

**Suppression of the Phosphorylation of Receptor Tyrosine Phosphatase- $\alpha$  on a Src-independent Site Tyrosine-789 by Reactive Oxygen Species**

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**a) *Running Title:*** Suppression of RPTP $\alpha$  Phosphorylation on Tyr-789 by ROS

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**d)** The abbreviations used are: PTPs, protein tyrosine phosphatases; RPTP $\alpha$ , receptor protein tyrosine phosphatase- $\alpha$ ; ROS, reactive oxygen species; HAECs, human aortic endothelial cells; HUVECs, human umbilical vein endothelial cells; PAO, phenylarsine oxide; PBS, phosphate-buffered saline.

### Abstract

Oxidation of receptor protein tyrosine phosphatase- $\alpha$  (RPTP $\alpha$ ) is emerging as an important, yet poorly characterized, regulatory mechanism for RPTP $\alpha$  signaling in cells. RPTP $\alpha$  has been shown to be reversibly oxidized and inhibited by reactive oxygen species. However, it is not known whether oxidative stress could regulate the phosphorylation of Tyr-789, a critical tyrosine residue for RPTP $\alpha$  signaling that modulates the function of Grb2 and the activation of Src family kinases. In the present study, we have taken advantage of a phospho-specific antibody against Tyr-789-phosphorylated RPTP $\alpha$  and characterized the phosphorylation of RPTP $\alpha$  Tyr-789 in various cultured cells, including SYF cells lacking all three ubiquitously expressed members (Src, Yes and Fyn) of Src family kinases. We have obtained substantial evidence indicating that the phosphorylation of RPTP $\alpha$  Tyr-789 is regulated predominantly by a Src kinase inhibitor PP1-sensitive but Src/Yes/Fyn-independent tyrosine kinase in cells. We further reported a novel finding that, besides the inhibition of RPTP $\alpha$ 's activity, H<sub>2</sub>O<sub>2</sub> at low to moderate concentrations (50-250  $\mu$ M) markedly suppressed the phosphorylation of RPTP $\alpha$  Tyr-789 and the association of RPTP $\alpha$  with Grb2 in cultured cells, which may result from inhibition of such a PP1-sensitive but Src/Yes/Fyn-independent tyrosine kinase. Since Tyr-789 plays an important role in RPTP $\alpha$  signaling, our findings may provide new insights into the functional regulation of RPTP $\alpha$  by oxidative stress in cells.

Protein tyrosine phosphorylation is a fundamental mechanism for many signal transduction pathways that control cell growth, differentiation, and motility (Hunter, 1995). Although it is generally agreed that tyrosine phosphorylation is regulated by the equal and balanced actions of protein tyrosine kinases and protein tyrosine phosphatases (PTPs), relatively little is known about the regulation and signal transduction of most PTPs (Neel and Tonks, 1997). The receptor-like PTP $\alpha$  (RPTP $\alpha$ ) is a widely expressed transmembrane PTP with a short heavily glycosylated extracellular domain and two tandem cytoplasmic PTP domains (Kaplan et al., 1990). The membrane-proximal domain contains most catalytic activity, whereas the membrane-distal domain is catalytically inactive but has a regulatory role (Blanchetot et al., 2002). Several lines of evidence indicate that RPTP $\alpha$  is a positive regulator of Src family kinases and is required for integrin-mediated cell spreading and migration (Ponniah et al., 1999; Su et al., 1999; Zeng et al., 2003). Overexpression of RPTP $\alpha$  results in Src activation and neoplastic transformation (Zheng et al., 1992). Conversely, deficiency of RPTP $\alpha$  markedly impairs the catalytic activities of Src and Fyn and the integrin signaling (Ponniah et al., 1999; Su et al., 1999). In addition, RPTP $\alpha$  interferes with insulin receptor signaling (Moller et al., 1995) and regulates the Kv1.2 potassium channel upon the activation of m1 muscarinic acetylcholine receptor (Tsai et al., 1999).

RPTP $\alpha$  is phosphorylated on a critical tyrosine (Tyr-789) located at the C-terminus. It has been estimated that about 20% of RPTP $\alpha$  in NIH3T3 cells is phosphorylated on Tyr-789 (den Hertog et al., 1994). The sequence on the C-terminal side of Tyr-789 (Y<sup>789</sup>ANF) fits the consensus binding site for the SH2 domain of adaptor protein Grb2 and Src family kinases (Songyang et al., 1993). Although Tyr-789 is not involved in the regulation of the intrinsic phosphatase activity of RPTP $\alpha$  (Zheng et al., 2000), a body of evidence indicates that phosphorylation of Tyr-789 negatively regulates Grb2-mediated signaling (den Hertog et al.,

1994; Su et al., 1996). Moreover, phosphorylation of Tyr-789 is required for RPTP $\alpha$  to dephosphorylate a negative regulatory site (Tyr-529 in mammalian Src) in Src C-terminus through a displacement of the phosphorylated Tyr-529 from the Src SH2 domain (Zheng et al., 2000). The regulation of Tyr-789 phosphorylation is not clear, although a previous study showed that Tyr-789 was an autodephosphorylation site and that co-expression of RPTP $\alpha$  with Src enhanced Tyr-789 phosphorylation in 293 cells (den Hertog et al., 1994).

Reactive oxygen species (ROS), such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide (O<sub>2</sub><sup>-</sup>), and hydroxyl radical (OH $\cdot$ ), are constantly produced in human body under physiological and pathophysiological conditions and are involved in the pathogenesis of cardiovascular diseases, cancer and Alzheimer diseases (Dreher and Junod, 1996; Knight, 1997; Madamanchi et al., 2005). PTPs are emerging as important redox sensors in cells. PTPs contain a catalytically essential cysteine residue in the signature active site motif, HCXXGXXR(S/T), which has a low pK<sub>a</sub> and can be reversibly oxidized by ROS to inactivate PTPs (Rhee et al., 2000). Recent studies have shown that RPTP $\alpha$  can be oxidized and inhibited by H<sub>2</sub>O<sub>2</sub> (Blanchetot et al., 2002). However, it is not known whether oxidative stress could regulate the phosphorylation of Tyr-789, a critical residue for RPTP $\alpha$  signaling. In the present study, we have reported a novel finding that H<sub>2</sub>O<sub>2</sub> at a low to moderate concentration (50-250  $\mu$ M) markedly suppresses the phosphorylation of RPTP $\alpha$  Tyr-789 in various cultured cells. Furthermore, our data suggest that inhibition of a Src/Yes/Fyn-independent tyrosine kinase may be involved in the suppression of Tyr-789 phosphorylation by H<sub>2</sub>O<sub>2</sub>.

