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Membrane-targeting potentiates guanine nucleotide exchange factor CDC25 and SOS1 activation of Ras transforming activity

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ABSTRACT **Growth factor-triggered activation of Ras proteins is believed to be mediated by guanine nucleotide exchange factors (CDC25/GRF and SOS1/2) that promote formation of the active Ras GTP-bound state. Although the mechanism(s) of guanine nucleotide exchange factor regulation is unclear, recent studies suggest that translocation of SOS1 to the plasma membrane, where Ras is located, might be responsible for Ras activation. To evaluate this model, we generated constructs that encode the catalytic domains of human CDC25 or mouse SOS1, either alone (designated cCDC25 and cSOS1, respectively) or terminating in the carboxyl-terminal CAAX membrane-targeting sequence from K-Ras4B (designated cCDC25–CAAX and cSOS1–CAAX, respectively; in CAAX, C is Cys, A is an aliphatic amino acid, and X is Ser or Met). We then compared the transforming potential of cCDC25 and cSOS1 with their membrane-targeted counterparts. We observed that addition of the Ras plasma membrane-targeting sequence to the catalytic domains of CDC25 and SOS1 greatly enhanced their focus-forming activity (10- to 50-fold) in NIH 3T3 transfection assays. Similarly, we observed that the membrane-targeted versions showed a 5- to 10-fold enhanced ability to induce transcriptional activation from the Ets/AP-1 Ras-responsive element. Furthermore, whereas cells that stably expressed cCDC25 or cSOS1 exhibited the same morphologies as untransformed NIH 3T3 cells, cells expressing cCDC25–CAAX or cSOS1–CAAX displayed transformed morphologies that were indistinguishable from the elongated and refractile morphology of oncogenic Ras-transformed cells. Thus, these results suggest that membrane translocation alone is sufficient to potentiate guanine nucleotide exchange factor activation of Ras.**

Ras proteins are GDP/GTP-binding proteins that function as molecular switches to mediate downstream signaling from a diverse variety of extracellular stimuli that influence cell growth and differentiation (1). Ras activity is controlled by a GDP/GTP cycle that is negatively regulated by GTPase-activating proteins (GAPs; p120 and NF1 GAP) that stimulate the intrinsic GTPase activity of Ras to promote formation of the inactive GDP-bound state of Ras and positively regulated by guanine nucleotide exchange factors (GEFs; CDC25/GRF and SOS1/2) that promote exchange of GDP for GTP to attain the active conformation (2). Mutations that activate Ras transforming potential render Ras proteins insensitive to GAP stimulation. Hence, oncogenic Ras proteins persist in the active GTP-bound state, thereby leading to constitutive activation of downstream effector targets. However, deregulated function of Ras GAPs (3, 4) or GEFs (5–7), in the absence of Ras mutations, may also lead to constitutive activation of Ras and, consequently, transformation.

A second critical requirement for Ras function is its association with the inner face of the plasma membrane (8). This association is mediated by a series of closely linked posttranslational modifications (farnesylation, proteolysis, and carboxylmethylation), which are signaled by a consensus carboxyl-terminal CAAX motif (where C is Cys, A is an aliphatic amino acid, and X is Ser or Met) present on all Ras proteins. Mutant Ras proteins that do not undergo this processing fail to associate with the plasma membrane and are completely impaired in their mitogenic and transforming properties. Although the precise role of membrane association in Ras transformation is not known, it is likely that this subcellular location is essential for Ras interaction with its upstream GDP/GTP regulators or downstream effector targets (e.g., GAPs and Raf) (2).

