DEPHOSPHORYLATION OF S259 REGULATES RAF-1 MEMBRANE ASSOCIATION

1Markus Kubicek, Margit Pacher1.Q, Dietmar Abraham1,¶, Klaus Podar1, §, Manfred Eulitz2, and Manuela Baccarini1, §§

1Department of Cell- and Microbiology, Institute of Microbiology and Genetics, Vienna Biocenter, Dr. Bohr Gasse 9, 1030 Vienna, Austria; §GSF - Forschungszentrum für Umwelt und Gesundheit, Institut für Klinische Molekularbiologie und Tumorgenetik, Marchioninistr. 25, D-81377 München, Germany

Running title: S259 dephosphorylation and Raf-1 activation
SUMMARY

Mitogenic stimulation of Raf-1 is a complex, yet incompletely understood process involving membrane relocalization and phosphorylation of activating residues. We recently reported that Raf-1-associated protein phosphatase 2A (PP2A) contributes to kinase activation, an effect mediated via S259 of Raf-1. Here, we show that mitogens stimulate S259 dephosphorylation and Raf-1/PP2A association concomitantly with membrane accumulation and activation of Raf-1. Blocking S259 dephosphorylation inhibits the two latter events, but it does not prevent activation of a S259A Raf-1 mutant, which is preferentially localized at the membrane independently of mitogenic stimulation. Inhibition of S259 dephosphorylation has no effect on the activation of membrane-tethered Raf-1 (Raf-1CAAX). These data show that S259 dephosphorylation contributes to Raf-1 activation by supporting its membrane accumulation rather than by increasing the specific activity of the kinase, and provide a mechanistic basis for the support of kinase activation by Raf-1-associated PP2A.
INTRODUCTION

The Raf-1 kinase is an important intermediate in the transduction of proliferative and anti-apoptotic signals. The best studied activator of Raf-1 is GTP-loaded Ras, which binds to the N-terminus of the kinase with high affinity (1) and recruits it to the plasma membrane, where activation takes place (2,3). Raf-1 artificially tethered to the cell membrane is partially activated but can be further stimulated by mitogens, suggesting that at the cell membrane Raf-1 is exposed to both constitutive and mitogen-regulated activators (4-7), including the kinases that phosphorylate serine 338 (4,7) and tyrosine 341 (7,8). Recently, phosphorylation of the activation loop of the kinase at residues T491 and S494 has been shown to be essential for Raf-1 activation by different stimuli (9).

Three serine residues whose phosphorylation negatively regulates kinase activation have been identified in Raf-1. All three (S43, S621, and S259) are phosphorylated to different degrees in resting fibroblasts (10). Phosphorylation of S43 was reported to interfere with Ras binding and consequently with Ras-mediated activation (2), but this conclusion has been recently challenged (11). Phosphorylated S259 and S621 represent binding sites for 14-3-3 adaptor proteins (12,13). Bivalent binding of 14-3-3 to pS259 and pS621 has been suggested to maintain Raf-1 in an inactive conformation (14,15). On the other hand, reversible association with 14-3-3 facilitates Ras-dependent Raf-1 activation in vivo and in vitro (16), and binding to the pS621 site appears to be necessary for kinase activity (15,17). However, phosphorylation of S621 site by PKA in vitro inhibits kinase activity (18). The significance of S621 phosphorylation is still in question and cannot be answered by the investigation of S621A mutants, since this residue cannot be replaced by other
amino acids without loss of kinase activity (18,19). In contrast, mutation of S259 moderately activates the Raf-1 kinase in cultured cells (15,20) and in Drosophila (14,19). Serine 259 can be phosphorylated by Akt/PKB, and this phosphorylation correlates with the down-regulation of kinase activity (21). We have recently shown that dephosphorylation of S259 by Raf-1-associated PP2A\(^1\) contributes to Raf-1 activation (22). PP2A has been shown to form a complex with a number of kinases, including Ca\(^++/\)Calmodulin-dependent Protein Kinase IV (23), PAK-1, PAK3 and p70S6kinase (24). The isolated catalytic subunit can associate with casein kinase 2\(\alpha\) (25). Where investigated (23), PP2A has been shown to contribute to the inactivation of the associated kinase. Raf-1 represents the first example of a PP2A-associated kinase activated by dephosphorylation.