## Materials and Methods

**Reagents.** H<sub>2</sub>O<sub>2</sub> was from Sigma (St. Louis, MO) and Fisher Scientific (Houston, TX), respectively. Inhibitors for protein kinase C (PKC) and tyrosine kinase were from EMD Calbiochem (San Diego, CA). Reagents for chemiluminescence detection were from Cell Signaling (Beverly, MA).

**Antibodies.** Antibodies against phospho-RPTP $\alpha$  (Tyr-789) and Tyr-410-phosphorylated p130Crk-associated substrate (p130Cas) were from Cell Signaling (Beverly, MA). Phospho-Src (Tyr-418) and phospho-Abl (Tyr-412) antibodies were from Biosource (Camarillo, CA). RPTP $\alpha$  antibody was from Upstate Signaling Solutions (Charlottesville, VA). Antibodies against Src family kinases (SRC-2) and PKC $\delta$  were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Culture.** Human aortic endothelial cells (HAECs), human umbilical vein endothelial cells (HUVECs), and bovine aortic endothelial cells were from Cambrex Bio Science (Walkersville, MD); they were cultured in EGM-2 or EGM-MV medium and used for experiments within 10 passages. SYF (deficient for Src, Yes, and Fyn) cells and 293 human embryonic kidney cells were from American Type Culture Collection (Manassas VA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Murine embryonic E6-RPTP $\alpha^{+/+}$  and E3-RPTP $\alpha^{-/-}$  fibroblasts (Su et al., 1999) were kindly provided by Dr. Jan Sap (University of Copenhagen, Denmark) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

**Cell Transfection.** SrcY529F (Tyr-529 mutation to Phe) (Polte and Hanks, 1997) was kindly provided by Dr. Steven K. Hanks (Vanderbilt University, TN), and v-Src (Wilkerson et al., 1985) was kindly provided by Dr. J. Thomas Parsons (University of Virginia Health System, VA). Transient expression of these constructs was performed using FuGENE 6 (Roche) according to the manufacture's protocol.

**Immunoblotting.** Immunoblotting was performed essentially as we described previously (Tang et al., 2000). Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed on ice in Nonidet P-40 lysis buffer (25 mM Tris-HCl, pH 7.5, 1% NP-40, 150 mM NaCl, 10 mM NaF, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml each of leupeptin and aprotinin). The extract was clarified by centrifugation. Whole cell lysates were subjected to SDS- PAGE and transferred to polyvinylidene difluoride membrane. The membrane was probed with various primary antibodies as indicated and detected using the ECL system with horseradish peroxidase-conjugated secondary antibodies according to the manufacturer's protocol.

**PTPs Assay.** Cells were washed twice with ice-cold PBS and then lysed on ice in PTP lysis buffer (25 mM sodium acetate, pH 5.5, 1% NP-40, 150 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml each of leupeptin and aprotinin). The phosphatase activity was measured essentially as we described recently (Tang et al., 2005). Briefly, the synthetic peptide Raytide (Oncogene) was labeled at its tyrosine residue using [ $\gamma$ -<sup>32</sup>P]ATP and Src tyrosine kinase. RPTP $\alpha$  immunoprecipitates were mixed with <sup>32</sup>P-labeled Tyr-Raytide in 50 µl of phosphatase reaction buffer (25 mM Hepes, pH 7.4, and 5 mM EDTA) and incubated at

37 °C for 15 min. The reaction was terminated by the addition of acidic charcoal mixture (0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM NaH<sub>2</sub>PO<sub>4</sub>, and 4% (w/v) Norit A). After centrifugation in a microcentrifuge, the amount of radioactivity present in the supernatant was determined by scintillation counting. The phosphatase activity was evaluated by the extent of Tyr-Raytide dephosphorylation *in vitro*.

**Statistical Analysis.** All data were expressed as mean ± SEM. Differences between mean values of 2 groups were analyzed by Student *t* tests. Differences between mean values of multiple groups were analyzed by 1-way ANOVA with a Newman-Keuls post hoc analysis. P<0.05 was considered statistically significant.



## Results

**H<sub>2</sub>O<sub>2</sub> Inhibits RPTP $\alpha$  Activity and Suppresses the Phosphorylation of RPTP $\alpha$  on Tyr-789.** H<sub>2</sub>O<sub>2</sub>, the most stable form of ROS, can easily diffuse across the membrane and has been widely used to study the role of ROS in cells (Finkel, 2003). To determine effect of H<sub>2</sub>O<sub>2</sub> on the catalytic activity of RPTP $\alpha$ , RPTP $\alpha$  was immunoprecipitated and phosphatase activity of the immune complexes towards <sup>32</sup>P-labeled Tyr-Raytide was measured. In agreement with a previous report (Blanchetot et al., 2002), we found that the activity of RPTP $\alpha$  was inhibited ~40% by H<sub>2</sub>O<sub>2</sub> at 5 min, then gradually recovered to basal level at 30 min (Fig. 1A). An early study suggests that Tyr-789 is an autodephosphorylation site of RPTP $\alpha$  (den Hertog et al., 1994). If so, inhibition of RPTP $\alpha$  activity by H<sub>2</sub>O<sub>2</sub> should increase the phosphorylation of Tyr-789. We next determined effect of H<sub>2</sub>O<sub>2</sub> on the phosphorylation of Tyr-789, a critical tyrosine for RPTP $\alpha$  signaling, using a phospho-specific antibody against Tyr-789-phosphorylated RPTP $\alpha$  (Cell Signaling). As shown in Fig. 1B, a 135 kDa protein band representing the phosphorylated RPTP $\alpha$  was detected with the phospho-specific antibody in E6-RPTP $\alpha^{+/+}$  but not in E3-RPTP $\alpha^{-/-}$  fibroblasts (Su et al., 1999), indicating that the phospho-RPTP $\alpha$  (Tyr-789) antibody specifically recognizes the phosphorylated RPTP $\alpha$  but not other receptor PTPs. Surprisingly, we found that the phosphorylation of RPTP $\alpha$  Tyr-789 was suppressed 95% by a subcytolytic concentration of H<sub>2</sub>O<sub>2</sub> (250  $\mu$ M) within 10 min and gradually returned to 50% of basal level by 120 min in E6 fibroblasts (Fig 1B). The time-dependent suppression of the phosphorylation of Tyr-789 by H<sub>2</sub>O<sub>2</sub> was also observed in HAECs and HUVECs, but with a lower recovery rate compared with E6 fibroblasts (Fig. 1C and 1D). We also found that the phosphorylation of RPTP $\alpha$  Tyr-789 was markedly suppressed by H<sub>2</sub>O<sub>2</sub> in bovine aortic endothelial cells and 293 cells (data not shown).

