Supporting Materials and Methods

Methylation-Specific PCR Analysis of the Protein Tyrosine Phosphatase Receptor-Type O CpG Island. Methylation-specific PCR (MS-PCR) analysis was performed on the bisulfite-converted DNA by using nonmethylation-specific primers hPTP-UF (5'-ATGTTTTTGGAGGATTTTGGGT-3') and hPTP-UR (5'-ATACCCCATCACTACACAAACA-3') and methylation-specific primers hPTP-MF (5'-CGTTTTTGGAGGATTTCGGGC-3') and hPTP-MR (5'-AAAACACGACTACGCTAACG-3'). These primers were designed to amplify 201- and 170-bp regions of the protein tyrosine phosphatase receptor-type O (PTPRO) CpG island, respectively. Each PCR was carried out in a 20-μl reaction containing ≈100 ng of bisulfite-converted DNA, 1× PCR buffer (Qiagen, Valencia, CA), 10 pmol each of forward and reverse primers, 0.2 mM dNTPs, and 0.75 units of *Taq*DNA polymerase (NEB, Beverly, MA). After an initial incubation at 94°C for 3 min, a touch-down PCR with 20 cycles of denaturation at 94°C for 30 sec, annealing at 60°C (Δ –0.4°C per cycle) for 1 min (Δ –0.02 sec per cycle), and annealing at 72°C for 30 sec was performed, followed by an additional 10 cycles of 30 sec of denaturing at 94°C, 15 sec of annealing at 50.4°C, and 30 sec of extension at 72°C. The reactions ended with a 72°C extension for 7 min, and 10-μl aliquots of the PCR were separated on a 2.5% agarose gel run in 1× TBE buffer (90 mM Tris/90 mM boric acid/1.0 mM EDTA, pH 8.3). The ethidium-bromide-stained DNA was visualized under UV light and captured by using a Kodak 1D imaging system.

Combined Bisulfite Restriction Analysis and Bisulfite Genomic Sequencing. The *PTPRO* CpG island from –208 to +236 bp with respect to the transcription start site was amplified from bisulfite-converted DNA using nested primers devoid of CpG to avoid methylation/nonmethylation bias. The first round of amplification was carried out in a 25-μl reaction with primers hPTP-BS-F1 (5'-AGGTTGTTGTTATTTTATGG-3') and hPTP-BS-R1 (5'-AAAACTCCCCTACCTTAAAC-3'). Each reaction contained ≈100 ng of bisulfite-converted DNA, 1× PCR buffer (Qiagen), 0.2 mM dNTP, 10 pmol each of primer, and 1 unit of *Taq*DNA polymerase (NEB). An initial incubation at 94°C for 3 min was followed by 35 cycles of amplification. Each cycle involved 1 min each of denaturation at 94°C, annealing at 50°C, and extension at 72°C followed by a final extension at 72°C for 7 min. An aliquot (2 μl) of this PCR was used in a 50-μl nested PCR with primers hPTP-BS-F2 (5'-CGGTAGTTAGAGTGAGAGTGTT-3') and hPTP-BS-R2 (5'-AAAAACCAAAACAAAAACAA-3'). Amplification with 35 total cycles was carried out in which each cycle involved 1 min each of 94°C denaturation, 51.5°C of annealing, and 72°C of extension followed by a single 72°C extension for 7 min. The PCR products were purified by using a gel-extraction kit (Qiagen). The purified PCR product was used for combined bisulfite restriction analysis (COBRA) and bisulfite sequencing. For COBRA, 5 μl of purified PCR product was incubated at 60°C in the presence or absence of *Taq*I for 1 h in a 15-μl reaction containing 1× React 2 (GIBCO). The entire digestion mix was loaded onto 2.5% agarose, and DNA visualized under UV light was captured by using a Kodak 1D imaging system. For bisulfite sequencing, the PCR product was either directly sequenced or cloned for automated sequencing. Direct

sequencing was performed with the Thermo Sequenase radiolabeled terminator cycle Sequenase kit (United States Biochemical) with the primers hPTP-BS-F2 (see above) and hPTP-BS-F3 (5'-TAGGGGGATTGGAAAGGTAG-3'). For automated sequencing (primary tumor and matching normal), the PCR product was cloned into pCR2.1-TOPO by using the TOPO-TA cloning kit (Invitrogen). Five clones each from the tumor and normal samples were selected randomly for sequencing.

