Phosphorylation of the Myosin-binding Subunit of Myosin Phosphatase by Raf-1 and Inhibition of Phosphatase Activity*

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Raf-1 serine/threonine protein kinase plays an important role in cell survival, proliferation, and migration; however, the specific targets of Raf-1 in diverse cellular processes are not clearly defined. Myosin phosphatase activity is critical to the regulation of cytoskeletal reorganization, cytokinesis, and cell motility. Here, we describe the association of Raf-1 with myosin phosphatase and phosphorylation of the regulatory myosin-binding subunit (MBS) of myosin phosphatase by Raf-1. Treatment of cells with phorbol 12-myristate 13-acetate has been shown to stimulate Raf-1 protein kinase. To determine the effect of enzymatic activation of Raf-1 on MBS phosphorylation, COS-1 cells were transiently transfected with FLAG-tagged full-length Raf-1. A significantly higher phosphorylation of purified glutathione S-transferase-tagged truncated MBS protein (amino acids 654–880) occurred in the presence of FLAG-Raf-1 immunoprecipitated from phorbol 12-myristate 13-acetate-treated cells compared with untreated cells (~3.0-fold). Using a sequential kinase-phosphatase assay and phosphorylated myosin light chain as substrate in the phosphatase reaction, we showed that Raf-1-associated protein phosphatase-specific activity was inhibited (relative phosphatase activity without and with adenosine 5’-O-(3-thiotriphosphate): 100 and ~30%, respectively). Previously, ionizing radiation has been shown to activate Raf-1 (Kasid, U., Suy, S., Dent, P., Ray, S., Whiteside, T. L., and Sturgill, T. W. (1996) Nature 382, 813–816). Exposure of cells to ionizing radiation resulted in the increased association of Raf-1 with MBS (3-6-fold versus unirradiated control) and inhibition of Raf-1-associated protein phosphatase-specific activity (relative phosphatase activity without and with ionizing radiation: 100 and ~54%, respectively). Our studies identify MBS as a new substrate of Raf-1 and implicate a role for Raf-1 in the regulation of pathways involving myosin phosphatase activity.

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1 The abbreviations used are: MBS, myosin-binding subunit; PP1, protein phosphatase-1; MLC, phosphorylated myosin light chain; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; PMA, phorbol 12-myristate 13-acetate; BOKa, Rho-associated kinase-α; ATPγS, adenosine 5’-O-(3-thiotriphosphate); MBP, phosphorylated myelin basic protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; IR, ionizing radiation.

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described. (20) A human MBS cDNA clone was isolated from a HeLa cell cDNA library using reverse transcription-PCR and inserted into a mammalian expression vector (pRK7) downstream of a Myc tag. The human MBS cDNA and its fragment were confirmed by the dideoxy sequencing method using the Biotronic Core Facility of the University of Virginia. The GST-MBS fusion protein was expressed in Esherichia coli strain BL21 (DE3) by incubation at 23 °C for 24 h using LB medium in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside and purified by glutathione-agarose column chromatography according to the manufacturer’s protocol (Amersham Biosciences, Inc.).

**MBS Identification Procedure**—MDA-MB 231 cells (2.5 × 10⁸) were lysed in Nonidet P-40 lysis buffer (100 mM HEPEs (pH 7.4), 10% glycerol, 150 mM NaCl, 1% Nonidet P-40, 50 mM NaF, 5 mM Na₂VO₃, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM phenylmethylsulfonly fluoride) as described. (17) The supernatant was precleared for 1–2 h with protein A-Sepharose CL-4B (Amersham Biosciences, Inc.) and incubated with monoclonal anti-Raf-1 antibody for 6 h at 4 °C. Rabbit anti-Myc-MBS sera were added, and the samples were incubated overnight by agitation incubation with protein A-Sepharose CL-4B at 4 °C. The beads were washed twice with Nonidet P-40 lysis buffer, and samples were pooled and electroblotted on 7.5% SDS-polyacrylamide gel. Following electrophoresis, the gel was stained without fixation with Coomassie Brilliant Blue R-250. The Coomassie Blue-stained bands (~130 and ~135 kDa) were excised, and radioactivity was determined in a scintillation counter.

