Raf-1-associated Protein Phosphatase 2A as a Positive Regulator of Kinase Activation*

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The Raf-1 kinase plays a key role in relaying proliferation signals elicited by mitogens or oncogenes. Raf-1 is regulated by complex and incompletely understood mechanisms including phosphorylation. A number of studies have indicated that phosphorylation of serines 259 and 621 can inhibit the Raf-1 kinase. We show that both serines are hypophosphorylated during early mitogenic stimulation and that hypophosphorylation correlates with peak Raf-1 activation. Concentrations of okadaic acid that selectively inhibit protein phosphatase 2A (PP2A) induce phosphorylation of these residues and prevent maximal activation of the Raf-1 kinase. This effect is mediated via phosphorylation of serine 259. The PP2A core heterodimer forms complexes with Raf-1 in vivo and in vitro. These data identify PP2A as a positive regulator of Raf-1 activation and are the first indication that PP2A may support the activation of an associated kinase.

The Raf-1 kinase is an important intermediate in the transduction of proliferative signals, and its activation may be a key event in the development of a wide range of tumors (1). Activated Raf-1 can regulate the mitogen-activated protein kinase network by phosphorylating and activating MEK1; within the mitogen-activated protein kinase cascade, Raf interacts physically with MEK-1 via its kinase domain and with GTP-loaded Ras via its N terminus (2). Activated Ras is the best studied effector of Raf-1 activation. It binds to Raf-1 with high affinity and mediates its translocation from the cytosol to the plasma membrane, where activation takes place (3, 4). Artificial tethering of Raf-1 to the cell membrane results in partial activation, which can be further enhanced by mitogenic stimulation, suggesting that Raf-1 to the cell membrane results in partial activation, which can be further enhanced by mitogenic stimulation, suggesting that at the cell membrane Raf-1 is exposed to both constitutive and mitogen-regulated activators (5–8).

Mitogenic stimulation of cells typically induces hyperphosphorylation of Raf-1 and a retardation of its migration on SDS gels. This hyperphosphorylation correlates with the down-regulation of Raf-1 kinase activity (9, 10) and may be implemented by a negative feedback mechanism depending on MEK activity (10, 11). Serines 43, 621, and 259 are phosphorylated in resting fibroblasts, albeit to different degrees (12). Phosphorylation of all three residues has been implicated in the negative regulation of Raf-1. Phosphorylation of serine 43 interferes with Ras binding and consequently with Ras-mediated activation (3). Phosphorylated serine 259 and serine 621 represent binding sites for 14-3-3 adaptor proteins (13, 14), whose function in Raf-1 activation is controversial. While bivalent binding to Ser259 and Ser621 has been suggested to maintain Raf-1 in an inactive conformation (15, 16), reversible association with 14-3-3 facilitates Ras-dependent activation in vivo and in vitro (17). In particular, binding to the Ser(P)621 site appears to be necessary for kinase activity (16, 18), finding that contrasts with the studies indicating that phosphorylation of this site by PKA in vitro is inhibitory (19). Therefore, the significance of Ser621 phosphorylation is still in question. Its investigation is hampered by the fact that Ser621 is essential for the catalytic function of Raf-1 and cannot be replaced by other amino acids without loss of kinase activity (19, 20). Serine 259 can be phosphorylated by protein kinase B, another Ras effector activated in parallel with Raf, and this phosphorylation correlates with the down-regulation of kinase activity (21). Consistent with an inhibitory role of Ser(P)259, mutation of this residue moderately activates the Raf-1 kinase in cultured cells (16, 22); the corresponding point mutants display a gain of function phenotype in Drosophila (15, 20). Taken together, these data raise the possibility that dephosphorylation of negative regulatory residues plays a role in Raf-1 activation.