Moreover, we found that the H<sub>2</sub>O<sub>2</sub>-induced suppression of Tyr-789 phosphorylation was blocked by an antioxidant N-acetyl cysteine (Ferrari et al., 1995) (Fig. 1E). Since RPTP $\alpha$  associates with Grb2 through binding of the phosphorylated Tyr-789 to Grb2 SH2 domain (den Hertog et al., 1994), we then determined the effect of H<sub>2</sub>O<sub>2</sub> on the association of RPTP $\alpha$  with Grb2. As shown in Fig. 1F, treatment of HUVECs with H<sub>2</sub>O<sub>2</sub> (250  $\mu$ M) caused a rapid dissociation of RPTP $\alpha$  from Grb2, further confirming the inhibitory effect of H<sub>2</sub>O<sub>2</sub> on Tyr-789 phosphorylation. These findings suggest that the phosphorylation of Tyr-789 by a kinase may be dominant over any Tyr-789 autodephosphorylation and that the tyrosine kinase may be inhibited by H<sub>2</sub>O<sub>2</sub> through a redox regulatory mechanism in cells.

We next determined the dose-dependent effect of H<sub>2</sub>O<sub>2</sub> using the phospho-RPTP $\alpha$  (Tyr-789) antibody. As shown in Fig. 2A, the Tyr-789 phosphorylation was suppressed 60% by 10 min treatment of E6 fibroblasts with as low as 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, 93% by 250  $\mu$ M H<sub>2</sub>O<sub>2</sub>, and 91% by 500  $\mu$ M H<sub>2</sub>O<sub>2</sub>, respectively. A similar dose-dependent inhibitory effect of H<sub>2</sub>O<sub>2</sub> on Tyr-789 phosphorylation was observed in HAECs (Fig. 2B). Thus, the maximal suppression of Tyr-789 phosphorylation can be achieved by 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> in both cells.

**PP1-sensitive but Src/Yes/Fyn-independent Phosphorylation of RPTP $\alpha$  on Tyr-789.** We next characterized the phosphorylation of RPTP $\alpha$  Tyr-789. As shown in Fig. 3A, the phosphorylation of Tyr-789 was inhibited 80% by H<sub>2</sub>O<sub>2</sub>, 85% by a Src kinase inhibitor PP1 (Hanke et al., 1996), and 70% by a PKC $\delta$  inhibitor rottlerin (Gschwendt et al., 1994) in HAECs, respectively. In contrast, a general PKC inhibitor GF109203X or a membrane-permeable calcium chelator BAPTA/AM had virtually no effect. Furthermore, the H<sub>2</sub>O<sub>2</sub>-induced

suppression of Tyr-789 phosphorylation was slightly enhanced by both PP1 and rottlerin in HAECs (Fig. 3B).

Although PP1 is an inhibitor of Src kinase (Hanke et al., 1996), it has been shown that PP1 also inhibits Kit and Bcr-Abl tyrosine kinases (Tatton et al., 2003). As shown in Fig. 4A, the phosphorylation of RPTP $\alpha$  Tyr-789 was inhibited 88% by PP1, whereas the phosphorylation of Src activation loop conserved tyrosine (Tyr-418 in mammalian Src) that represents Src activation (Yamaguchi and Hendrickson, 1996) was inhibited only 40% by PP1 in HAECs, suggesting involvement of a Src-independent mechanism for Tyr-789 phosphorylation. Interestingly, the phosphorylation of RPTP $\alpha$  Tyr-789 and Src Tyr-418 was inhibited 82% and 90% by H<sub>2</sub>O<sub>2</sub> in HAECs, respectively (Fig. 4A), which may result from the H<sub>2</sub>O<sub>2</sub>-induced inhibition of a novel kinase other than Src as we hypothesized (Tang et al., 2005). To determine whether Src kinase is involved in the phosphorylation of RPTP $\alpha$  Tyr-789, HAECs were transiently transfected with SrcY529F (Polte and Hanks, 1997), a constitutively activated form of Src, and lysates were immunoblotted with the phospho-RPTP $\alpha$  (Tyr-789) antibody. As shown in Fig. 4A, transient expression of the SrcY529F mutant had virtually no effect on the phosphorylation of Tyr-789 in HAECs, although it apparently enhanced (2.65-fold) Src activation detected with a phospho-Src (Tyr-418) antibody that recognizes the activated form of Src (Biosource). We next performed similar experiments using 293 cells that can be transfected with a high efficiency. As shown in Fig. 4B, transient expression of v-Src (Wilkerson et al., 1985) virtually did not affect the phosphorylation of RPTP $\alpha$  Tyr-789 in 293 cells, although it apparently enhanced (4-fold) Src activation detected with the phospho-Src (Tyr-418) antibody (Biosource). Furthermore, activation of Src to an extremely high level (>30-fold) with expression of the SrcY529F mutant only slightly (1.3-fold) increased the phosphorylation of RPTP $\alpha$  Tyr-789 in 293 cells (Fig. 4B).

