overcome Rap1’s inability to activate Raf-1. This may be due to the lack of specific Y341 kinases within Rap1 domains. Therefore, we propose that Rap1 prevents Raf-1 activation by positioning it away from tyrosine kinases that are required for Y341 phosphorylation. One of the functions of Y341 phosphorylation might be to provide a regulatable interaction with proteins or lipids to participate in proper targeting of Ras/Raf-1 (62,63).

B-Raf’s lack of dependence on tyrosine phosphorylation accounts for its activation by Rap1. The Y341D mutation in Raf-1 resembles the naturally occurring sequences within the Raf isoform, B-Raf. B-Raf lacks a tyrosine at the site corresponding to Y341 in Raf-1 (448 in B-Raf). Like Raf-1Y341D, B-Raf is constitutively phosphorylated on the nearby serine (S338 in Raf-1, S445 in B-Raf). Although B-Raf was constitutively active in *in vitro* kinase assays, we show that membrane recruitment was required to permit B-Raf to activate MEK and ERKs *in vivo*. Moreover, targeting of B-Raf chimeras via either Rap1-CAAX or Ras-CAAX was sufficient. The ability of B-Raf-Rap1 chimeras to activate ERKs confirms that the requirement for membrane localization for B-Raf activation by small G proteins is less stringent than that of Raf-1. The mutant of B-Raf in which D447D448 was replaced by tyrosines (B-RafYY) behaved like Raf-1; it was no longer activated by Rap1, but retained the ability to be activated by Ras. The unique specificity of Rap1 for B-Raf activation, but not Raf-1 activation, can be largely explained by the distinct requirements of each kinase for specific membrane targeting for phosphorylation and activation. Future studies examining the ability of Rap1 to support additional critical
phosphorylations, including T491 and S494 in Raf-1 (T598 and S601 in B-Raf) (28,64) may be informative as well.

It has been proposed that sequences within the cysteine-rich domain (CRD) of Raf-1 and B-Raf dictated the contrasting actions of Rap1 on each Raf isoform (44). However, the ability of Rap1 to activate RafY341D, as well as the ability of Rap/Ras chimeras to activate wild type Raf-1, both argue strongly that the interactions between Rap1 and Raf-1 are not the only determinants of Raf-1 inhibition. It should be noted that Rap1/Ras chimeras were not as effective as Ras in activating/phosphorylating Raf-1, suggesting that Ras also provides an activation function that is distinct from localization (22,59,65). Furthermore, the lack of activation of B-RafYY by Rap1 suggests that interactions between the B-Raf CRD and Rap1 are also not sufficient to promote activation, although they may be important (44). We propose that the carboxy-terminal domain of Rap1 provides specificity to Rap1 signaling in addition to that provided through the interaction between Rap1’s effector loop and the Raf CRDs.

In conclusion, we show that Raf-1 phosphorylation at S338 requires membrane targeting of Raf-1 to specific raft microdomains. We propose that tyrosine phosphorylation of Y341 potentiates S338 phosphorylation by facilitating proper membrane localization. This two-step mechanism is outlined in Fig. 10 and may explain the contrasting actions of Ras and Rap1 on Raf isoforms.
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FIGURE LEGENDS