This report focuses on the specific role of S259 dephosphorylation in Raf-1 kinase activation in vivo. It shows that preventing mitogen-induced S259 dephosphorylation results in inefficient accumulation of endogenous as well as overexpressed Raf-1 to the plasma membrane, and that both a S259A mutation and artificial tethering of Raf-1 to the membrane bypass the requirement for phosphatase activity. Our results indicate that S259 phosphorylation is involved in the relocation of Raf-1 to the cytosol and that PP2A supports full-fledged Raf-1 activation by dephosphorylating this residue and increasing the efficiency with which Raf-1 is retained at the membrane of mitogen-stimulated cells.
EXPERIMENTAL PROCEDURES

Cell culture, transfection, and growth factor stimulation. BAC-1.2F5 cells (26) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 0.63 nM purified mouse recombinant CSF-1 or 15% L-cell conditioned medium (27) as a source of murine CSF-1. COS-1 cells and 3T3 like fibroblasts (28) were grown in DMEM supplemented with glutamine and 10% FCS. COS cells were transfected by electroporation (0.5 - 1x10^7 cells/cuvette, 240V, 960 µF, 10µg plasmid DNA). The plasmids used were pCMV5-Raf-1, a S259A and a S621A Raf-1 mutant (pCMV5-S259A and pCMV5-S621A), a Raf-1 mutant artificially tethered to the cell membrane via the Ki-Ras membrane localization signal (pCMV5-Raf-1CAAX) and a control with a mutated localization signal (pCMV5-Raf-1SAAX), as well as a plasmid encoding activated Ras (pCIS-Ha-RasG12V). Cells were harvested two days after transfection. Under these conditions protein expression increased linearly between 1 and 10µg of transfected plasmid DNA. Confluent cultures were starved for 18 h prior to stimulation with recombinant EGF (33 nM, 10 min). In selected experiments, cells were incubated with okadaic acid (100 nM, 45 min) prior to growth factor stimulation.

Cell lysis, immunoprecipitation and western blotting. Cells were lysed in solubilization buffer (10 mM Tris-Cl, 50 mM NaCl, 1% Triton X-100, 30 mM sodium pyrophosphate, 100 µM Na3VO4, 1 mM PMSF, 100 nM okadaic acid). Insoluble material was removed by centrifugation (15,000 rpm, 30 min, 4°C). For subcellular fractionation cells were washed and scraped on ice into 0.5 ml hypotonic buffer (10 mM Tris-Cl, pH 8.0, 5 mM MgCl₂, 1 mM EGTA, 1 mM dithiotreitol, 100
µM Na₃VO₄, 1 mM PMSF, 100 nM okadaic acid). After 30 min on ice, cells were homogenized by 30 strokes through a 26G needle. Nuclei were removed by low speed centrifugation. The post nuclear supernatants were centrifuged at 100,000 x g. The supernatant (S100) was removed, and the sedimented fraction (P100) was resuspended in resuspension buffer (0.1 M Tris-Cl, pH 7.4, 30 mM Sodium-pyrophosphate, 50mM NaCl, 1% Triton X-100, 1 mM dithiotreitol, 100 µM Na₃VO₄, 1 mM PMSF, 100 nM okadaic acid) after washing with PBS. A rabbit polyclonal antiserum raised against a carboxy-terminal peptide of v-raf (SP63, CTLTTSRPLPVF) was used to immunoprecipitate Raf-1 molecules. A mouse monoclonal antibody against PP2AC (F2 8F5) was used to immunoprecipitate PP2A (22). Immunocomplexes were collected following incubation (1-3 h at 4°C) with Protein A-Sepharose beads (Sigma) and washed thrice with lysis buffer prior to western blotting or immune complex kinase assay. For western blotting, cell lysates (25 µg/lane) or immunoprecipitates were separated by 7.5% SDS-PAGE prior to electrophoretic transfer onto Hybond C super (Amersham). The blots were probed with monoclonal antibodies against Raf-1 (29) or PP2AC (22), prior to incubation with horseradish peroxidase conjugated secondary antibodies and exposure to the ECL substrate. Rabbit polyclonal antisera against Raf-1 phosphopeptides (anti-pS259, QRQRSTS*TPNVHC; and anti pS621, KINRSAS*EPSLHRC) were produced in our lab. Their specificity is shown in Figure 1A. The blots were subsequently incubated for 1h at room temperature with donkey-anti-rabbit horseradish peroxidase conjugated antibodies prior to exposure to the ECL substrate. All blotting reagents were from Amersham. The blots were stripped according to the
manufacturer’s instruction.