Remarkably, we found that RPTP $\alpha$  Tyr-789 was strongly phosphorylated in SYF cells lacking all three ubiquitously expressed members (Src, Yes and Fyn) of Src family kinases (Klinghoffer et al., 1999) (Fig. 4C). Reintroducing the activated Src (SrcY529F) into SYF cells only slightly (1.2-fold) increased the phosphorylation of RPTP $\alpha$  Tyr-789, whereas it markedly augmented the phosphorylation of p130Cas Tyr-410, a Src phosphorylation site (Tang et al., 2005). The phosphorylation of p130Cas Tyr-410 was unable to detect in vector-transfected (control) SYF cells (Fig. 4C). In addition, we found that the phosphorylation of RPTP $\alpha$  Tyr-789 was suppressed 51% by genistein (Spinozzi et al., 1994), a general inhibitor for protein tyrosine kinases, 70% by PP1, but only 10% by another Src kinase inhibitor herbimycin A (Ogino et al., 2004) in SYF cells, respectively (Fig. 4D). In contrast, piceatannol (Oliver et al., 1994), a Syk tyrosine kinase inhibitor, AG-1295 (Levitzki and Gazit, 1995), a selective inhibitor of platelet-derived growth factor receptor, AG-1478 (Levitzki and Gazit, 1995), a selective inhibitor of epidermal growth factor receptor, GTP-14564 (Murata et al., 2003), a selective inhibitor of class III receptor tyrosine kinases including Kit, and terreic acid (Kawakami et al., 1999), a selective inhibitor of Bruton's tyrosine kinase, did not significantly affect the phosphorylation of Tyr-789 in SYF cells (Fig. 4D and 4E). Interestingly, we found that AG-957 (Anafi et al., 1992), a potent inhibitor of Bcr-Abl and c-Abl, caused a mobility shift of RPTP $\alpha$  and apparently suppressed the phosphorylation of Tyr-789 in a dose-dependent manner in SYF cells (Fig. 4E). Taken together, these findings demonstrated that, in contrast to Tyr-410 of p130Cas, Tyr-789 of RPTP $\alpha$  is not a primary target site of Src kinase. Thus, the phosphorylation of RPTP $\alpha$  Tyr-789 is predominantly regulated by a PP1-sensitive but Src/Yes/Fyn-independent tyrosine kinase in cells.

### **H<sub>2</sub>O<sub>2</sub> Suppresses the Phosphorylation of RPTP $\alpha$ Tyr-789 in Src/Yes/Fyn-deficient SYF**

**Cells.** Consistent with the data obtained using fibroblasts and endothelial cells (Fig. 1), we found that the phosphorylation of RPTP $\alpha$  Tyr-789 was also markedly suppressed by H<sub>2</sub>O<sub>2</sub> in SYF cells lacking all three ubiquitously expressed members (Src, Yes and Fyn) of Src family kinase (Fig. 5A). The phosphorylation of Tyr-789 was inhibited 85% by H<sub>2</sub>O<sub>2</sub> at 10 minutes, then gradually recovered to near basal level at 60 min. Of note, the phosphorylation of c-Abl on the kinase activation loop Tyr-412 (Brasher and Van Etten, 2000), which represents c-Abl activation, was not altered by H<sub>2</sub>O<sub>2</sub> treatment in SYF cells (Fig.5A, third panel). Interestingly, the H<sub>2</sub>O<sub>2</sub>-induced suppression of Tyr-789 phosphorylation was mimicked by a PTP inhibitor phenylarsine oxide (PAO) (Garcia-Morales et al., 1990), but not by the serine/threonine phosphatase inhibitor NaF, in SYF cells (Fig. 5B). Furthermore, pretreatment of SYF cells with PAO enhanced the suppression of Tyr-789 phosphorylation by H<sub>2</sub>O<sub>2</sub>. These findings suggest that a novel tyrosine kinase (but not Src, Yes, Fyn, or c-Abl) phosphorylating RPTP $\alpha$  Tyr-789 may be inhibited by H<sub>2</sub>O<sub>2</sub> treatment in cells and that the inhibition of a PTP(s) may be involved in the process.

## Discussion

In the present study, we have characterized the phosphorylation of RPTP $\alpha$  on Tyr-789 using different approaches and obtained substantial evidence indicating that the phosphorylation of RPTP $\alpha$  Tyr-789 is regulated by a PP1-sensitive but Src/Yes/Fyn-independent tyrosine kinase in cells. We further show that, besides the inhibition of RPTP $\alpha$  activity, H<sub>2</sub>O<sub>2</sub> at low to moderate levels (50-250  $\mu$ M) markedly suppresses the phosphorylation of Tyr-789 and the association of RPTP $\alpha$  with Grb2 in cultured cells, which may be through inhibition of such a Src/Yes/Fyn-independent tyrosine kinase. Since Tyr-789 plays an important role in RPTP $\alpha$  signaling, our findings may provide new insights into the functional regulation of RPTP $\alpha$  by H<sub>2</sub>O<sub>2</sub>, a stable form of ROS.

It has been demonstrated that phosphorylation of RPTP $\alpha$  Tyr-789 modulates Grb2-mediated signaling and the activation of Src family kinases, although Tyr-789 is not involved in the regulation of the intrinsic phosphatase activity of RPTP $\alpha$  (den Hertog et al., 1994; Su et al., 1996; Zheng et al., 2000). However, the regulation of RPTP $\alpha$  Tyr-789 phosphorylation remains unclear. An early study suggests that Tyr-789 is an autodephosphorylation site of RPTP $\alpha$  (den Hertog et al., 1994). If so, inhibition of RPTP $\alpha$  activity should increase the phosphorylation of Tyr-789. Surprisingly, we found that besides the inhibition of RPTP $\alpha$  activity, H<sub>2</sub>O<sub>2</sub> markedly suppressed the phosphorylation of Tyr-789 in cells, leading to the dissociation of RPTP $\alpha$  from Grb2. These data suggest that the phosphorylation of Tyr-789 by a tyrosine kinase may be dominant over any Tyr-789 autodephosphorylation and that the tyrosine kinase may be inhibited by H<sub>2</sub>O<sub>2</sub>. A model for the RPTP $\alpha$ -mediated Src activation describes that binding of the

phosphorylated Tyr-789 to Src SH2 domain induces displacement of the Src negative regulatory site (Tyr-529) from its SH2 domain, which facilitates the dephosphorylation of Tyr-529 by RPTP $\alpha$  and thereby promotes Src activation (Zheng et al., 2000). If the phosphorylation of Tyr-789 is mediated by Src in cells as suggested by a early study in which co-expression of RPTP $\alpha$  with Src enhanced Tyr-789 phosphorylation in 293 cells (den Hertog et al., 1994), the activated Src will phosphorylate RPTP $\alpha$  Tyr-789 and the phosphorylated Tyr-789 will bind to Src SH2 domain to activate Src, thereby causing hyperactivation of Src and hyperphosphorylation of RPTP $\alpha$  through cycles of the positive-feedback loop. Apparently, this is against the fact that only a small fraction of Src is activated in cells, suggesting involvement of a Src-independent mechanism for Tyr-789 phosphorylation. In the present study, we have provided substantial evidence indicating that the phosphorylation of RPTP $\alpha$  Tyr-789 is regulated predominantly by a Src/Yes/Fyn-independent tyrosine kinase. Transient expression of SrcY529F (Polte and Hanks, 1997), a constitutively activated form of Src, or v-Src (Wilkerson et al., 1985), which led to a moderate Src activation (2.6-4 fold), had virtually no effect on the phosphorylation of Tyr-789 in HAECs and 293 cell, respectively. The phosphorylation of Tyr-789 was only slightly increased (1.3-fold) in 293 cells when Src was activated to an extremely high level (>30-fold) with expression of SrcY529F mutant. It should be noted that RPTP $\alpha$  Tyr-789 was strongly phosphorylated in SYF fibroblasts lacking Src, Yes and Fyn (Klinghoffer et al., 1999). Furthermore, reintroducing the activated Src (SrcY529F) into SYF cells only slightly (1.2-fold) increased the phosphorylation of RPTP $\alpha$  Tyr-789, whereas it remarkably augmented the phosphorylation of a Src substrate p130Cas on Tyr-410 that was not detected in control SYF cells. These findings demonstrated that, in contrast to Tyr-410 of p130Cas, the Tyr-789 of RPTP $\alpha$  is not a primary target site of Src family kinases in cells. It is likely that the