**Immunoprecipitation Assay**—MDA-MB 231 cells were lysed in Nonidet P-40 lysis buffer for 30 min in a cold room and centrifuged at 14,000 × g for 20 min. Two mg of protein A-Sepharose CL-4B and over protein A/Plus-agarose (Santa Cruz Biotechnology) for 1–2 h; the beads were removed by centrifugation; and the supernatant was incubated with a desired antibody overnight at 4 °C. The antigen-antibody complex was captured by incubation with 25 µl of protein A/Plus-agarose for 1.5–3 h. The beads were washed three times with ice-cold Nonidet P-40 lysis buffer and once with 50 mM Tris-HCl (pH 7.4). The immunocomplex was eluted with trypsin and analyzed by liquid chromatography-tandem mass spectrometry. The resulting peptides were separated by reversed-phase chromatography and analyzed by liquid chromatography-tandem mass spectrometry using SEQUEST molecular recognition system software. The peptides obtained from mass spectrometric analysis of ~130- and ~135-kDa proteins co-immunoprecipitating with Raf-1 and nonimmunoprecipitated human MBS were identified using the SEQUEST molecular recognition system software.

**GST Phosphorylation Assay**—Purified GST-MBS protein was used as substrate in vitro kinase reactions performed using purified GST-Raf-1 fusion protein or immunoprecipitated FLAG-tagged full-length Raf-1 as detailed below.

**Cos-1 cells** were transiently transfected with FLAG-Raf-1 or FLAG-Raf-1 (K375M). Thirty-six h after transfection, cells were serum-starved overnight and stimulated for 20 min with 200 nM phorbol 12-myristate 13-acetate (PMA) or vehicle (Me₆SO). Cells were washed twice with ice-cold PBS and lysed in Nonidet P-40 lysis buffer (200 mM NaCl). Lysates were centrifuged for 20 min at 16,000 × g. The supernatant (~1 mg of protein) was precleared with protein A/G-agarose for 2 h. Exogenous Raf-1 was immunoprecipitated overnight at 4 °C with monoclonal anti-FLAG antibody M2 (Sigma). The immune complex was captured by protein A/G-agarose for 1 h. The beads were washed three times with ice-cold PBS and once with FLAG/HEPES (pH 7.4, 1 mM dithiothreitol, 10 mM MnCl₂, 5 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) containing 0.5 mg/mlGST-Raf-1 fusion protein or GST (as a negative control). Bound GST-Raf-1 protein was observed by incubation of the membrane with 0.5 µg/ml anti-GST antibody for 1 h, followed by ECL detection.

**Raf-1 was immunoprecipitated from whole cell lysates of logarithmically growing MDA-MB 231 cells using monoclonal anti-Raf-1 antibody and analyzed by SDS-PAGE**. The Coomassie Blue-stained ~130- and ~135-kDa bands were excised and individually digested with trypsin. The resulting peptides were separated by reverse-phase chromatography and analyzed by liquid chromatography-tandem mass spectrometry using SEQUEST molecular recognition system software.
In vitro phosphorylation of purified GST-MBS protein in the presence of purified GST-Raf-1 was performed using the kinase reaction conditions described above. In vitro phosphorylation of purified GST-MBS by catalytically active, recombinant Rho-associated kinase-α (ROKα, 66 kDa) (Upstate Biotechnology, Inc.) was performed exactly as described (15).

**MBS Peptide Phosphorylation Assay**—Purified GST-Raf-1 or endogenous Raf-1 immunoprecipitated from MDA-MB 231 cells was used to phosphorylate an MBS-specific peptide, P3, representing amino acids 683–701 of the MBS protein (NH2-KARSRQARQSRRSTQGVTL-COOH) (23). P3 containing a single amino acid mismatch, P3m (Thr696 → Val, NH2-KARSRQARQSRRSVQGVTL-COOH), and two other MBS peptides, P1 (NH2-VTTPTVSSGQATPTSPIK-COOH, amino acids 395–412) and P2 (NH2-ISPKEEERKDESPATWRLGLRK-COOH, amino acids 442–462) (23), were also designed and tested. GST-Raf-N containing only the NH2 terminus of Raf-1 was used as a negative control. Immunoprecipitated Raf-1 (~750 μg of total protein) or GST-Raf-1-Sepharose (20 ng) was incubated with the peptide (150 μM) for 15–20 min at 30 °C in 0.5 μl of the kinase reaction mixture, and peptide-associated radioactivity was quantified by liquid scintillation. Unless otherwise indicated, data were plotted using the radioactivity values obtained with P1 as a base-line control.