**Assay of kinase activity.** Raf-1 kinase activity was measured as the ability of immunoisolated Raf-1 to activate recombinant MEK-1 in coupled assays using MBP as the endpoint of the assay (30).
RESULTS

Serine 259 is dephosphorylated upon mitogenic activation of Raf-1. We have previously shown that okadaic acid inhibits peak Raf-1 kinase activation by the mitogens CSF-1 and EGF. In the case of EGF, S259 was identified as the site relevant for inhibition (22). Since this residue is constitutively phosphorylated in quiescent cells (10), these data predict mitogen-induced dephosphorylation of S259. To test this hypothesis, we determined the effect of EGF stimulation of COS-1 cells expressing wt Raf-1 on the phosphorylation of S259 by immunoblotting with phosphospecific antisera recently produced in our laboratory (Figure 1A). Consistent with the postulated negative regulatory role of this residue, we observed a rapid mitogen-induced S259 dephosphorylation which persisted up to 10 min after EGF stimulation (Figure 1B). In contrast, dephosphorylation of S621, a second putative inhibitory site (18), was transient. After initial dephosphorylation (2 min), this residue was quickly rephosphorylated and appeared to be actually hyperphosphorylated 10 min after EGF stimulation, a time when the Raf-1 kinase was activated. Therefore, dephosphorylation of S259, but not of S621, correlates with peak Raf-1 activation by EGF. Okadaic acid inhibited mitogen-induced dephosphorylation of both residues (Figure 1B) and full-fledged Raf-1 activation (Figure 1C; 37% and 40% inhibition after 5 and 10 min of EGF treatment). We next examined whether dephosphorylated Raf-1 was found preferentially in the membrane or in the cytosol of COS cells. In untreated cells, both the cytosol and the membrane compartment contained Raf-1 phosphorylated on S259 (Figure 1D). 2 min after EGF stimulation, dephosphorylation was evident in the cytosol and, considering the increased amount of Raf-1 protein due to the beginning of recruitment, even more evident in the membrane fraction. Okadaic
acid blocked dephosphorylation in both compartments. Interestingly, the EGF-mediated translocation of wt Raf-1 to the membrane seemed to be impaired by the drug. Considering the impairment in membrane translocation, the specific activity of membrane-recruited kinase was comparable in okadaic acid-treated and untreated cells. The bulk of the Raf-1 protein was cytosolic, and, at this early time point, had a very low specific activity which was not reduced by okadaic acid pretreatment (Figure 1D).

Mitogen treatment of different cell types increases the association between endogenous Raf-1 and PP2A. We next investigated whether mitogens induced S259 dephosphorylation by promoting physical association between Raf-1 and PP2A. We had previously addressed this question in macrophages and shown that PP2A is present in Raf-1 immunoprecipitates (22). These experiments were performed under extremely stringent washing conditions, and did not clearly show whether growth factor treatment influenced the association between Raf-1 and PP2A. We re-examined this issue by determining the amount of Raf-1 present in PP2AC immunoprecipitates obtained under less stringent conditions from quiescent and growth factor-stimulated cells. Raf-1 was recovered in PP2AC immunoprecipitates from both the cytosolic and the membrane fraction of COS-1 cells. Treatment with EGF stimulated the Raf-1/PP2A association in both compartments, although the increase was more evident in the cytosolic fraction (Figure 2A). The kinetics of mitogen-stimulated complex formation were similar to those of kinase activation (compare Figure 2A and Figure 1C). Okadaic acid did not disrupt the association, and in fact appeared to stabilize the complex, possibly due to substrate trapping by the inactive phosphatase (Figure 2A). Raf-1 was also detectable in PP2A
immunoprecipitates from EGF-treated wt, but not Raf-1-deficient (28) 3T3 cells (Figure 2B). In BAC-1.2F5 cells, CSF-1 stimulated Raf-1/PP2A association both in the cytosolic and in the membrane fraction. The amount of Raf-1 recovered in PP2A i.p.s was maximal at 2 min and decreased after 15 min of treatment (Figure 2C). In BAC-1.2F5 cells, Raf-1 kinase activation reaches a peak around 2 min and subsides by 15 min (22,31). Thus, the kinetics of Raf-1/PP2A association correlated with those of activation in CSF-1-treated macrophages. These data indicate that mitogens can stimulate association between endogenous Raf-1 and PP2A in different cell types.