While mitogen-stimulated elevations in Ras-GTP are believed to occur primarily as a consequence of the activation of Ras GEFs, the precise mechanism for this activation remains unresolved (9). Recent studies identifying the role of SOS1 in mediating epidermal growth factor (EGF)-triggered activation of Ras have suggested a mechanism whereby recruitment to the plasma membrane may be sufficient to cause Ras activation (10–17). In quiescent Rat-1 fibroblasts, SOS1 was found almost exclusively in the cytosolic fraction. However, upon EGF stimulation, Buday and Downward (12) observed that the majority of SOS1 was translocated to the particulate fraction and was found to be associated with the EGF receptor. This association was mediated via the Grb-2 adaptor protein that is composed exclusively of a Src homology (SH) 2 domain flanked by two SH3 domains (18). While the SH3 domains promote Grb-2 association with proline-rich sequences in SOS1, the SH2 domain recognizes a specific phosphorylated tyrosine residue on the stimulated EGF receptor (12, 14, 16, 17). Since no change was observed in the intrinsic enzymatic activity of SOS1 upon association of the SOS1–Grb-2 complex with the EGF receptor (12), it has been proposed that this translocation event presumably causes an increase in the local concentration of SOS1 at the plasma membrane to then trigger the activation of Ras (9, 12, 13). To evaluate this model, we have determined whether membrane translocation of CDC25 and SOS1 potentiates the ability of these Ras GEFs to activate Ras and to cause cellular transformation. Our observation that addition of the Ras plasma membrane-targeting sequence to the catalytic domains of CDC25 and SOS1 greatly enhanced their transforming (10- to 50-fold) and transactivation (5- to 10-fold) activities suggest that membrane translocation alone is sufficient to potentiate GEF activation of Ras.

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Abbreviations: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; EGF, epidermal growth factor; CAT, chloramphenicol acetyltransferase; RRE, Ras-responsive element.

MATERIALS AND METHODS

Molecular Constructs of CDC25 and SOS1. DNA sequences encoding the catalytic domains of human CDC25/GRF (residues 863–1275) or mouse SOS1 (residues 559–1071) (19, 20) were generated by *Taq* polymerase chain reaction (PCR) DNA amplification of partial cDNA sequences of the respective cDNAs to incorporate *Bam*HI restriction sites along with 5′ initiating ATG and 3′ stop codons (designated cCDC25 and cSOS1, respectively). Generation of the membrane-targeted versions of cCDC25 and cSOS1 was performed by four-primer PCR procedures to add the carboxyl-terminal 18 codons of K-*ras4B* onto the carboxyl termini of cCDC25 and cSOS1 to generate chimeric genes encoding cCDC25–CAAX and cSOS1–CAAX, respectively. All sequences were verified by dideoxynucleotide sequencing and introduced into the pZIP-NeoSV(x)1 retrovirus vector or the pcDNA mammalian expression vector for expression from the Moloney long terminal repeat or cytomegalovirus promoters, respectively.

Cell Culture and Transformation Assays. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) calf serum. DNA transfections were done using the calcium phosphate precipitation technique as described (21). Transformed foci were quantitated after 14–16 days. Transfected cells were also selected in growth medium containing G418 at 400 μg/ml (Geneticin, GIBCO/BRL) to establish cell lines that stably expressed each CDC25 or SOS1 protein. The growth properties of NIH 3T3 cells expressing stably transfected Ras GEFs were compared in soft agar (0.3%) as described (21).

Chloramphenicol Acetyltransferase (CAT) Assays. To determine the ability of CDC25 or SOS1 proteins to induce normal Ras transcriptional activation, NIH 3T3 cells were transiently cotransfected with 2–5 μg of pZIP DNA encoding the catalytic domains of CDC25 and SOS1 plus 1 μg of the pB4X-CAT CAT reporter plasmid driven by minimal promoter sequences that contain the Ets/AP-1 Ras-responsive element (RRE). pB4X-CAT contains four tandem copies of the RRE from the polyomavirus enhancer (Ets/AP-1 sequences) (provided by B. Wasylyk, Institut Chimie Biologique, Strasbourg, France) (22). Forty-eight hours after transfection, cell lysates were prepared, and the CAT activity was assayed as described (23, 24).

RESULTS

To evaluate the role of membrane translocation in Ras GEF activation of Ras, we have determined whether artificial membrane targeting of the isolated catalytic domains of mammalian CDC25 and SOS1 potentiates the ability of these Ras GEFs to activate Ras and to cause cellular transformation of NIH 3T3 cells. Isolated catalytic fragments were used for these studies to enable the effects of membrane translocation of GEFs to be examined in the absence of any potential regulatory effects of the noncatalytic sequences. We generated pZIP retrovirus constructs that encode the catalytic domains of human CDC25 (residues 863–1275) (19) or mouse SOS1 (residues 559–1071) (20), either alone (designated cCDC25 and cSOS1, respectively) or terminating in the carboxyl-terminal CAAX membrane targeting sequence from K-Ras4B (designated cCDC25–CAAX and cSOS1–CAAX, respectively) (Fig. 1). We (23) and others (25–27) have shown that this 18-amino acid sequence is sufficient to specifically target heterologous proteins to the plasma membrane. Since recent studies have shown that human or yeast CDC25 and *Drosophila* SOS can transform rodent fibroblasts via activation of endogenous Ras (7, 13, 28), we compared the transforming potential of cCDC25 and cSOS1 with their membrane-targeted counterparts.