**Sequential Kinase-Phosphatase Assay**—Raf-1 was immunoprecipitated from MDA-MB 231 cell lysates (2 mg of protein) prepared in Nonidet P-40 lysis buffer without the serine/threonine phosphatase inhibitors as described above. The beads were resuspended in 50 mM HEPES (pH 7.4), 12 mM MgCl2, 1 mM dithiothreitol, and 20 or 100 μM nonradioactive ATP-S (Sigma). The control reaction did not contain ATP-S. In addition, a kinase reaction was also performed in the presence of an ROKα inhibitor, HA-1077 (Calbiochem) or Y-27632 (Upstate Biotechnology, Inc.). The reaction was carried out at 30 °C for 30 min, followed by microcentrifugation. Phosphatase activity was assayed using the serine/threonine protein phosphatase assay system and phosphorylated myelin basic protein (MBP32, 32P-labeled MBP) according to the manufacturer’s protocol (New England Biolabs Inc.). Phosphatase activity in Raf-1 immunoprecipitates was also assayed using MLC3 (4 μM, 32P-labeled MLC) as substrate. The phosphatase reaction was carried out at 30 °C for 5 min (MLC3) or 10 min (MBP32). The radioactivity released in the supernatant was measured by liquid scintillation counting. The reaction was performed in duplicate per data point per experiment. Proteins in the pellet (Raf-1–immunocomplex, 1.0–1.5 mg of protein) were resolved by 7.5% SDS-PAGE, followed by sequential immunoblotting of the same blot with anti-MBS, anti-PP1, and anti-Raf-1 antibodies and ECL to detect MBS, PP1, and Raf-1 protein expression, respectively. The ECL signals were computer-scanned using ImageQuant software. The relative amounts of Raf-1–associated PP1β protein in various samples were determined by dividing the PP1β arbitrary scanner value by the Raf-1 value for that lane. Phosphatase-specific activity was then calculated using the following formula: absolute radioactivity (cpm)/relative amount of Raf-1–associated PP1β protein. The phosphatase-specific activity data were plotted as -fold base-line control reaction, i.e. without ATP-S.

**RESULTS**

**Physical Interaction of Raf-1 and MBS**—To identify new substrates of Raf-1 protein kinase, the whole cell lysates of MDA-MB 231 human breast carcinoma cells were examined for proteins associated with Raf-1. Two proteins (~130 and ~135 kDa) co-immunoprecipitated with Raf-1 and were readily visible on a Coomassie Blue-stained gel. These proteins were purified by sequential fractionation on SDS-polyacrylamide gel, followed by tandem mass spectrometric analysis. Four peptides from the 130-kDa band (peptides 1–4) and two peptides from the 135-kDa band (peptides 5 and 6) matched with MBS human myosin phospha-tase (Table I). In addition, three mass spectra from the 130-kDa band matched with myosin phosphatase protein from rat and chicken (data not shown).

To confirm the *in vitro* association of Raf-1 and MBS, immunoprecipitation and immunoblot experiments were performed. Co-immunoprecipitation of endogenous Raf-1 and MBS was observed in MDA-MB 231 cells (Fig. 1, A and B). To determine whether exogenous MBS interacts with Raf-1, COS-1 cells were transiently transfected with the expression vector containing Myc-tagged full-length MBS. The expression of Myc-MBS (~140 kDa) in COS-1 transfectants was verified by immuno-
blotting with anti-Myc epitope antibody (Fig. 1C). The interaction of Mys-MBS with GST-Raf-1 fusion protein (~100 kDa) (8) was observed by two independent approaches, the GST-Raf-1 pull-down assay (Fig. 1C) and the overlay assay (Fig. 1D). These data established a direct association between Raf-1 and MBS in MDA-MB 231 and COS-1 cells.

Phosphorylation of MBS by Raf-1 Protein Kinase—To address that the physical association of Raf-1 and MBS means that Raf-1 phosphorylates MBS, in vitro kinase assays were performed using purified GST-tagged truncated MBS protein (amino acids 654–880, GST-MBS, ~55 kDa) as a substrate of Raf-1. This fragment of MBS includes an inhibitory phosphorylation site (Thr696) (27). In a direct in vitro kinase assay, followed by SDS-PAGE and autoradiography, GST-Raf-1 protein kinase was observed to phosphorylate GST-MBS (Fig. 2, left panels). As a positive control, the catalytic domain of purified ROKα protein (66 kDa) was shown to phosphorylate GST-MBS (Fig. 2, right panels).