Fig. 1. EGF stimulation relocalizes Rap-1 to raft microdomains.
(A) Schematic illustration of the sucrose density gradient assay. Isopycnic centrifugation is used to achieve an equilibrium gradient. The lipid-associated proteins migrate to buoyant densities, where they can be detected within the top fractions of the gradient. The localization of Raf-1 in EGF-treated cells is shown as an example. (B) Sucrose density gradient profiles of Cos-7 cells. Cells were lysed and proteins prepared for sucrose equilibrium density gradient centrifugation as described in Experimental Procedures. The top twelve 0.43 ml fractions are shown (open squares). The protein content of each fraction is shown as black circles. (C) Growth factor stimulation recruits Raf-1 to lipid rafts. Cells were treated with or without EGF as indicated, and proteins separated into the indicated fractions by equilibrium centrifugation on a sucrose density gradient (1 = top). Fractions one through ten were pelleted and proteins separated by SDS-PAGE. The position of caveolin-1 within the gradient is shown in the upper panel using caveolin-1 antisera. The presence of endogenous Raf-1 within fractions (1-10) of each gradient is shown in the middle and lower panels, using antisera to Raf-1. (D) Sucrose density gradients of protein fractions containing Raf-1 and Raf mutants. Cells were transfected with wild type Flag-tagged Raf-1 (Raf-1 WT), treated with or without EGF or CD, as indicated, and proteins separated into the indicated fractions by sucrose density gradients. The presence of FlagRaf-1 constructs within fractions 1-10 of each gradient is shown in the lower two panels, using Flag antibody. (E) Activated Ras proteins recruit Raf-1 into raft microdomains. Cos-7 cells were transfected with Flag-Raf-1 and either vector, HaRasV12 (top panel), KiRasV12 (second panel), NRasV12 (third panel), or vector (bottom panel) as indicated, and proteins separated into the indicated fractions by sucrose density centrifugation. The presence of Flag-Raf-1 within fractions (1-10) of each gradient is shown using the Flag antibody.
FIG. 2. Ras isoforms are localized to raft domains.

(A) Western blot of Ras isoforms expressed in Cos-7 cells. Cells were left untransfected or transfected with Flag-HaRas, Myc-KiRas, or HA-NRas. Proteins were assayed by Western blot for endogenous and transfected Ras proteins, using isoform-specific Ras antibodies directed against Ha-Ras, Ki-Ras, and N-Ras as indicated. (B) Sucrose density gradients of membrane fractions from unstimulated Cos-7 cells. The presence of endogenous Ki-Ras (top panel) and N-Ras (bottom panel) are shown using Ki-Ras or N-Ras antisera, respectively. (C) Sucrose density gradients of membrane fractions containing transfected Ras proteins. Cells were transfected with wild type (WT) Flag-HaRas, myc-KiRas, or HA-NRas as indicated, and proteins separated into the indicated fractions by sucrose density gradients. The presence of Ha-Ras (top panel), Ki-Ras (middle panel), and N-Ras (lower panel) within fractions 1-10 of each gradient were analyzed using Flag, myc, and HA antibodies respectively. (D) Sucrose density gradients of membrane fractions containing transfected Ras proteins. Cells were transfected with constitutively active Flag-HaRasV12, myc-KiRasV12, or HA-NRasV12, as indicated. The presence of HaRasV12 (top panel), KiRasV12 (middle panel), and NRasV12 (lower panel) within fractions 1-10 of each gradient were analyzed using Flag, myc, and HA antibodies respectively.

FIG. 3. S338 phosphorylation and activation requires intact raft domains.

(A) Raf-1 kinase assays. HaRasV12 and either Flag-tagged Raf-1, RafS338, RafY341A, or pcDNA3 vector was transfected into Cos-7 cells. Equivalent amounts of Raf protein were immunoprecipitated using the Flag antibody (Flag I.P.) and assayed for the ability to phosphorylate MEK (pMEK) in vitro (top panel). The levels of FlagRaf (middle panel) and MycRasV12 (lower panel) are shown. (B) Cells were transfected with Flag Raf-1 wild type (WT) and treated with EGF and/or CD as indicated. Lysates were subjected to Flag I.P. and assayed for p338 (upper panel) and the ability to phosphorylate MEK (pMEK) in vitro (middle panel). The levels of FlagRaf-1 expression are shown in the lower panel, using Flag antibody. (C) Cells
were transfected with wild type FlagRaf-1 (WT) or FlagRaf-Y341A, and treated with EGF and/or CD as indicated. Lysates were subjected to Flag I.P. and assayed for tyrosine phosphorylation with pTyr antibody (pTyr) (upper panel). The levels of FlagRaf-1 expression are shown in the lower panel, using Flag antibody.