phosphorylation of RPTP $\alpha$  Tyr-789 is regulated predominantly by a Src/Yes/Fyn-independent tyrosine kinase. Nine members of Src family kinases have been identified to date. In contrast to Src, Yes and Fyn that are expressed ubiquitously, Hck, Fgr, Blk, Yrk and Lck are restricted to hematopoietic cell lineages, Lyn is expressed in both hematopoietic and neuronal cells (Brown and Cooper, 1996). The defective integrin signaling phenotype of the SYF cells also argues against expression of other cell type-specific Src family members in SYF cells (Klinghoffer et al., 1999). Indeed, no band was detected in SYF fibroblasts by using a SRC-2 antibody that recognizes the C-terminus of Src family kinases (data not shown). It seems that a non-Src family kinase may be responsible for the phosphorylation of RPTP $\alpha$  Tyr-789.

Interestingly, we found that the phosphorylation RPTP $\alpha$  Tyr-789 was profoundly inhibited by a Src kinase inhibitor PP1 (Hanke et al., 1996) in HAECs and even in SYF cells lacking Src family kinases, but was not affected by another Src kinase inhibitor herbimycin A (Ogino et al., 2004) in SYF cells. Of note, the phosphorylation of RPTP $\alpha$  Tyr-789 was inhibited 88% by PP1, whereas the phosphorylation of Src activation loop conserved tyrosine Tyr-418 that represents Src activation (Yamaguchi and Hendrickson, 1996) was inhibited only 40% by PP1 in HAECs, suggesting involvement of a Src-independent mechanism. Indeed, PP1 also inhibits other tyrosine kinases, including Kit and Bcr-Abl tyrosine kinases (Tatton et al., 2003). We found that AG-957 (Anafi et al., 1992), a potent inhibitor of Bcr-Abl and c-Abl, but not GTP-14564 (Murata et al., 2003), a selective inhibitor of class III receptor tyrosine kinases including Kit, markedly inhibited the phosphorylation of Tyr-789 in SYF cells through a distinct mechanism from PP1 by causing a mobility shift of RPTP $\alpha$ . Whether c-Abl is directly involved in Tyr-789 phosphorylation remains to be investigated. Moreover, the pharmacological studies revealed that platelet-derived growth factor receptor kinase, epidermal growth factor receptor kinase, Syk and



Bruton's tyrosine kinases may not be involved in the phosphorylation of RPTP $\alpha$  Tyr-789. These findings suggest that a PP1-sensitive but Src-independent tyrosine kinase (probably not Kit) may be responsible for the phosphorylation of RPTP $\alpha$  Tyr-789. This notion was further supported by the finding that the phosphorylation of Tyr-789 was inhibited by a general tyrosine kinase inhibitor genistein in SYF cells. In addition, we found that the phosphorylation of RPTP $\alpha$  Tyr-789 was also sensitive to rottlerin in cells. Although rottlerin is originally identified as an inhibitor of PKC $\delta$  (Gschwendt et al., 1994), it readily suppresses cellular ATP levels (Soltoff, 2001). We found that neither PKC $\delta$  wild type nor the kinase-dead mutant (kindly provided by Dr. Trevor Biden) affected the phosphorylation of RPTP $\alpha$  Tyr-789, indicating that the phosphorylation of Tyr-789 is regulated by rottlerin in a PKC $\delta$ -independent mechanism (data not shown).

RPTP $\alpha$  that can be oxidized and inhibited by H<sub>2</sub>O<sub>2</sub> is emerging as an important redox sensor in cells (Blanchetot et al., 2002). However, it is not known whether oxidative stress could regulate the phosphorylation of Tyr-789, a critical residue for RPTP $\alpha$  signaling that modulates the function of Grb2 and the activation of Src family kinases. In the present study, we reported a novel finding that, besides the inhibition of RPTP $\alpha$  activity, H<sub>2</sub>O<sub>2</sub> at low to moderate concentrations (50-250  $\mu$ M) markedly suppressed the phosphorylation of RPTP $\alpha$  Tyr-789 in endothelial cells, E6 fibroblasts, and Src/Yes/Fyn-deficient SYF cells. Remarkably, the phosphorylation of Tyr-789 was inhibited 65% by 5 min treatment of HAECs with as low as 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> and was almost abolished by 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> in all cells examined. Thus, H<sub>2</sub>O<sub>2</sub> suppresses RPTP $\alpha$  signaling not only through inhibition of its catalytic activity but also through a reduction in the phosphorylation of Tyr-789. Because the phosphorylation of Tyr-789 is predominantly regulated by a PP1-sensitive but Src/Yes/Fyn-independent tyrosine kinase, it

seems that such a tyrosine kinase phosphorylating RPTP $\alpha$  Tyr-789 may be inhibited by H<sub>2</sub>O<sub>2</sub>, leading to a reduction in Tyr-789 phosphorylation. Moreover, our data suggest that inhibition of a PTP(s) may be involved in the process since the H<sub>2</sub>O<sub>2</sub>-induced suppression of Tyr-789 phosphorylation was mimicked by a PTP inhibitor PAO (Garcia-Morales et al., 1990). The hypothesis merits further investigation.

In summary, we have obtained substantial evidence indicating that H<sub>2</sub>O<sub>2</sub> suppresses the phosphorylation of RPTP $\alpha$  on a Src-independent phosphorylation site Tyr-789, which may offer new insights into the redox regulation of RPTP $\alpha$  signaling.