Treatment of cells with the protein kinase C activator PMA has been shown to decrease Raf-1 mobility and to enhance Raf-1-associated serine/threonine kinase activity (24) (data not shown). We investigated whether PMA-activated Raf-1 is more efficient in phosphorylating GST-MBS protein. COS-1 cells were transiently transfected with the expression vector containing FLAG-tagged wild-type Raf-1 or FLAG-tagged Raf-1(K375M). Following PMA treatment, exogenous Raf-1 was immunoprecipitated with anti-FLAG antibody, and the immune complex was used in an in vitro kinase assay. Representative data demonstrate that a significantly higher phosphorylation of GST-MBS occurred with PMA-stimulated FLAG-Raf-1 compared with the unstimulated counterpart (~3.0-fold) (Fig. 3B, WT/PMA versus WT). The final stoichiometry of phosphorylation of GST-MBS using the FLAG-Raf-1 immune complex as a source of Raf-1 protein kinase was ~0.1 mol of phosphate/mol of substrate (Fig. 3C). The reason for the slight activity seen in the presence of FLAG-Raf-1(K375M) immunoprecipitates is unclear and may represent some background activity. However, PMA treatment had a negligible effect on mutant Raf-1 kinase activity (Fig. 3B, K375M versus K375M/PMA). In addition, as would be expected, phosphorylation of GST-MBS by wild-type Raf-1 was found to be significantly higher compared with mutant Raf-1 (3.96-fold) (Fig. 3B, WT/PMA versus K375M/PMA). Similar observations were made when we used His6-MEK1 protein (4.44-fold), a known physiological substrate of Raf-1 (data not shown).

We next designed three MBS-specific peptides (23) designated as P1 (amino acids 395–412), P2 (amino acids 421–442), and P3 (amino acids 683–701) and tested whether these are the novel peptides phosphorylated by Raf-1. GST-Raf-1 specifically phosphorylated P3, but not P2 or P1 (P3 versus P1 or P2, ~8-fold) (Fig. 4A). Furthermore, P3 containing a single amino acid mismatch (P3m, Thr696 to Val) exhibited significantly diminished phosphorylation compared with P3 (~4-fold), and GST-Raf-N fusion protein (containing only the amino terminus of Raf-1) was far less effective in phosphorylating P3 compared with GST-Raf-1 (~4.0-fold) (Fig. 4A). ROKα has been shown to cause phosphorylation of MBS (13). HA-1077 (100 μM), a chemical compound previously shown to inhibit ROKα activity (25), did not inhibit Raf-1 kinase activity and Raf-1-mediated phosphorylation of P3 (data not shown). Cellular Raf-1 also phosphorylated MBS peptide P3 compared with control MBS peptide P1 (~6.0-fold), and phosphorylation of a single amino acid
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Fig. 4. Raf-1 phosphorylates an MBS-specific peptide. A, an in vitro kinase reaction was performed using GST-Raf-1-Sepharose (20 ng) and 150 μM MBS peptide P3 (amino acids 683–701). The radioactivity values obtained were normalized to control MBS peptide P1 (amino acids 395–412). The data shown represent two to three independent experiments (mean ± S.D.), each data point performed in triplicate per experiment. Other peptides used include P2, another MBS-specific peptide (amino acids 421–442), and P3m, peptide P3 with a single amino acid mismatch. GST-Raf-N is the fusion protein of GST and the Raf-1 amino terminus. B, Raf-1 was immunoprecipitated (IP) from lysates of logarithmically growing MDA-MB 231 cells (750 μg of protein), and an in vitro kinase reaction was performed in the presence of 150 μM P3, P1, or P3m and with or without ROKα inhibitor HA-1077 (100 μM). Data shown represent one to three independent experiments, each data point performed in triplicate per experiment. C, mitotic Raf-1 was immunoprecipitated with monoclonal anti-Raf-1 antibody from MDA-MB 231 cells treated with nocodazole (40 μg/ml, 9 h). Raf-1 immunoprecipitates (750 μg of protein) were used in in vitro kinase reactions in the presence of 150 μM P3 or P2. Data shown are from a representative experiment, each data point performed in triplicate or quadruplicate (mean ± S.D.). ML, mock lysate.

Fig. 5. Sequential kinase-phosphatase assay showing that Raf-1-associated protein phosphatase is inhibited. An in vitro kinase reaction was initiated using Raf-1 immunoprecipitated (IP) from logarithmically growing MDA-MB 231 cell lysates (2 mg of protein) and nonradioactive ATP—S (100 μM) in the presence or absence of ROKα inhibitor HA-1077 (100 μM) or Y-27632 (20 μM), followed by the phosphatase assay using MBP—P (MyBP—P, 32P-labeled MBP; A) or MLC—P (32P-labeled MLC; B) as substrate. Control reactions were performed in the absence of nonradioactive ATP—S. Phosphatase-specific activity was calculated based on the radioactivity value normalized against the level of Raf-1-associated PP18 protein determined by immunoblotting and quantification as explained under “Experimental Procedures.” Data from representative experiments are shown.