**FIG. 4.** Interfering mutants of caveolin do not disrupt Ras function.  
(A) Epifluorescent micrographs of CAV/WT and CAV/DGV. Cos-7 cells were transfected with myc-CAV/WT (WT; panels 2, 4, and 6) or myc-CAV3/DGV (DGV; panels 1, 3, 5, and 7). The expression of myc-CAV/DGV is shown in the panels 1 and 2 (anti-myc; red). The expression of endogenous caveolin-1 is shown in panels 3 and 4 (anti-caveolin; green). Merges of the red and green images are shown in panels 5 and 6. Panel 7 shows a magnification of the boxed area in panel 5. Bar = 10 µM. The zoom field is 10x10 µM.  
(B) Cells were transfected with FlagRaf-1 and either vector (-) or myc-CAV/DGV (+), and treated with EGF or left untreated, as indicated. Lysates were subjected to Flag I.P. and assayed for p338 (top panel) and the ability to phosphorylate MEK (pMEK) *in vitro* (second panel). The levels of FlagRaf-1 expression are shown in the third panel, using Flag antibody. The levels of myc-CAV/DGV expression are shown in the fourth panel, using myc antibody.  
(C) Cells were transfected with FlagRaf-1 along with HaRasV12 (Ha), KiRasV12 (Ki), or NRasV12 (N), and vector (V), with CAV/DGV; (+) or without CAV/DGV; (-). Lysates were subjected to Flag I.P. and assayed for p338 (top panel) and the ability to phosphorylate MEK (pMEK) *in vitro* (second panel). The levels of FlagRaf-1 expression are shown in the third panel, using Flag antibody. The levels of myc-CAV/DGV expression are shown in the fourth panel, using myc antibody.

**FIG. 5.** The requirement for Y341 can be overcome by targeting Raf-1 to raft domains.  
(A) Sucrose density gradients of membrane fractions containing Raf-1 mutants. Cells were transfected with Flag-tagged RafY341A, and RafS338A, treated with EGF as indicated, and proteins separated into the indicated fractions by sucrose density
gradients, as in Fig. 1C. The presence of FlagRaf constructs within each gradient is shown in both panels, using Flag antibody. (B) Phosphorylation and activation of Raf-1 mutants by EGF. Flag-tagged Raf-1, RafY341A, or RafY341D were transfected into Cos-7 cells, and cells were either treated with EGF or left untreated, as indicated. Lysates were subjected to Flag I.P. and assayed for p338 (upper panel) and the ability to phosphorylate MEK (pMEK) in vitro (middle panel). The levels of FlagRaf proteins are shown in the lower panel, using Flag antibody. (C) Immunofluorescence of Raf-Ras chimeras. Cos-7 cells were transfected with Flag-tagged Raf-1 WT, Raf-HaCAAX(+hvr), and Raf-HaCAAX(-hvr), as indicated. Cells were prepared for epifluorescent microscopy as described in Experimental Procedures, and representative cells are shown. (D) Sucrose density gradients of Raf-Ras chimeras. Cells were transfected with Flag-tagged Raf-HaCAAX(+hvr), Raf Y341AHaCAAX(+hvr), and Raf S338AHaCAAX(+hvr), and left untreated. Proteins were separated as in Fig. 1C and the presence of the chimera within each fraction is shown, using Flag antibody. (E) Lack of requirement of Y341 for S338 phosphorylation in targeted chimeras. Cells were transfected with Raf-1 WT, Raf-HaCAAX(+hvr), RafS338A-HaCAAX(+hvr), or RafY341AHaCAAX(+hvr), and treated with or without EGF, as indicated, and assayed for p338 (upper panel), or Raf-1 kinase activity (pMEK, middle panel). The levels of Flag-containing proteins are shown in the lower panel.

**Fig. 6.** Ectopic targeting of Raf-1 to raft domains is required for constitutive S338 phosphorylation, activation of Raf-1, and activation of ERKs. (A) Localization of Raf-Ras chimeras. Cells were transfected with Flag-tagged Raf-1 WT and the Raf-Ras chimeras as indicated and fractionated into S100 and P100 fractions, and Flag-containing proteins detected by western blot. (B) Sucrose density gradients of Raf-HaCAAX(-hvr). Cells were transfected with Flag-tagged Raf-HaCAAX(-hvr), and left untreated. Proteins were separated as in Fig. 1C and the presence of the chimera within each fraction is shown (upper panel), using Flag antibody. Gradients of lysates expressing Raf-HaCAAX(+hvr) are shown as a control (lower panel). (C) Raft-targeted
chimeras show constitutive activity. Cells were transfected with MycERK2 and individual Raf-1 chimeras. Cells transfected with wild type Raf-1 were also treated with EGF as indicated. Flag-containing proteins were recovered by I.P. and examined for S338 phosphorylation (p338, first panel), and phosphorylation of MEK \textit{in vitro}, as in Fig. 1B (pMEK, second panel). The position of Flag Raf proteins is shown in the third panel (FlagRaf). In the lower two panels, the lysates were subjected to Myc I.P. and the recovered MycERK2 examined for phosphorylation (pMycERK2) or total MycERK levels (MycERK2).