**Okadaic acid induces S259 phosphorylation and impairs membrane accumulation of endogenous Raf-1 in different cell types.** We next investigated the relevance of S259 dephosphorylation in the activation of endogenous Raf-1. In untreated COS-1 cells, S259 phosphorylation of Raf-1 could be observed in the cytosol (Figure 3A; pS259 immunoblot, middle left panel). 10 min after EGF stimulation, okadaic acid-sensitive dephosphorylation was evident in the cytosol. Inhibition of S259 dephosphorylation by the drug was accompanied by a 30% reduction in EGF-stimulated kinase activity (Figure 3A, top left panel). S259 phosphorylation of membrane associated Raf-1 could not be detected under any of the conditions used (Figure 3A; pS259 immunoblot, middle right panel), likely due to the limited amount of Raf-1 protein present in this compartment. In particular, in the presence of okadaic acid EGF-mediated membrane recruitment of Raf-1 was completely abrogated (Figure 3A; Raf-1 immunoblot, bottom right panel), while Raf-1 kinase activation was reduced by 48% but not entirely prevented (Figure 3A, top right panel). It would thus appear that the main effect of okadaic acid is to decrease Raf-1 membrane translocation rather than to reduce the specific activity of
the kinase. We next investigated the effect of okadaic acid treatment on Raf-1 S259 phosphorylation and membrane translocation in CSF-1-induced BAC-1.2F5 macrophages. In contrast to COS cells, phosphorylation of S259 cannot be demonstrated by \textit{in vivo} labeling of quiescent macrophages (22), but is readily detectable in okadaic acid-pretreated, CSF-1-stimulated cells. Immunoblotting with anti-pS259 antibodies confirmed our original result (Figure 3B). Thus, phosphorylation of S259 can be constitutive or mitogen-induced, depending on the cellular context. In BAC-1.2F5 macrophages, Raf-1 activation occurs with much faster kinetics than in COS cells (peak at 2 min; 22), and cannot be blocked by transfection with a dominant negative Ras mutant (31). Despite these differences, Raf-1 activation in macrophages was paralleled by membrane translocation of the kinase. Pretreatment of quiescent cells with okadaic acid increased the amount of membrane-associated Raf-1, but abrogated the membrane translocation in CSF-1-stimulated cells (Figure 3C).

\textbf{Okadaic acid impairs membrane translocation of wild type Raf-1, but not of a S259A Raf-1 mutant.} Based on these data, we investigated the effect of okadaic acid on the EGF-induced membrane translocation of wt Raf-1 and of a S259 Raf-1 mutant in COS-1 cells in more detail. Subcellular fractionation showed that low amounts of Raf-1 are detectable in the membrane fraction of quiescent cells, and that EGF treatment caused steady membrane accumulation of the kinase over the time course analyzed. In okadaic acid-treated cells, early translocation (2 min) was slightly impaired. At later time points, instead of accumulating, Raf-1 eventually disappeared from the membrane fraction (Figure 4A, left panel). In contrast, a S259A Raf-1 mutant, whose activation is not impaired by okadaic acid (Figure 4B; and 22), was
constitutively present in the membrane fraction of quiescent cells, and neither EGF nor okadaic acid altered its localization significantly (Figure 4A, middle panel). Wt Raf-1 and the S259A mutant were expressed at comparable levels in COS cells. This rules out the possibility that the preferential localization of S259A to the membrane was due to overexpression of the mutant as compared to the wt protein and therefore to non-specific membrane association (Figure 4A, right panel). Thus, S259 dephosphorylation appears to play a role in Raf-1 membrane accumulation. Paradoxically, in most experiments okadaic acid caused a detectable increase in the accumulation of wt Raf-1 to the membrane of unstimulated cells (Figure 4A, left panel; and Figures 2A and 3C). It is conceivable that this effect of okadaic acid might be mediated via phosphorylation of S621, which has been implicated in the translocation of Raf-1 to the membrane (17). This explanation, however, would not fit well with the transient dephosphorylation of this residue observed early in the kinetics of EGF stimulation. To address the significance of S621 in Raf-1 membrane translocation, we compared the behavior of wt kinase and of a S621A mutant in EGF-stimulated cells, pretreated or not with okadaic acid. The S621A mutant associated with the membrane of unstimulated cells slightly more efficiently than wt Raf-1, and was translocated to the membrane upon EGF stimulation. Okadaic acid treatment of unstimulated cells increased the membrane pool of S621A Raf-1 molecules, but it reduced their EGF-induced translocation. This is behavior is identical to that displayed by wt Raf-1. Thus, S621 phosphorylation is dispensable for EGF-induced Raf-1 membrane translocation.