A mutant peptide (P3m) was relatively less (~2.0-fold) (Fig. 4B). The presence of HA-1077 in the reaction mixture did not affect the level of P3 phosphorylation (Fig. 4B). Cellular ROKα was also found to phosphorylate P3, and HA-1077 inhibited this mode of P3 phosphorylation (data not shown). From these observations, it appears that Thr406 of MBS is an important phosphorylation site for Raf-1 protein kinase, although the presence of additional site(s) in the P3 peptide cannot be ruled out.

Phosphorylation of Ser430 of chick MBS (which corresponds to Thr435 of human MBS) (26) during mitosis has been associated with activation of myosin phosphatase, and the P2 peptide contains Thr435. To further confirm Raf-1 selectivity for the P3 site, the phosphorylation of MBS peptides P3 and P2 was compared using Raf-1 immunoprecipitated from nocodazole-arrested MDA-MB 231 cells enriched in the mitotic phase (G2/M > 93%). Consistent with the data shown in Fig. 4B, Raf-1 phosphorylated P3, but not P2 (Fig. 4C).

Inhibition of Raf-1-associated Protein Phosphatase—We used a sequential kinase-phosphatase assay to unequivocally demonstrate the inhibition of Raf-1-associated myosin phosphatase activity. We used nonradioactive ATP—S in the kinase reaction and MBP—P or MLC—P as substrate in the phosphatase reaction. Thio-phosphorylation of cellular MBS (co-immunoprecipitated with Raf-1) was performed because it is resistant to phosphatase activity of the myosin phosphatase holoenzyme (27). In the presence of ATP—S (100 μM), endogenous protein phosphatase activity associated with Raf-1 immune complexes decreased by ~55 and ~70% with MBP—P and MLC—P as substrates, respectively (Fig. 5, A and B). Similar results were obtained when the ATP—S concentration was reduced to 20 μM (data not shown). The presence of ROKα inhibitors HA-1077 (100 μM) and Y-27632 (20 μM) (26) did not prevent reduction of phosphatase activity, establishing that the observed phosphorylation of MBS and inhibition of myosin phosphatase activity are not due to ROKα.

Stimulation of Raf-1 and MBS Interaction by Ionizing Radiation and Concomitant Inhibition of Raf-1-associated Protein Phosphatase—Previously, we demonstrated that exposure of human tumor cells (PCI-04A and MDA-MB 231) to ionizing radiation (IR) results in tyrosine phosphorylation, membrane translocation, and activation of Raf-1 (17, 18). To determine whether myosin phosphatase is a target of Raf-1 protein kinase in irradiated cells, we first examined the effect of IR on the association of MBS and PP1 with cellular Raf-1. IR treatment of MDA-MB 231 cells caused a significant increase in the association of Raf-1 with MBS (~3–6-fold) and PP1 (~3-fold) (Fig. 6, A–C). No change in the total amount of Raf-1, MBS, or PP1 protein per se was detected after irradiation (data not shown). These results suggest that activated Raf-1 selectively...
associates with myosin phosphatase.

We next measured protein phosphatase-specific activity in Raf-1 immune complexes from irradiated cells. As shown in Fig. 6, protein phosphatase-specific activity in Raf-1 immunoprecipitates from irradiated cells was inhibited compared with unirradiated MDA-MB 231 cells (IR, 100%; +IR, 54%). Additional metabolic labeling and immunoprecipitation experiments indicated that irradiation of MDA-MB 231 cells also led to a modest increase (50%) in the total pool of phosphorylated MBS (data not shown).

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Concur with these observations in the sense that Raf-1 protein kinase targets Thr696 in MBS; we cannot, however, rule out the presence of other sites in MBS preferentially phosphorylated by Raf-1. Previously, MBS-specific peptide P2 has been shown to be phosphorylated (MBS Ser430) by a mitotic kinase, resulting in the activation of myosin phosphatase activity (26). Our present observations that the P2 peptide is not phosphorylated by Raf-1 emphasize the importance of Raf-1-specific inhibition of myosin phosphatase. In addition, Raf-1 does not phosphorylate another MBS-specific peptide, P1. Whether regulation of myosin phosphatase by Raf-1 can lead to a biological response distinct from other known and unknown MBS kinases remains to be seen. Our data appear to support the general notion that, depending on the cell type and stimulation, physiological compensatory mechanisms including regulation of myosin phosphatase activity are governed by overlapping and complementary pathways.

Dynamic reorganization of the actin cytoskeleton is an integral aspect of cellular responses to environmental signals. The small GTPase Rac is required for the formation of lamellipodia at the front of migrating cells, whereas at the rear of the cell, phosphorylation of MLC produces actomyosin contractility and de-adhesion necessary for cell movement (11, 12, 32, 33). Interestingly, Rac influences the cell migration process by selec-
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