FIG. 7. Mutation of Y341D restores phosphorylation of S338, activation and raft localization of inactive Raf/Ras(-hvr) chimeras. (A) Raft localization of RafY341DHaCAAX(-hvr). Cells were transfected with RafY341DHaCAAX(-hvr) and proteins fractionated as in Fig. 1C. RafS338D-HaCAAX(-hvr) is shown as a control (lower panel). The presence of Flag-containing proteins within each fraction is shown. (B) S338 phosphorylation, and Raf-1 activity assays. Cells were transfected with either Flag-tagged Raf-HaCAAX(-hvr), RafY341DHaCAAX(-hvr), Raf-HaCAAX(+hvr), or RafY341DHaCAAX(+hvr) and immunoprecipitated using Flag antibody and assayed for S338 phosphorylation (upper panel) and the ability to phosphorylate MEK (pMEK) \textit{in vitro} (middle panel). The levels of FlagRaf-1 expression are shown in the lower panel, using Flag antibody. (C) Raf-1 assays from three independent experiments, as in Fig. 4B. The data are shown as fold activation above that seen for Raf-1 WT, with standard error.

FIG. 8. The inability of Rap1 to activate Raf-1 is due to the inability of Rap1 to induce S338 phosphorylation. (A) GFP epifluorescence. Cells were transfected with GFP (a), GFP-RapE63 (b), GFP-HaRasV12 (c), and GFP-RapE63-HaRas-CAAX (d). The locations of the transfected proteins were examined by epifluorescent microscopy, and representative cells shown. (B) Rap1 supports neither Raf-1 activation nor S338 phosphorylation. Cells were transfected with Raf-1 WT along with pcDNA3, RapE63,
RapE63-HaCAAX(+hvr) or HaRasV12 and examined for S338 phosphorylation (p338) and Raf-1 activity (pMEK). (C) S338 phosphorylation and activation of RafY341D by Rap1. Cells were transfected with RafY341D along with either pcDNA3, RapE63, RapE63-HaCAAX(+hvr) or HaRasV12 and examined for S338 phosphorylation (p338) and kinase activity of Raf-1 (pMEK). (D) Raf-Rap1 chimeras are not constitutively active. Cells were transfected with either Flag Raf-1 wild type (WT), Raf-Rap1CAAX, RafY341D-Rap1CAAX or Raf-HaCAAX(+hvr). Cells were treated with EGF (+) or left untreated (-) and lysates immunoprecipitated using Flag antibody and assayed for both S338 phosphorylation (upper panel) and Raf-1 activity in vitro (middle panel). The levels of FlagRaf expression are shown in the lower panel, using Flag antibody. (E) GFP epifluorescence. Cells were transfected with GFP-Raf-Rap1CAAX (a) or GFP-RafY341-Rap1CAAX (b). The locations of the transfected proteins were examined by epifluorescent microscopy, and representative cells are shown.