RT-PCR. Semiquantitative PCR (for tissues) was performed by using cDNA equivalent to 75 ng (for 18S rRNA) or 300 ng (for PTPRO) RNA. The 20-μl reaction mix contained 1× ThermoPol buffer (NEB), 0.2 mM dNTP, 2 pmol each of forward (^{32}P-labeled) and reverse primers, and 0.75 units of *Taq*DNA polymerase (NEB). PCR amplification (20 cycles for 18S rRNA and 28 cycles for PTPRO) was performed with 30 sec each at 94°C denaturation, 65°C (18S) or 54.5°C (PTPRO-FL) annealing, and 72°C extension. The reaction ended with a final extension step of 72°C for 7 min. The PCR products were separated on a 6% native polyacrylamide gel and identified by autoradiography. The qualitative PCR (cell lines) was performed by using cDNA equivalent to 150 ng (for 18S rRNA) and 300 ng (for PTPRO) of RNA. Each 25-μl reaction mix consisted of 1× ThermoPol buffer (NEB), 0.2 mM dNTP, 10 pmol each primer and 1 unit *Taq*DNA Polymerase (NEB). The cycling conditions were as follows: 30 sec each for denaturation at 94°C, annealing at 54.5°C (PTPRO-FL and PTPROt), 57.2°C (hPTPRO), or 65°C (18S rRNA), and extension at 72°C for 25 (18S rRNA) or 32 (all PTPRO) cycles. The PCR products were separated on an agarose (1.5%) gel and stained with ethidium bromide. The DNA visualized under UV light was imaged by using Kodak DIGITAL SCIENCE 1D software. The gene-specific primers used for amplification of the respective cDNA are: 18S rRNA (F-5'-TCAAGAACGAAAGTCGGAGG-3' and R-5'-GGACATCTAAGGGCATCACA-3'); hPTPRO-FL (F-5'-GGGGATGATACAACGGACTT-3' and R-5'-ACCATTGTTGAGACGGCTATGAACG-3'); hPTPROt (F-5'-GGGGATGCTTCACCTGCTTA-3' R-5'-ACCATTGTTGAGACGGCTATGAACG-3'); and hPTPRO (F-5'-CTCCACCCAAATCACTCTTCGCAG-3' and R-5'-ACCATTGTTGAGACGGCTATGAACG-3').

Immunofluorescence. Immunostaining of the selected A549 cells was performed essentially as described [Matsuo, Y., Akiyama, N., Nakamura, H., Yodoi, J., Noda, M. & Kizaka-Kondoh, S. (2001) *J. Biol. Chem.* **276,** 10032–10038] with minor modifications. Briefly, the cells were plated on coverslips 24 h before staining. They were fixed with 4% paraformaldehyde in PBS at room temperature for 1 h followed by permeabilization with 0.2% Triton X-100 in PBS for 2 min on ice. After rinsing with PBS, the cells were blocked with 2% BSA for 1 h at room temperature. The cells were rinsed again with PBS and incubated with 20 μg/ml α-Flag M2 antibody (Sigma) at 4°C for 16 h. The primary antibody was rinsed off with PBS, and the coverslips were incubated with tetramethylrhodamine B isothiocyanate-conjugated α-mouse antibody (Sigma) diluted 1:50 in PBS for 1 h at room temperature. After a final PBS wash, the coverslips were mounted with 4',6-diamidino-2-phenylindole for nuclear staining. Immunofluorescence was analyzed under a confocal microscope.

Anchorage-Independent Colony-Formation Assay in Soft Agar. A 4-ml base agar of 0.5% final concentration was prepared in 60-mm dishes using a 4% agarose stock (noble agar, Sigma) diluted with A549 culture medium (RPMI medium 1640 supplemented with 10% FBS and penicillin-streptomycin) and containing 1 mg/ml G418 (except for A549/control cells). After the base agar was set, a top agar prepared similarly but with 0.33% final concentration of agar and 1×10^4 cells was seeded over the base agar, and the plates were incubated at 37°C for up to 2 weeks. Triplicate plates were prepared for each transfectant, i.e., control, vector alone, PTPRO/WT, and PTPRO/CS (catalytic site mutant) and the experiment was repeated twice. The cell colonies were visualized by staining with 0.005% crystal violet. The colonies were photographed under a phase-contrast microscope using a Nikon digital camera and were also counted under the same microscope after dividing the plates into grids. Up to 200 colonies were counted from each plate for statistical analysis.

BrdUrd-Incorporation Assay. A549 cells (vector alone, PTPRO/WT, and PTPRO/CS) were synchronized at the G_0/G_1 and G_1/S phases of the cell cycle by using serum starvation and double-thymidine block, respectively. For serum starvation, 1.25×10^5 cells seeded in 12-well plates were grown in the presence of medium containing 0.2% serum for 30 h followed by release into complete growth medium for 4 h. Double-thymidine block was performed by incubating 4×10^4 cells in 12-well plates with 2 mM thymidine for 16 h followed by growth in complete medium for 10 h and further incubation with 2 mM thymidine for an additional 16 h. After completion of thymidine block, cells were rinsed and released into complete growth medium for 4 h. One set of cells immediately after release (from serum starvation and thymidine block) and another 4 h after release were pulsed with 10 μM BrdUrd (Sigma) for 2 h, fixed with 70% ethanol, denatured with 2 M HCl and stained with anti-BrdUrd antibody (Sigma) for 2 h at 37°C followed by staining with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia). Color was developed with diaminobenzidine (Sigma) and counter-stained with eosin Y (Sigma). Cells were photographed with a Nikon digital camera under a phase-contrast microscope.