Protein phosphatase 2A (PP2A) is a major form of serine/threonine phosphatase involved in the regulation of signal transduction, growth, and development (23). This class of enzymes consists of a heterotrimer that exists in multiple forms. The core components of all trimeric forms are the 36-kDa catalytic subunit (PP2AC) and the 65-kDa regulatory subunit (A subunit, PR65). This core heterodimer is ubiquitous, and it forms complexes with “variable” subunits of cellular origin (some of which are expressed in a tissue- and/or development-restricted manner) as well as with transforming viral antigens (24–26). Association with variable subunits of cellular and viral origin occurs via the N-terminal leucine-rich repeats of PR65 (27) and confers distinct properties to the enzyme (28). Recently, PP2A has been shown to form a complex with Ca2+/
calmodulin-dependent protein kinase IV (29) as well as with PKA1, PKA3, and p70 S6 kinase (30). The isolated catalytic subunit can associate with casein kinase 2α (31). Where investigated (29), PP2A has been shown to contribute to the inactivation of the associated kinase. Here we show that the PP2A inhibitor okadaic acid inhibits full fledged Raf-1 activation. This effect is mediated by a change in the phosphorylation of Ser259 of Raf-1. In addition, Raf-1 forms stable complexes with PP2A heterodimers. Our results are the first indication that PP2A may support the activation of an associated kinase and highlight the intimate relationship between kinases and phosphatases, which we are just beginning to understand.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and Growth Factor Stimulation**—BAC-1.2F5 cells (32) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 0.63 nm purified murine recombinant mitogen colony-stimulating factor-1 (CSF-1) or 15% L-cell conditioned medium (33) as a source of murine CSF-1. COS-1 and COS-7 cells were grown in RPMI 1640 or Dulbecco's modified Eagle's medium supplemented with glutamine and 10% fetal calf serum. COS cells were transfected by electroporation (0.5–1 × 10^6 cells/euflage, 240 V, 960 microfarads, 10 μg of plasmid DNA). The plasmids used were the pcDNA3/HA tag (a kind gift of Dr. Lisa Ballou (I.M.P., Vienna)), pCMV-HA-ERK (courtesy of Michael Karin, UCSD), pCMV-Raf1, and a S259A Raf1 mutant (pCMV-S259A). Cells were harvested 2 days after transfection. Under these conditions, protein expression increased linearly between 1 and 10 μg of transfected plasmid DNA. Confluent cultures were starved for 18 h prior to stimulation with recombinant CSF-1 (BAC-1.2F5 cells, 6.3 nm mouse recombinant CSF-1 or 63 nm human recombinant CSF-1 (Chiron Co.) or EGF (COS-7 cells). The blots were probed with rabbit poly-

somal RNA from BAC-1.2F5 cells and from the myeloma line Sp2/0-Ag14 by polyethylene glycol treatment (29), PP2A has been shown to contribute to the inactivation of the associated kinase. Here we show that the PP2A inhibitor okadaic acid inhibits full fledged Raf-1 activation. This effect is mediated by a change in the phosphorylation of Ser259 of Raf-1. In addition, Raf-1 forms stable complexes with PP2A heterodimers. Our results are the first indication that PP2A may support the activation of an associated kinase and highlight the intimate relationship between kinases and phosphatases, which we are just beginning to understand.

**In Vivo Labeling of Cells and Phosphotryptic Peptide Mapping**—32P-Labeling of cells was performed as described previously (36). Cell lysis and immunoprecipitation of Raf-1 proteins were performed as described above.32P-Labeled proteins were resolved by 7.5% SDS-PAGE, ex-tracted from the gels, and subjected to digestion with sequencing grade trypsin (Promega) according to the manufacturer’s instructions prior to phosphopeptide mapping. Tryptic peptides were separated in the first dimension by electrophoresis using pH 8.9 buffer and in the second dimension (chromatography) using a buffer containing n-butanol/pyri-dine/acetic acid/water (12:10:3:15). Chromatography was allowed to proceed for 20 h. The amount of Raf-1 contained in the immunoprecipi-
tates used for the mapping of the phosphotryptic peptides was deter-
mined by immunoblotting an aliquot of the immunoprecipitates, and it was equal in all samples. Phosphotryptic peptide mapping was repeated twice with comparable results.