**Phosphorylation of S259 does not decrease the activity of membrane bound Raf-1.** The data mentioned above indicate that the phosphorylation of S259 impairs
Raf-1 membrane accumulation. To test whether this is the main function of S259 phosphorylation, or whether, in addition, phosphorylation of this residue has a direct effect on kinase activity, we investigated the effect of okadaic acid on the activation and S259 phosphorylation of Raf-1CAAX (Raf-1 artificially tethered to the membrane by the Ki-ras membrane targeting signal). As previously described, the basal activity of Raf-1CAAX was modestly increased with respect to a mutant with a non-functional membrane-targeting signal (Raf-1SAAX). Both mutants were efficiently stimulated by EGF. Pretreatment with okadaic acid prevented EGF-induced S259 dephosphorylation and decreased activation of Raf-1SAAX by 40%. Raf-1CAAX activation, in contrast, was fully refractory to okadaic acid (Figure 5). These data confirm that S259 phosphorylation \textit{per se} does not have a negative effect on the activity of membrane-targeted Raf-1, and indicate that S259 dephosphorylation supports full-fledged Raf-1 activation solely by increasing accumulation of the kinase at the membrane.
DISCUSSION

We have previously shown that Raf-1-associated PP2A is a positive regulator of Raf-1 activation in COS cells and macrophages, and that this function is mediated via S259 of Raf-1. PP2A might either prevent inactivation of Raf-1 or facilitate its activation. Although our former work did not distinguish between these two possibilities, we favored the idea that PP2A counteracted the inhibitory effect of a mitogen-stimulated S259 kinase (possibly Akt/PKB (21). By using a phosphospecific antiserum, we could confirm this hypothesis in macrophages. In quiescent BAC-1.2F5 cells, in fact, S259 phosphorylation is undetectable even in the presence of okadaic acid. S259 phosphorylation occurs simultaneously with activation, but it is so efficiently counteracted by PP2A that it can only be revealed by okadaic acid pretreatment. In COS cells, in contrast, S259 phosphorylation is constitutive as determined by both western blotting with phosphospecific antiserum (Figure 1 B and D) and in vivo radiolabeling (not shown), indicating a turnover of this site in the absence of mitogen. Dephosphorylation occurs early upon EGF stimulation, and it precedes (and contributes to) peak activation. Thus, depending on the cellular context, constitutively active as well as mitogen-induced kinases can phosphorylate S259. In either case, however, dephosphorylation is necessary to stabilize Raf-1 activation and its association with the membrane.

S259 phosphorylation affects mainly mitogen-induced membrane localization of Raf-1, but not its specific activity. Consistently, S259A Raf-1 localizes to the membrane significantly more than the wt, and is refractory to okadaic acid pretreatment (Figure 4). Furthermore, a membrane-tethered Raf-1 (Raf-1CAAX) is activated by EGF regardless of S259 phosphorylation (Figure 5). In COS cells,
inhibition of dephosphorylation leads to a dramatic impairment in EGF-induced membrane accumulation of wt Raf-1, but only to a 40-50% reduction in kinase activation. S259 dephosphorylation is apparent 2 min after EGF stimulation; at this time point, however, phosphatase inhibition has only marginal effects on Raf-1 membrane translocation and kinase activation (Figures 1 and 4). Furthermore, the few Raf-1 molecules in the membrane of okadaic acid-pretreated cells appear to be extremely active (Figure 3A). This higher specific activity of membrane-recruited Raf-1 explains why the okadaic acid fails to inhibit kinase activation completely. Thus, inhibition of dephosphorylation appears to play a dual role in Raf-1 activation: a negative role in membrane accumulation, but a positive role on the specific activity of membrane-recruited Raf-1. These results are reminiscent of previous observations made by Hancock and co-workers in an in vitro system of Ras-mediated Raf-1 activation and recruitment. In those experiments, the addition of recombinant 14-3-3 proteins to the system caused an increase in the specific activity of membrane-recruited Raf-1 but a decrease in the amount of Raf-1 associated with the Ras membranes (16).