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### Footnotes

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### Legends for figures

**Fig. 1.** H<sub>2</sub>O<sub>2</sub> inhibits RPTP $\alpha$  activity and suppresses the phosphorylation of RPTP $\alpha$  on Tyr-789 in various cultured cells. A, time-dependent effect of H<sub>2</sub>O<sub>2</sub> (250  $\mu$ M) on the catalytic activity of RPTP $\alpha$  in HUVECs. The phosphatase activity was evaluated by the extent of <sup>32</sup>Pi release from <sup>32</sup>P-labeled Tyr-Raytide. Values are mean  $\pm$  SEM. \*P<0.05 vs control. B-D, E6-RPTP $\alpha$ <sup>+/+</sup> and E3-RRPTP $\alpha$ <sup>-/-</sup> fibroblasts (B), HAECs (C), and HUVECs (D) were treated with H<sub>2</sub>O<sub>2</sub> (250  $\mu$ M) for the indicated time periods. E, E6-RPTP $\alpha$ <sup>+/+</sup> cells were left untreated (control) or pretreated with N-acetyl cysteine (NAC, 6 mM) for 1 h and then incubated without or with H<sub>2</sub>O<sub>2</sub> (250  $\mu$ M) for 10 min. Lysates from B to E were subjected to immunoblotting (IB) with RPTP $\alpha$  (pY789) antibody that recognizes Tyr-789-phosphorylated RPTP $\alpha$ . The phosphorylation of Tyr-789 is also shown as percentage of untreated individual control cells determined by densitometric analysis from three independent experiments. The same blot was stripped and reprobed with a RPTP $\alpha$  antibody to show the equal loading. F, HUVECs were treated without (-) or with (+) H<sub>2</sub>O<sub>2</sub> (250  $\mu$ M) for 10 min. RPTP $\alpha$  was immunoprecipitated (IP) and subjected to immunoblotting with Grb2 or RPTP $\alpha$  antibodies. Representative immunoblots of three (B-E) or two (F) independent experiments are shown.

**Fig. 2.** Dose-dependent suppression of RPTP $\alpha$  Tyr-789 phosphorylation by H<sub>2</sub>O<sub>2</sub>. E6-RPTP $\alpha$ <sup>+/+</sup> fibroblasts (A) or HAECs (B) were treated with various concentrations of H<sub>2</sub>O<sub>2</sub> for 10 min, and lysates were subjected to immunoblotting (IB) with RPTP $\alpha$  (pY789) antibody. The phosphorylation of Tyr-789 is also shown as percentage of untreated control cells determined by

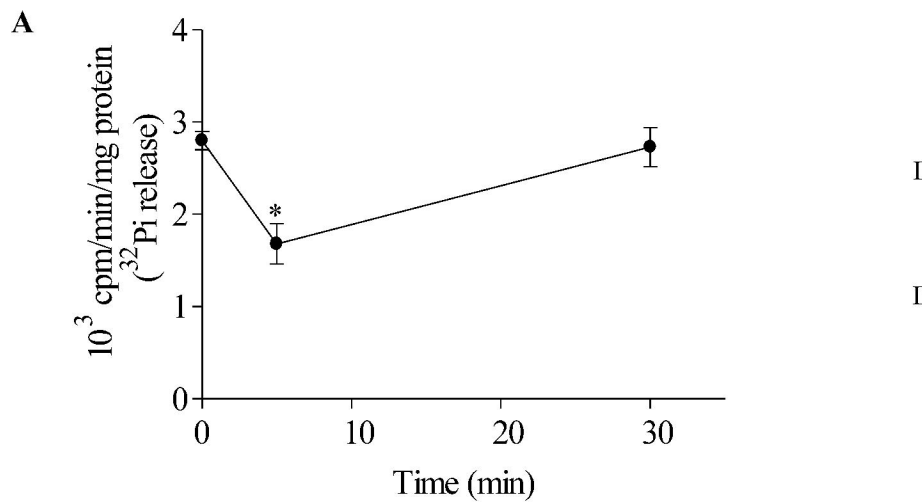
densitometric analysis. The same blot was stripped and reprobed with a RPTP $\alpha$  antibody to show the equal loading. Representative immunoblots of three independent experiments are shown.

**Fig. 3.** PP1-sensitive phosphorylation of RPTP $\alpha$  on Tyr-789. A, HAECs were treated 15 min with H<sub>2</sub>O<sub>2</sub> (250  $\mu$ M) or 30 min with PP1 (10  $\mu$ M), GFX109203X (GFX, 10  $\mu$ M), rottlerin (10  $\mu$ M), or BAPTA-AM (50  $\mu$ M). B, HAECs were left untreated (-) or pretreated with various inhibitors as shown in Fig. 3A and then incubated without (-) or with H<sub>2</sub>O<sub>2</sub> (250  $\mu$ M) for 15 min. Lysates were subjected to immunoblotting (IB) with RPTP $\alpha$  (pY789) antibody. The phosphorylation of Tyr-789 is also shown as average percentage of untreated control cells determined by densitometric analysis from two independent experiments. The same blot was stripped and reprobed with a RPTP $\alpha$  antibody to show the equal loading. Representative immunoblots of two independent experiments are shown.

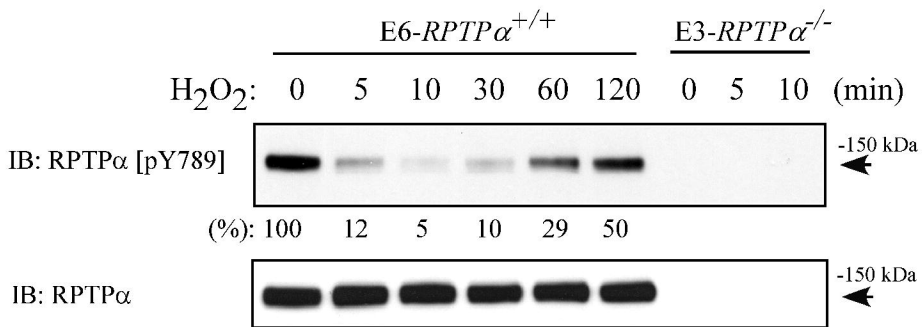
**Fig. 4.** PP1-sensitive but Src/Yes/Fyn-independent phosphorylation of RPTP $\alpha$  on Tyr-789. A, HAECs were transiently transfected with vector alone or SrcY529F mutant and then treated without or with PP1 (10  $\mu$ M) for 30 min or H<sub>2</sub>O<sub>2</sub> (250  $\mu$ M) for 10 min. B and C, 293 cells or SYF cells were transiently transfected with vector alone, v-Src or SrcY529F as indicated. D and E, SYF cells were left untreated (control) or treated for 30 min with PP1 (10  $\mu$ M), herbimycin A (10  $\mu$ M), AG-1295 (50  $\mu$ M), AG-1478 (250 nM), genistein (300  $\mu$ M), peceatannol (50  $\mu$ M), GTP-14564 (1 or 5  $\mu$ M), AG-957 (10 or 20  $\mu$ M), or terreic acid (50  $\mu$ M) as indicated. Lysates from A to E were subjected to immunoblotting (IB) with antibodies against RPTP $\alpha$  (pY789), Src (pY418), Cas (pY410) or RPTP $\alpha$ . The phosphorylation of RPTP $\alpha$  Tyr-789 or Src Tyr-418 is also shown as mean percentage or fold over untreated control cells determined by densitometric

analysis from three independent experiments. Representative immunoblots of three independent experiments are shown. \*P<0.05 vs control.