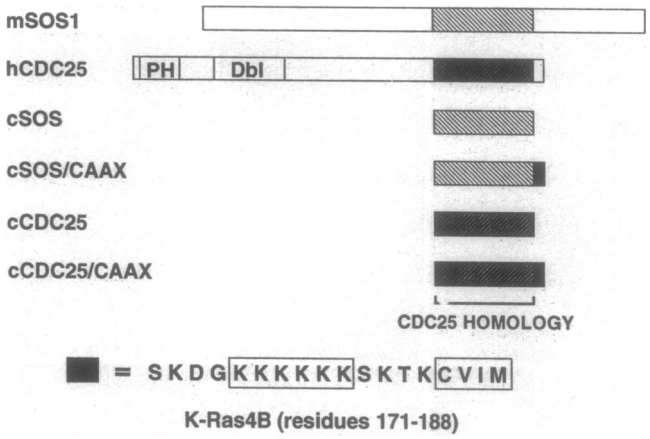

FIG. 1. Linear representation of CDC25 and SOS1 indicating the catalytic domains and K-Ras4B plasma membrane targeting sequence. Sequences encoding the catalytic domains of human CDC25/GRF (residues 863–1275) or mouse SOS1 (residues 559–1071) (19, 20) were generated. Membrane-targeted versions of cCDC25 and cSOS1 were generated by addition of the carboxyl-terminal 18 codons of K-*ras4B* onto the carboxyl termini of cCDC25 and cSOS1 to generate chimeric genes encoding cCDC25–CAAX and cSOS1–CAAX, respectively. PH, pleckstrin homology domain; Dbl, Dbl oncoprotein homology domain.

While no transformed foci were observed in cultures transfected with up to 1 μg of pZIP-cCDC25, morphologically transformed foci (20–30 foci per μg of DNA) were readily observed in cultures transfected with 1 μg of pZIP-cCDC25–CAAX plasmid DNA (Table 1). Cotransfection with normal Ras further potentiated cCDC25–CAAX-induced focus-forming activity (2- to 3-fold), whereas no significant enhancement of cCDC25 focus-forming activity was observed. When compared to the nontargeted version of cSOS1, we also observed a significant (10- to 20-fold) enhancement of transforming activity induced by cSOS1–CAAX. Thus membrane targeting greatly potentiated cSOS1 and cCDC25 activation of Ras transforming activity (10- to 50-fold).

The enhanced transforming potential of the membrane-targeted forms of cCDC25 and cSOS1 was also evident in the morphology of stably transfected NIH 3T3 cells, where only cells expressing the membrane-targeted GEFs displayed transformed morphologies, which were indistinguishable from oncogenic Ras-transformed cells (Fig. 2). Furthermore, both cCDC25–CAAX- and cSOS1–CAAX-transformed cells displayed the same transformed growth properties (e.g., colony formation in soft agar) as oncogenic Ras-transformed cells (Fig. 3). To further characterize the consequences of membrane targeting for Ras GEF function, we evaluated the

Table 1. Transforming activity of mammalian CDC25/GRF and SOS1 constructs

Plasmid construct	Transformed foci, no. per dish	
	Alone	+ Ras(WT)
pZIP-NeoSV(x)1	0.0	0.0
pZIP-cCDC25	0.0	1.0
pZIP-cCDC25–CAAX	23.0	58.0
pZIP-cSOS1	0.0	2.5
pZIP-cSOS1–CAAX	0.0	24.5

NIH 3T3 cells were transfected with pZIP plasmid DNAs of cCDC25 or cSOS1 (1 and 2 μg per dish, respectively) alone or with 500 or 250 ng of H-*ras*(WT) DNA in 60-mm dishes, and transformed foci were quantitated after 14–18 days. Values represent the average of four dishes and are representative of at least three experiments. Under these conditions, pZIP-ras(61L) typically induced $>4 \times 10^3$ foci per μg of plasmid DNA.