The essential role of 14-3-3 as a stimulator of Raf-1 activity is most likely mediated by S621 (15,17). Like S259, this residue is dephosphorylated early upon EGF stimulation (Figure 1A). Interestingly, a S621A mutant associates with the membrane of quiescent cells somewhat more efficiently than wt Raf-1 (Figure 4C), suggesting that dephosphorylation of S621, too, might promote membrane translocation of Raf-1. It is possible that dephosphorylation of both residues is necessary for the dissociation of 14-3-3 proteins and membrane translocation of Raf-1 (16). Unlike S259, which remains dephosphorylated throughout activation,
S621 is quickly rephosphorylated. This is in line with the proposed autophosphorylation of this residue (17) and would allow, in theory, quick re-binding of 14-3-3 to S621 and stimulation of Raf-1 (15,17). In contrast to S259A Raf-1, the S621A mutant behaves like wt Raf-1 in okadaic acid-treated, EGF-stimulated cells; thus, phosphorylation of S259, but not of S621, is responsible for the impairment in mitogen-induced membrane accumulation of Raf-1.

In principle, S259 dephosphorylation might increase Raf-1 membrane association either by promoting recruitment (priming Raf-1 for activation) or by allowing the Raf-1 to remain at the membrane after activation. We favor the latter hypothesis for two reasons. Firstly, okadaic acid leads to the complete disappearance of Raf-1 from the membrane fraction of EGF-treated cells, but it actually increase the amount of membrane-associated Raf-1 in quiescent cells (Figure 3A and C, Figure 4). At present, the molecular basis of this paradoxical effect, which is not affected by mutation of S259 or S621, and which is somewhat variable in its extent, is unknown. However, these data indicate that only activated Raf-1 can be earmarked for removal from the membrane by S259 phosphorylation. Secondly, small amounts of S259-phosphorylated Raf-1 can be detected in the membrane of untreated COS-1 cells overexpressing Raf-1 and can be activated by EGF even in the presence of okadaic acid (Figure 1D). Thus, S259 phosphorylation does not absolutely preclude membrane localization or activation, but should rather be envisioned as a safety catch decreasing the pool of active Raf-1 molecules that can accumulate in this compartment (Figure 6). How could phosphorylation of S259 favor the removal of Raf-1 from the membrane of activated cells? S259 phosphorylation might, for instance, render membrane association more transient by masking the CRD (Raf-1...
cysteine-rich domain) and thereby destabilizing its interaction with membrane lipids and/or with Ras (32,33). Indeed, the S259A Raf-1 mutant shows increased Ras binding (data not shown). An alternative, attractive possible explanation comes from the recently published work by Jaumot and Hancock (34). Using an in vitro system that relies on Ras binding to activate Raf-1, these authors have shown that phosphatase inhibitors block Raf-1 activation and promote the accumulation of inactive Raf-1/14-3-3 complexes in membrane microdomains that contain endocytic markers. The authors suggest that these complexes might represent a pool of deactivated molecules on their way to the cytosol. It is likely that these intermediates can only be detected in the in vitro system, while in vivo one would only observe an increased removal of Raf-1 from the membrane, as seen here. Finally, it is possible that membrane-associated, pS259 Raf-1 is recognized and removed from the membrane by a factor(s) activated by EGF. This would explain why, in unstimulated cells, Raf-1 is not removed from the membrane when phosphorylated on S259 as a result of okadaic acid treatment.

Be that as it may, mitogens deactivate the safety catch by promoting Raf-1/PP2A association and the dephosphorylation of S259 (Figures 1-3). By so doing, they ensure efficient membrane accumulation of active Raf-1 and allow a larger pool of molecules to be retained at this site. In vivo, such a mechanism might have a remarkable impact on the strength and duration of kinase activation, as recently shown in C. elegans. In this system, elimination of the inhibitory phosphorylation is as effective as mimicking activating phosphorylation in conferring constitutive biological activity on lin-45 Raf (9). By showing that S259 dephosphorylation supports membrane accumulation of Raf-1, our findings assign a specific function to
S259 phosphorylation/dephosphorylation in Raf-1 activation in vivo and provide a mechanistic explanation for the positive regulation of the kinase by PP2A.