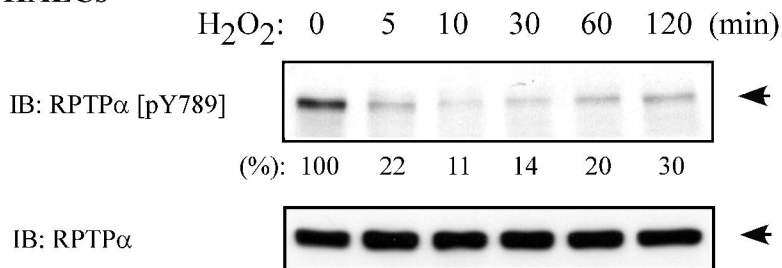
**Fig. 5.** H<sub>2</sub>O<sub>2</sub> suppresses the phosphorylation of RPTP $\alpha$  Tyr-789 in SYF Cells lacking Src/Yes/Fyn. A, SYF cells lacking Src/Yes/Fyn were treated with H<sub>2</sub>O<sub>2</sub> (250  $\mu$ M) for the indicated time periods. B, SYF cells were left untreated (-) or treated 10 min with H<sub>2</sub>O<sub>2</sub> (250  $\mu$ M) or 20 min with PAO (20  $\mu$ M) or NaF (10 mM). PAO+H<sub>2</sub>O<sub>2</sub> indicates that SYF cells were pretreated 10 min with PAO and then incubated with H<sub>2</sub>O<sub>2</sub> (250  $\mu$ M) for 10 min in the presence of PAO. Lysates were subjected to immunoblotting (IB) with RPTP $\alpha$  (pY789) or Abl (pY412) antibodies. The phosphorylation of Tyr-789 is also shown as mean percentage of untreated control cells determined by densitometric analysis from three independent experiments. The same blot was stripped and reprobed with a RPTP $\alpha$  antibody to show the equal loading. Representative immunoblots are shown.



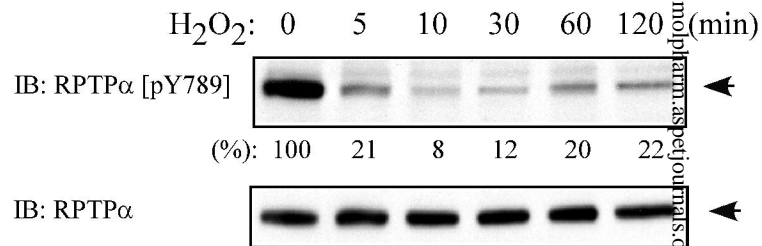
**B: E6/E3 fibroblasts**



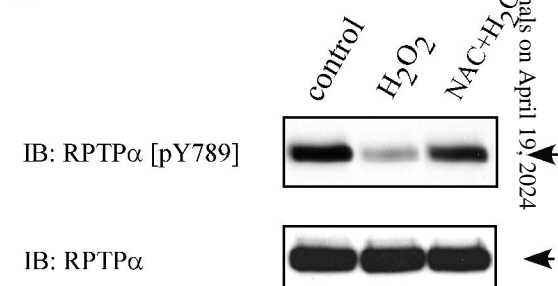
**C: HAECs**



**D: HUVECs**



**E**



**F**

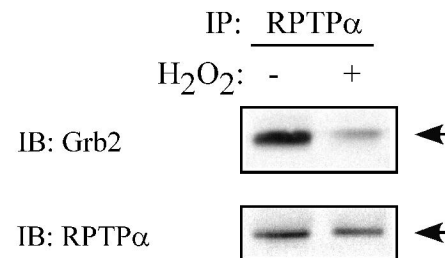
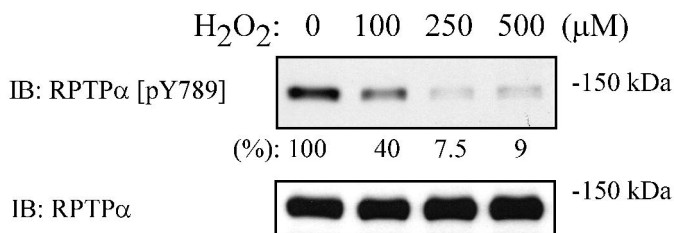


Figure 1

### A: E6 fibroblasts



### B: HAECs

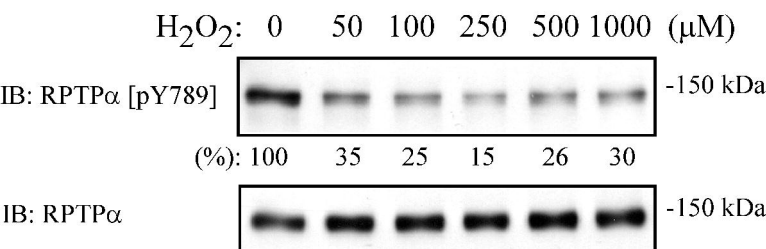


Figure 2

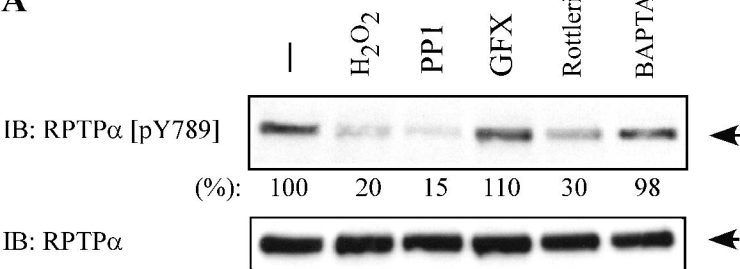
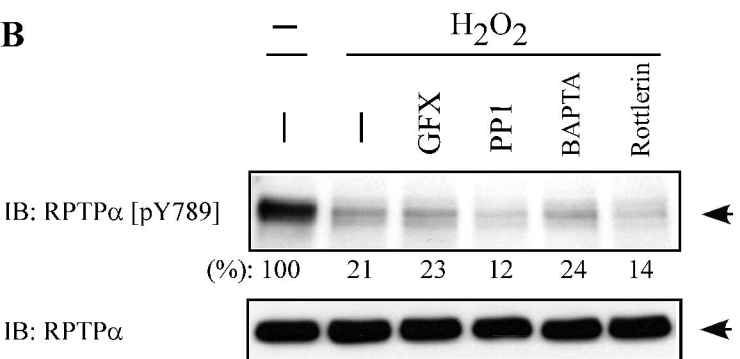
**A****B**