FIG. 9. The ability of B-Raf to be activated by Rap1 requires aspartic acid residues at positions D447/D448 in B-Raf. (A) p445 phosphorylation and B-Raf activity assays. Cells were transfected with FlagB-Raf wild type (B-Raf WT) or FlagB-RafYY (B-RafYY) along with either pcDNA3, HaRasV12, or RapE63, as indicated. Lysates were subjected to Flag I.P. and assayed for both p338 (upper panel) and Raf-1 activity in vitro (pMEK, middle panel). The levels of FlagB-Raf expression are shown in the lower panel, using Flag antibody. (B) ERK activation by B-Raf requires targeting to either Rap1- or Ras-containing membranes. Cells were transfected with MycERK2 and either Flag B-Raf wild type (WT), B-Raf-HaCAAX(+hvr), or B-Raf-Rap1CAAX and immunoprecipitated using Flag antibody and assayed for S445 phosphorylation (p445, upper panel). In the middle panel (pMycERK2), the total lysates were assayed for phosphorylation of MycERK2. The levels of FlagB-Raf expression are shown in the lower panel, using Flag antibody.
FIG. 10. A model of sequential phosphorylation of Raf-1 for full activation. EGF induces the recruitment of Raf-1 to plasma membrane-bound Ras. Following this association of Ras with Raf-1 two sequential modifications occur. The first modification is the phosphorylation of Y341 by membrane-bound Y341 kinases, whose activities are induced by EGF and/or Ras activation. This phosphorylation may relocalize the Ras/Raf-1 complex within specialized plasma membrane microdomains where a second phosphorylation on S338 can occur that renders the Raf-1 molecule competent in phosphorylating downstream effectors like MEK/ERK. Rap1 signaling is depicted in the lower portion of the figure. Rap1 activation does not lead to phosphorylation of Raf-1 or activation of MEK/ERK, although Raf-1 is recruited to Rap1-containing membranes. In contrast, B-Raf is constitutively phosphorylated on S445 (S338 equivalent site), and the adjacent tyrosines in Raf-1 are replaced with aspartate residues (447D,448D), and therefore does not need to be phosphorylated upon recruitment to Rap1. In this case, Rap1 is capable of coupling B-Raf to MEK/ERK signaling. We suggest that the lack of Y341 activity within Rap1 domains is the limiting step in Rap1’s inability to activate Raf-1. Gray and white circles represent Y341 kinases and S338 kinases, respectively.
A. Isolation of Lipid Rafts

Cells

Gradient

Analysis

B.

\[ \% \text{ sucrose} \quad \% \text{ total protein} \]

C.

Caveolin-1

Raf-1 (-EGF)

Raf-1 (+EGF)

D.

Raf-1 WT (-EGF)

Raf-1 WT (+EGF)

Raf-1 WT (+EGF) plus CD

E.

vector

HaRasV12

KRasV12

N RasV12

Carey et al, Fig. 1
Carey et al. Fig. 2
Carey, et al. Fig. 3
A

anti-myc  anti-cavolin  merge  zoom

DGV

1  3  5  7

WT

2  4  6

B

mycCAV3/DGV  -  +  -  +

-p338
-pMEK
-Flag-Raf-1
-mycCAV/DGV

EGF  -  -  +  +

C

V  Ha  Ki  N

-western blot -p338

-kinase assay -pMEK

-anti-Flag WB -FlagRaf WT

-anti-myc WB -mycCAV/DGV

CAV/DGV  -  +  -  +  -  +  +

Carey, et al. Fig. 4
Carey, et al. Fig. 5
Carey, et al. Fig. 7
A

| pDNA3 | H-Ras V12 | RapE63 | pDNA3 | H-Ras V12 | RapE63 |
|-------|-----------|--------|-------|-----------|--------|
|       |           |        |       |           |        |

- p445
- pMEK
- FlagB-Raf

B

| pDNA3 | B-Raf WT | B-Raf-hiCAAX (+24h) | B-Raf Rapt-CAAX |
|-------|----------|---------------------|-----------------|
|       |          |                     |                 |

- p445
- pMycERK2
- FlagB-Raf

Carey, et al. Fig. 9
Ras activation

Raf-1

EGF

Y341 kinase

Ras

Raf-1

S338 kinase

Y341 kinase

Y341

Ras

Raf-1

S338

Raf-1

Y341

S338 kinase

Y341 kinase

MEK

ERK

Rap1 activation

Raf-1

B-Raf

No Y341 kinase

Raf-1

MEK

ERK

Raf-1

B-Raf

S415

447/448

MEK

ERK

Carey, et al. Fig. 10
The requirement of specific membrane domains for Raf-1 phosphorylation and activation
Kendall D. Carey, Robert T. Watson, Jeffrey E. Pessin and Philip J. S. Stork

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