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FOOTNOTES

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QPresent address: Department of Obstetrics and Gynecology, University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna Austria

¶Present address: Department of Anatomy, University of Vienna, Währinger Straße 13, A-1090 Vienna Austria

Present address: Dana Farber Cancer Institute, 133 Brookline Avenue, Boston, MA 02115, USA

§§To whom correspondence should be addressed. Tel.: ++431 4277-5460; fax: ++431 4277-9546; E-mail: manuela@gem.univie.ac.at

1The abbreviations used are: PP2A, protein phosphatase 2A; MEK, mitogen-activated protein kinase/extracellular signal regulated-kinase kinase; CSF-1, colony stimulating factor 1; EGF, epidermal growth factor; MBP, myelin basic protein; CRD, cystein-rich domain
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FIGURE LEGENDS

Figure 1 - Dephosphorylation of S259, but not of S621, correlates with Raf-1 kinase activation in EGF-stimulated COS-1 cells. A, COS-1 cells were transfected with a vector encoding human wt Raf-1 (pCMV5c-raf) or S259A and S621A Raf-1 mutants (pCMV5-S259A and -S621). Raf-1 immunoprecipitates prepared 48 hrs after transfection were immunoblotted with phosphospecific antisera against the peptides containing pS259 or pS621. The specificity of the antisera is shown by their failure to recognize phosphorylation site mutants. B and C, COS-1 cells overexpressing human wt Raf-1 were treated with okadaic acid (OA, 100 nM, 45 min) prior to stimulation with EGF (33 nM) for the indicated time periods. Raf-1 immunoprecipitates were immunoblotted with phosphospecific antisera (B) or assayed for Raf-1 kinase activity (duplicates) in a coupled assay (C). The experiment was repeated twice with comparable results. D, COS-1 cells overexpressing human wt Raf-1 were treated with okadaic acid (100 nM, 45 min) prior to stimulation with EGF (33 nM, 2 min) and hypotonic lysis. 25% of the total cytosolic proteins and 50% of the total membrane proteins were used for Raf-1 immunoprecipitation. The immunoprecipitates were immunoblotted with phosphospecific antisera against the peptides containing pS259 or with an anti-Raf-1 serum or assayed for kinase activity (duplicates) in a coupled assay.

Figure 2 – EGF stimulates Raf-1/PP2A association. A, COS-1 cells were treated with okadaic acid (OA, 100 nM, 45 min) prior to stimulation with EGF (33 nM) for the indicated time periods and hypotonic lysis. 25% of the total cytosolic proteins and 50% of the total membrane proteins were used for PP2AC immunoprecipitation. B, wild-tpe (wt) and Raf-1 knock-out (KO) 3T3-like
fibroblasts were stimulated with EGF (33 nM) for 10 min. PP2AC was immunoprecipitated from 180 µg whole cell lysate. The immunoprecipitates were analyzed by western blotting with anti-Raf-1 and anti-PP2AC monoclonal antibodies. C, BAC-1.2F5 cells were treated with CSF-1 (6.3 nM) for the indicated time periods prior to hypotonic lysis. 25% of the total cytosolic proteins and 33% of the total membrane proteins were used for PP2AC immunoprecipitation.

**Figure 3 – S259 phosphorylation correlates with reduced membrane accumulation of endogenous Raf-1 in different cell types.** A, COS-1 cells were treated with okadaic acid (100 nM, 45 min) prior to stimulation with EGF (33 nM, 10 min) and hypotonic lysis. 25% of the total cytosolic proteins and 50% of the total membrane proteins were used for Raf-1 immunoprecipitation. The immunoprecipitates were immunoblotted with phosphospecific antisera against the peptides containing pS259 or with an anti-Raf-1 serum or assayed for kinase activity (duplicates) in a coupled assay. B, BAC-1.2F5 cells were treated with okadaic acid prior to stimulation with CSF-1 (6.3 nM) for the indicated time periods. Raf-1 i.p.s from whole cell lysates were immunoblotted with phosphospecific antisera against the peptides containing pS259 or with an anti-Raf-1 serum. In C, untreated or okadaic acid-treated BAC-1.2F5 cells were stimulated with CSF-1 (2 min) prior to hypotonic lysis. The membrane and the cytosolic fraction were subjected to immunoblotting with anti-Raf-1 antibodies. The experiments were repeated twice with comparable results.