Figure 3

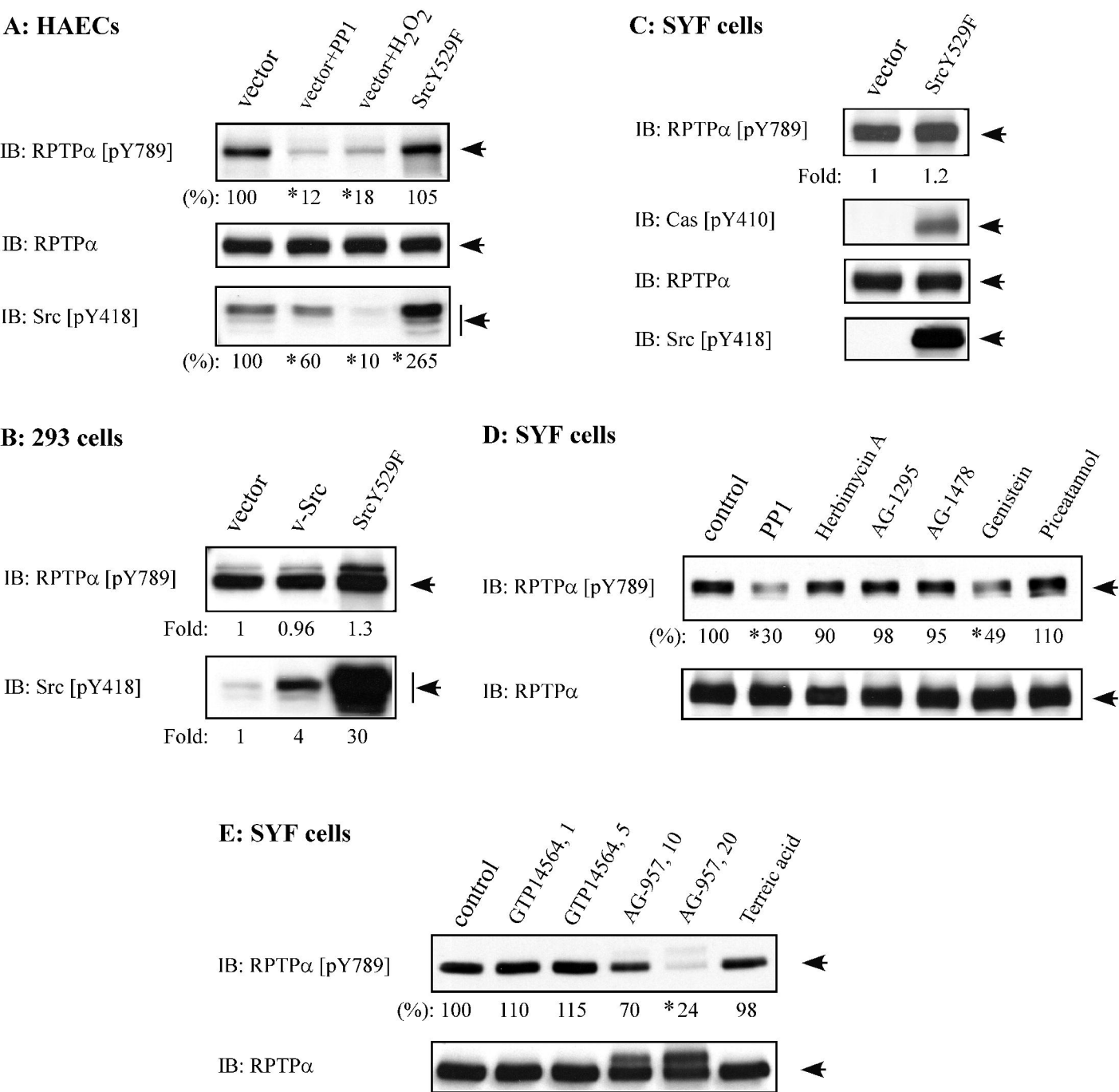


Figure 4

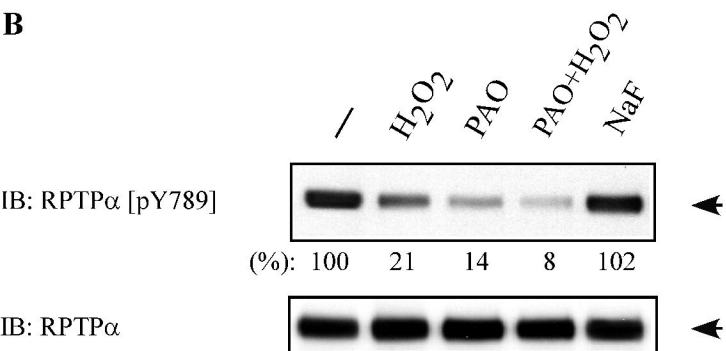
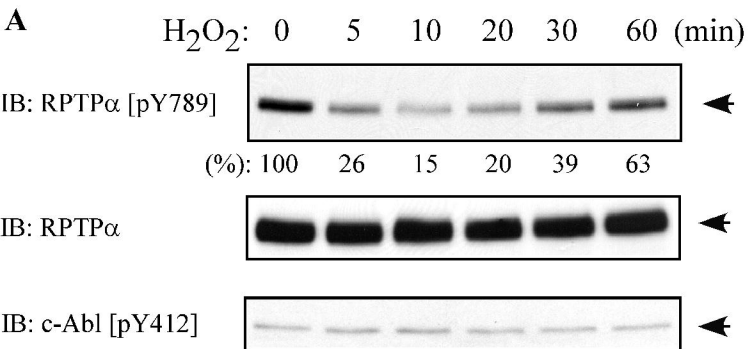


Figure 5