**RESULTS AND DISCUSSION**

The PP2A Inhibitor Okadaic Acid Decreases Mitogen-induced Raf-1 Activation and Dephosphorylation: Role of Ser259—We have studied Raf-1 phosphorylation and activation in BAC-1.2F5 macrophages stimulated by CSF-1 (45, 46). The addition of CSF-1 to quiescent BAC-1.2F5 cells induced robust induction of Raf-1 activation and dephosphorylation: Role of Ser259. This suggests that both positive and negative regulatory phosphorylation sites are targets of an inhibitory system and purified as described previously (40) with the exception that 1% Triton X-100 was added before binding to GST-agarose. Recombi-

nant MEK-1 in coupled assays using MBP (41) as the end point of the assay. This additional 100 μg of fusion protein in phosphate-buffered saline plus 0.1% SDS was added 2–4 h after CSF-1 stimulation; by this time, Raf-1 activation had decayed (45, 46). The addition of CSF-1 to quiescent BAC-1.2F5 cells induced robust induction of Raf-1 activation and dephosphorylation: Role of Ser259. This suggests that both positive and negative regulatory phosphorylation sites are targets of an inhibitory system and purified as described previously (40) with the exception that 1% Triton X-100 was added before binding to GST-agarose. Recombin-

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ppptides, as well as of spot 1 (Ser 621 partial digest) and of the uniden-
cated BAC-1.2F5 cells were left untreated (open circles) or were pre-
treated with okadaic acid (100 nM, 45 min; closed squares) prior to cell lysis and Raf-1 immunoprecipitation. 

The scheme shows the positions of Ser43-, Ser259-, and Ser621-containing peptides. Phosphotryptic peptide mapping was repeated twice with corresponding synthetic peptides phosphorylated by PKA and treated with okadaic acid (100 nM, 45 min; closed squares). In addition, significant amounts of Raf-1 were present in immunoprecipitates prepared with a monoclonal antibody against PP2AC but not with nonimmune mouse IgG (Fig. 3B), demonstrating the specificity of the interaction. The ubiquitously expressed PP2A core heterodimer binds to different variable subunits of cellular or viral origins, which are involved in regulating its substrate specificity and/or localization. None of the cellular variable subunits tested (p55b and p72 plus p130) was present in Raf-1 immunoprecipitates (not shown). Consistent findings were obtained in fibroblasts stimulated with EGF (not shown).

We next verified the interaction between Raf-1 and the PP2A heterodimer in COS-7 cells transfected with vectors directing the expression of HA-tagged PP2A subunits (Fig. 4A). Anti-HA immunoprecipitates from cells transfected with the HA-tagged PP2AC contained low amounts of endogenous PR65 and Raf-1 (Fig. 4A, lane 1). Anti-HA immunoprecipitates from cells transfected with the HA-PR65, on the other hand, contained significant amounts of endogenous PP2AC subunit and more Raf-1 (Fig. 4A, compare lanes 1 and 2). Therefore, the amount of Raf-1 detected correlated with the amount of heterodimer pres-
With okadaic acid (100 nM, 45 min) prior to stimulation with EGF (33 nM, 10 min), lysis, and immunoprecipitation. Raf-1 immunoprecipitates (duplicates) were assayed for kinase activity in a coupled assay. The amount of Raf-1 in the immune complexes was determined by immunoblotting. One representative kinase assay out of three is shown. Differences between samples were below 5% in all cases.

FIG. 2. EGF-stimulated activation of wild type Raf-1, but not of a S259A Raf-1 mutant, is decreased by okadaic acid in COS-1 cells. COS-1 cells were transfected with a vector encoding human wild type Raf-1 (pCMV5c-raf) or S259A Raf-1 mutant (pCMV5-S259A). 48 h after transfection, cells were treated with okadaic acid (100 nM, 45 min) prior to stimulation with EGF (33 nM, 10 min), lysis, and immunoprecipitation. Raf-1 immunoprecipitates (duplicates) were assayed for kinase activity in a coupled assay. The amount of Raf-1 in the immune complexes was determined by immunoblotting. One representative kinase assay out of three is shown. Differences between samples were below 5% in all cases.