**Figure 4 – Okadaic acid decreases EGF-induced membrane translocation of wt Raf-1, but not of S259A mutant.** COS-1 cells were transfected with a vector encoding human wt Raf-1 (pCMV5c-raf), a S259A (pCMV5-S259A), or a S621A
(pCMV5-S621A) mutant. 48 h after transfection cells were treated with okadaic acid (100 nM, 45 min) prior to stimulation with EGF (33 nM) for the indicated time periods. A and C, cells were subjected to hypotonic lysis and subcellular fractionation. 10% of total cytosolic proteins (C) or 20% of the total membrane proteins (M) were analyzed by SDS-PAGE prior to immunoblotting with an anti-Raf-1 serum. The exposure time for the cytosol immunoblot was shorter than that for the membrane immunoblot. The experiment was repeated twice with comparable results. B, Raf-1 (wt or S259A) immunoprecipitates from whole cell lysates were assayed for kinase activity. The experiment was repeated twice with comparable results. The results are expressed as fold stimulation, where 1=activity of wt Raf-1 immunoprecipitated from untreated cells.

Figure 5 – S259 phosphorylation does not affect the activity of membrane-tethered Raf-1. COS-1 cells were transfected with a vector encoding Raf-1 modified by the addition of the Ki-Ras membrane targeting signal (pCMV5 Raf-1CAAX) or a control containing a non-functional targeting signal (pCMV5 Raf-1SAAX). 48 h after transfection cells were treated with okadaic acid (100 nM, 45 min) prior to stimulation with EGF (33 nM, 10 min) and hypotonic lysis. Raf-1 immunoprecipitates (duplicates) were assayed for kinase activity in a coupled assay. The amount of Raf-1 in the immune complexes and the phosphorylation status of S259 were determined by immunoblotting. The experiment was repeated twice with comparable results.

Figure 6 – Role of S259 phosphorylation in Raf-1 activation – a working model. In quiescent COS cells (left), Raf-1 is phosphorylated on S259 and S621, and is not efficiently recruited to the membrane. Mitogens increase the efficiency of Raf-
S259 dephosphorylation and Raf-1 activation

1 translocation to the membrane, where the kinase is exposed to constitutive as well as mitogen-induced activators, and stimulate Raf-1 association with PP2A and S259 dephosphorylation (1., 2.). S259 phosphorylation appears to earmark activated Raf-1 molecules (indicated by a star) for removal from the membrane and deactivation (3.). This is counteracted by PP2A, which dephosphorylates S259 and allows the accumulation of activated Raf-1 at the membrane.
Figure 2

A

| EGF (min) | 0 | 2 | 15 | 0 | 15 |
|-----------|---|---|----|---|----|
| OA        |   |   |    |   |    |

B

| EGF (min) | 0 | 10 | 0 | 10 |
|-----------|---|----|---|----|
| CSF-1 (min) | 0 | 2 | 15 | 0 | 2 | 15 |

C

| cytosol | membrane |
|---------|----------|
| Raf-1   |          |
| PP2AC   |          |

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| cytosol | membrane |
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| Raf-1   |          |
| PP2AC   |          |

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Figure 3

A

Fold activation

|     | - | + | - | + |
|-----|---|---|---|---|
| cytosol |   |   | 10 |   |
| membrane | 10 |   |   |   |

EGF

OA

pS259

Raf-1

B

pS259

Raf-1

CSF-1 (min) 0 2 15 0 2 15

OA

C

CSF-1

OA
Figure 4

A

Raf-1

wt Raf-1

S259A

Raf-1

EGF (min) 0 2 10 15

OA

C

B

Fold stimulation

0 5 10 15 20 25 30 35

EGF (min)

0 2.5 10 15

wt

wt+OA

S259A

S259A+OA

C

V wt S621A

EGF (min) 0 2 10 15

OA
Figure 6

1. Increased PP2A association

2. Activation at the membrane

3. Deactivation (S259 Kinase)
Dephosphorylation of S259 regulates Raf-1 membrane association
Markus Kubicek, Margit Pacher, Dietmar Abraham, Klaus Podar, Manfred Eulitz and Manuela Baccarini

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