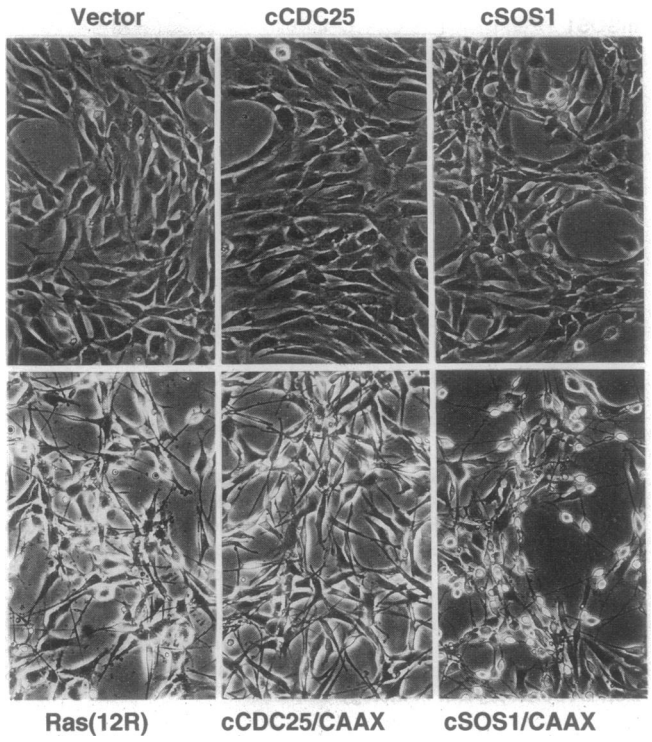

FIG. 2. Morphologic transformation of NIH 3T3 cells expressing membrane-targeted forms of cCDC25 and cSOS1. NIH 3T3 cells were transfected with pZIP plasmid vector constructs that encode the indicated proteins by using the calcium phosphate precipitation procedure as described (29). Stably transfected cells were isolated in growth medium supplemented with G418 (Geneticin) at 400 μg/ml, and drug-resistant colonies were pooled to establish cell lines expressing each protein. (×75.)

abilities of the GEFs to promote transcriptional activation from RREs that regulate CAT gene expression in the pB4X-CAT reporter plasmid. Although oncogenic, but not normal, Ras proteins can efficiently (10- to 20-fold) stimulate transcription from this reporter construct (24, 28), we (24) and others (28) have shown that Ras GEFs can stimulate the activity of normal Ras to activate transcription from RREs. While transfection of 1 μg of pZIP-cCDC25 DNA caused limited stimulation of transcription activation from pB4X-CAT (Fig. 4*A*), 1 μg of pZIP-cCDC25–CAAX efficiently (6- to 10-fold) promoted CAT activity. Lower concentrations of pZIP-cCDC25–CAAX (0.25 μg) acted synergistically with cotransfected normal Ras to cause efficient stimulation. Similarly, whereas 1 μg

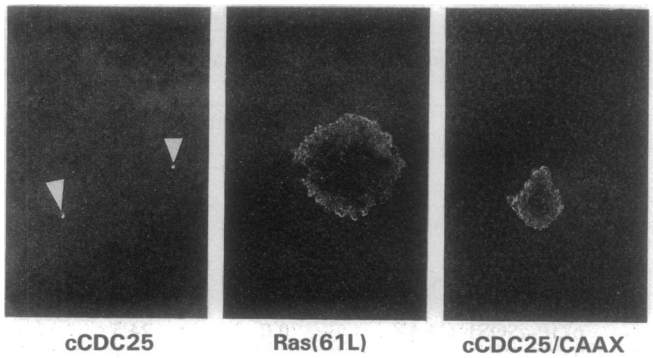

FIG. 3. Enhanced growth of cCDC25–CAAX-transformed cells in soft agar. NIH 3T3 cells stably expressing cCDC25, Ras(61L), and cCDC25–CAAX were suspended in 0.33% top agar and overlaid onto a 0.5% agar bottom layer and grown at 37°C for up to 3 weeks to assay for the appearance of colonies. Arrowheads indicate single cells expressing cCDC25. (×20.)