FIG. 3. Endogenous Raf-1 interacts with PP2A heterodimers in vivo. A, the PP2A core heterodimer is present in Raf-1 immunoprecipitates from quiescent and mitogen-stimulated BAC-1.2F5 cells. Quiescent BAC-1.2F5 cells were stimulated with 6.3 nM mouse recombinant CSF-1 at 37 °C for different times prior to solubilization. Raf-1 immunoprecipitates from 1 mg of whole cell lysates were analyzed by Western blotting with antisera directed against the 36-kDa catalytic subunit of PP2A, PR65α, and Raf-1. 25 μg of whole cell lysates (WCL) were loaded as a control. Neither PP2AC nor PR65α were detected in immunoprecipitates prepared using nonimmune rabbit sera (NI) or protein A beads (A) instead of the Raf-1-specific antiserum. The background bands observed in the PP2AC Westerns could also be detected by anti-rabbit antibody alone and represent IgG heavy chains. B, Raf-1 is present in PP2AC immunoprecipitates. BAC-1.2F5 were solubilized, and 1 mg of whole cell lysates were subjected to immunoprecipitation using a monoclonal antibody against PP2AC (PP2AC), nonimmune mouse IgG (NI), or an anti-Raf-1 serum. The immunoprecipitates were analyzed with monoclonal antibodies directed against PP2AC, PR65α, or Raf-1.

The Raf-1-PP2A complex formation was further analyzed by in vitro reconstitution experiments with purified proteins (Fig. 4C). GST-tagged Raf was expressed in SF-9 cells alone or in combination with v-Ras plus Lek in order to activate it (Raf*). Raf proteins immobilized on glutathione-Sepharose beads were incubated with PR65, PP2AC, or the heterodimer PR65-PP2AC. Consistent with the lack of effect of mitogens on in vivo complex formation, we did not observe significant differences between PP2A binding to Raf or Raf*, PP2AC displayed only weak binding to Raf-1. In contrast, both PR65 and the core heterodimer associated strongly. Thus, as it is the case for cellular and viral subunits (28), PR65 probably plays the key role in the association between Raf-1 and the PP2A heterodimer. This association, however, is not likely to be direct. Raf-1 does not interact with PR65, PP2AC, or p55 in the yeast two-hybrid system,2 and size fractionation experiments indicate that in vivo Raf-1 and PP2A are part of a large protein complex (data not shown). Additional proteins, and possibly a variable subunit not detected in our experiments, might also be present in small amounts in the purified enzyme preparations used in the GST pull-down experiments and might be facilitating or even mediating the interaction observed in vitro. In this context, a variable subunit of PP2A has been recently shown to positively regulate Ras signaling upstream of raf during vulval development in Caenorhabditis elegans (50).

Conclusion—Concentrations of okadaic acid that specifically affect PP2A reduced Raf-1 activation, and PP2A was found in Raf-1 immunoprecipitates from quiescent and mitogen-treated cells. Therefore, while an effect of the drug on other phosphatases cannot be formally excluded, PP2A presumably represents the okadaic acid-sensitive phosphatase involved in Raf-1 regulation. Our current working model is that Raf-1-associated PP2A facilitates kinase activation by maintaining Ser259 in a dephosphorylated state and thereby preventing the formation of inactive 14-3-3-Raf-1 complexes (14–18). This may permit the activation of a larger number of Raf-1 molecules and prolong it by counteracting the mitogen-induced kinase (probably protein kinase B) that phosphorylates Ser259. Ultimately, the Ser259 kinase must outpace PP2A to terminate Raf-1 activation. Inhibition of PP2A by okadaic acid has been reported to selectively impair Raf-dependent transformation (51). Furthermore, in genetically dissectable organisms, hypomorphic alleles of PP2A suppress the effects of activated Raf (52) or enhance the loss of function phenotype of Raf mutations (50). On the basis of these results, PP2A might have been considered either a Raf-1 effector or a positive regulator of Raf-1 activation. Our findings provide a mechanistic explanation for these observations. By identifying PP2A as a positive regulator of Raf-1, our data define a new function for this phosphatase and add a new facet to the complexity of Raf-1 regulation.

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