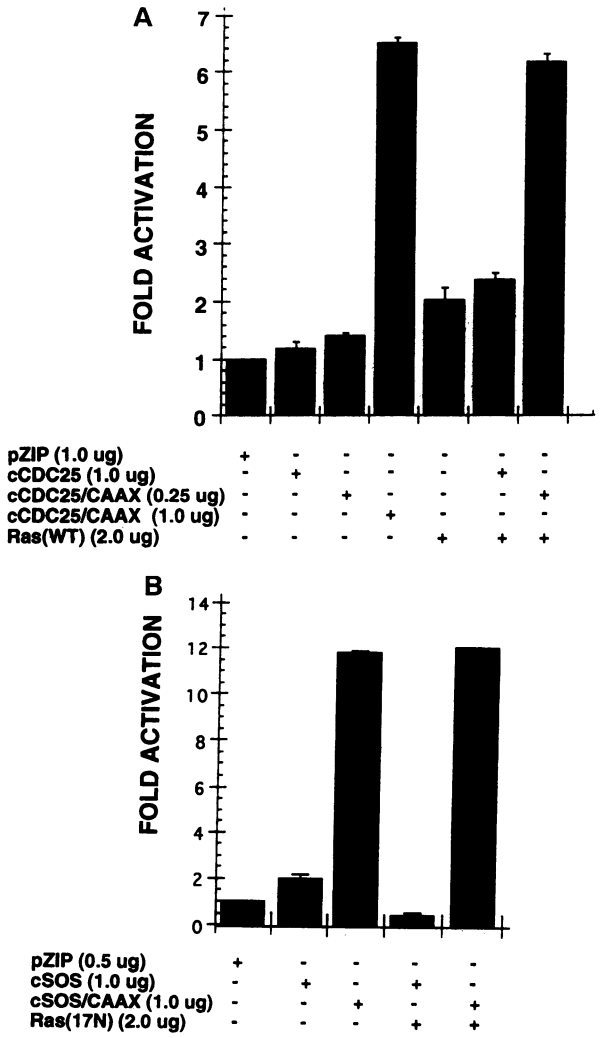

FIG. 4. Membrane-targeting enhances Ras GEF stimulation of transcriptional activation of RREs. NIH 3T3 cells were cotransfected with the indicated cCDC25 (*A*) and cSOS1 (*B*) DNAs plus 1 μg of pB4X-CAT reporter construct and CAT activity was determined by enzymatic assay as described (23, 24). Results are expressed as mean ± range for duplicate samples and are representative of at least three experiments. +, Added; −, not added; ug, μg; WT, wild type.

of pZIP-cSOS1 showed limited stimulatory activity, 1 μg of pZIP-cSOS1–CAAX caused a 10-fold stimulation of transcription (Fig. 4*B*). We also compared the sensitivity of the membrane-targeted version of cSOS1 to inhibition by the Ras(17N) dominant inhibitory mutant, which is believed to form nonfunctional complexes with Ras GEFs (24, 30, 31). Although cSOS1 activity was completely inhibited by coexpression with 1 μg of Ras(17N), cSOS1–CAAX activity was considerably less sensitive to Ras(17N) action (Fig. 4*B*). Thus, membrane-targeted versions of SOS1 and CDC25 showed greatly increased abilities to activate transcription from RREs and decreased sensitivity to Ras(17N) inhibitory action.

We (23) and others (25–27) have shown that addition of the carboxyl-terminal 18 amino acids of K-Ras4B onto heterologous proteins (e.g., the p120 GAP catalytic domain) triggers their association with the plasma membrane. To determine whether the subcellular location of cCDC25 was similarly redirected, G418-selected NIH 3T3 cells expressing the introduced sequences were separated into soluble and particulate fractions and analyzed on immunoblots with a rabbit polyclonal antibody generated against human CDC25 recombinant protein. As shown in Fig. 5, cCDC25 was found predominantly in the cytosolic fraction whereas the cCDC25–CAAX chimera

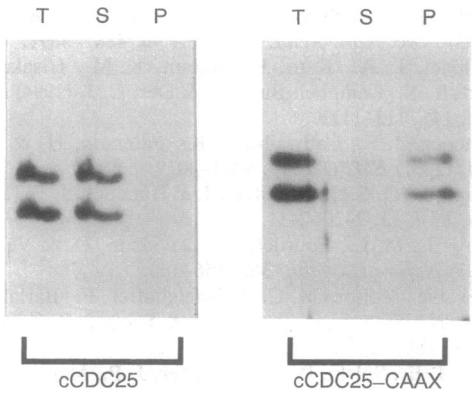

FIG. 5. Membrane association of cCDC25 and cCDC25–CAAX. NIH 3T3 cells expressing both truncated CDC25 constructs were separated into crude membrane (P100) and cytosolic (S100) fractions by centrifugation at 100,000 × *g* as described (29). Each fraction was then subjected to SDS/PAGE on 10% gels, transferred to poly(vinylidene fluoride) membranes, and then analyzed on an immunoblot using a CDC25-specific rabbit polyclonal antibody. Lanes: T, total lysate; S, soluble fraction; P, particulate fraction. The presence of an ≈45-kDa doublet is presumably due to the use of an alternate initiation site or amino-terminal proteolysis. The cCDC25–CAAX chimeric protein migrated slower than cCDC25 due to its carboxyl-terminal extension.

was detected exclusively in the membrane fraction. Therefore, the enhanced transforming and transactivating activities of the versions of cCDC25 and cSOS1 that contain the K-Ras4B plasma membrane-targeting sequence are likely to be due to their enhanced association with the plasma membrane.

DISCUSSION

The translocation of a Grb-2–SOS1 complex to the plasma membrane, via Grb-2 association with phosphotyrosine residues on the activated EGF receptor, has been proposed to increase the local concentration of SOS in the vicinity of Ras-GDP without increasing the intrinsic enzymatic activity of SOS1 (9, 10, 12). Our demonstration that addition of the Ras membrane targeting sequence onto the isolated catalytic domains of CDC25/GRF and SOS1 greatly potentiated their abilities to activate Ras provides strong support for this mechanism of GEF activation of Ras. The essential requirement for membrane association to promote Ras interaction with Ras GEFs is further supported by observations that the Ras(17N) dominant-inhibitory mutant, which competes with endogenous Ras for GEF interaction, also requires membrane association for its growth-inhibitory phenotype (24, 30, 31).

In contrast to SOS1, no analogous Grb-2-mediated translocation has been described for CDC25 regulation. However, our observation that membrane targeting greatly potentiated cCDC25 activation of Ras suggests that such a mechanism may also be important for controlling CDC25 function. Further support for this possibility is provided by recent studies on CDC25-mediated transformation of NIH 3T3 cells. Cen *et al.* (7) observed that, while CDC25 was associated with both the membrane and cytosolic fractions, cytosol- and membrane-derived CDC25 displayed equivalent abilities to stimulate Ras guanine nucleotide exchange *in vitro*. Furthermore, serum-induced elevation of Ras-GTP levels was dependent on Ras membrane association. Whether a membrane-targeting adaptor molecule is also involved in CDC25 activation remains to be determined.

Although we have observed that membrane translocation alone is sufficient to achieve significant Ras activation by truncated Ras-GEFs, other mechanisms of regulation may also exist that are conferred by the noncatalytic sequences. These putative regulatory sequences could potentially positively regulate GEFs by directing membrane association or negatively regulate the catalytic domain. For example, it has been observed that phosphorylation of SOS1 occurs after EGF stimulation of rodent fibroblasts (14, 32). However, the slower time course for SOS1 phosphorylation vs. Ras-GTP elevation suggests that phosphorylation is not involved in SOS1 activation but instead may be part of an inhibitory feedback mechanism. Indeed, the recent observation that cAMP-induced inactivation of mitogen-activated protein kinases also prevents SOS1 phosphorylation supports the notion that a feedback loop might terminate mitogen activation of Ras (32). Whether membrane association is the major physiological mechanism of growth factor-mediated Ras GEF activation or whether additional levels of regulation exist will require further investigation with the full-length molecules.

Our observation that membrane-targeting potentiates Ras GEF activation of Ras is consistent with the proposal that Ras membrane association is required for proper regulation of Ras GDP/GTP cycling by SOS1 and CDC25. We (23) and others (26) have observed that membrane targeting potentiates the abilities of Ras GAPs to negatively regulate Ras activity. Furthermore, we have recently observed that Ras membrane association may also be critical for its interaction with the Raf-1 serine/threonine kinase (unpublished observation). Therefore, while Ras GAPs, GEFs, and Raf-1 can interact with nonprenylated Ras proteins *in vitro*, the prenylation-triggered translocation of Ras to the plasma membrane may still be critical to facilitate Ras interaction with these components in the context of the cell. Although it has been speculated that a dynamic interaction of Ras itself with the plasma membrane may be involved in regulating Ras function, there is presently no evidence to support this notion (8). Instead, Ras function may be controlled by the dynamic interaction of its regulatory proteins with the plasma membrane.

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