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Regulation of Insulin Signaling through Reversible Oxidation of the Protein-tyrosine Phosphatases TC45 and PTP1B*

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Many studies have illustrated that the production of reactive oxygen species (ROS) is important for optimal tyrosine phosphorylation and signaling in response to diverse stimuli. Protein-tyrosine phosphatases (PTPs), which are important regulators of signal transduction, are exquisitely sensitive to inhibition after generation of ROS, and reversible oxidation is becoming recognized as a general physiological mechanism for regulation of PTP function. Thus, production of ROS facilitates a tyrosine phosphorylation-dependent cellular signaling response by transiently inactivating those PTPs that normally suppress the signal. In this study, we have explored the importance of reversible PTP oxidation in the signaling response to insulin. Using a modified in-gel PTP assay, we show that stimulation of cells with insulin resulted in the rapid and transient oxidation and inhibition of two distinct PTPs, which we have identified as PTP1B and TC45, the 45-kDa spliced variant of the T cell protein-tyrosine phosphatase. We investigated further the role of TC45 as a regulator of insulin signaling by combining RNA interference and the use of substrate-trapping mutants. We have shown that TC45 is an inhibitor of insulin signaling, recognizing the β-subunit of the insulin receptor as a substrate. The data also suggest that this strategy, using ligand-induced oxidation to tag specific PTPs and using interference RNA and substrate-trapping mutants to illustrate their role as regulators of particular signal transduction pathways, may be applied broadly across the PTP family to explore function.

The reversible phosphorylation of tyrosyl residues in proteins, catalyzed by the coordinated actions of protein tyrosine kinases and protein-tyrosine phosphatases (PTPs),[1] is of paramount importance to the control of such fundamental physiological functions as cell proliferation, differentiation, survival, metabolism, and motility (1, 2). The phosphorylation of a target protein alters its function, including changes in enzymatic activity or its ability to associate with other proteins. In response to a stimulus, such as a growth factor or hormone, multiple phosphorylation and dephosphorylation reactions are coordinated in signal transduction cascades that culminate in the physiological response (3, 4). A characterization of the enzymes responsible for the regulation of protein tyrosine phosphorylation *in vivo* will be essential for an understanding of the control of signal transduction under normal and pathophysiological conditions and would be expected to identify important new targets for therapeutic intervention in human disease. We are focusing on this process from the perspective of the PTP family of enzymes.

A substantial body of information has been accumulated to describe the role of protein tyrosine kinases in the regulation of signal transduction. In contrast, we are only now beginning to appreciate in mechanistic detail the role of some members of the PTP family in fine-tuning the signaling response to extracellular stimuli. Analysis of the human genome sequence revealed the existence of 38 PTP genes in humans (5, 6). These PTPs compose receptor-like proteins, which have the potential to regulate signaling directly through ligand-controlled protein dephosphorylation, as well as nontransmembrane, cytoplasmic enzymes. In addition, there are ~60 dual-specificity phosphatases, which are members of the PTP family that recognize Ser/Thr and Tyr residues in proteins (3).[2] This structural diversity in the family is indicative of their functional importance in the control of cell signaling, and it is now apparent that the PTPs have the potential to display exquisite substrate, and functional, specificity *in vivo*. The substrate specificity of PTPs is determined both by targeting the enzymes to defined subcellular locations, via their noncatalytic domains (7), and through structural features intrinsic to their catalytic domains (8). Moreover, the activity of PTPs themselves is regulated through post-translational modifications in response to extracellular stimuli. The combination of these mechanisms and the control of protein tyrosine kinases govern the tyrosine phosphorylation of PTP substrates in a spatial and time-dependent manner.

Recently, a novel tier of control of tyrosine phosphorylation-dependent signaling, and PTP function in particular, has been revealed. The production of reactive oxygen species (ROS) in response to a diverse array of physiological stimuli is currently viewed as an important mechanism for fine-tuning tyrosine phosphorylation-dependent signaling (9–12). Attention has been drawn to the PTPs as targets of ROS because the signature motif of this family, [I/V]HC*X*X*G*X*X*R[S/T], contains an invariant Cys residue that, because of the unique environment of the PTP active site, is characterized by an extremely low pK_a

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[1] The abbreviations used are: PTP, protein-tyrosine phosphatase; ROS, reactive oxygen species; TC-PTP, T-cell PTP; siRNA, small interfering RNA; RNAi, RNA interference; IAA, iodoacetic acid; DCF, 2′,7′-dichlorofluorescein; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; Nox, NADPH oxidase.

[2] H.-H. Chen, J. Faith, R. Sachidanandam, and N. K. Tonks, unpublished data.

(13, 14). The low pK_a promotes the function of this Cys residue as a nucleophile in catalysis but renders it highly susceptible to oxidation with concomitant inhibition of PTP activity (15–17). It is now known that multiple PTPs are transiently oxidized by H_2O_2 (18–20) and also in response to certain cellular stimuli (17, 21–24), illustrating that this mode of regulation may apply broadly across the enzyme family (25).

In the present study, we have investigated the importance of ROS production and concomitant PTP inhibition in the context of insulin-mediated signal transduction. We demonstrate that ectopic expression of catalase impaired both insulin signaling and tyrosine phosphorylation of the β-subunit of the insulin receptor. Using a modified in-gel PTP assay (19), we observed that two PTPs were rapidly and transiently oxidized in response to insulin. We identified these PTPs as PTP1B and the 45-kDa spliced variant of T-cell PTP (TC-PTP, TC45). We explored further the role of TC45 in regulating insulin-stimulated signal transduction using a combination of RNA interference and substrate-trapping mutant forms of the enzyme. We have demonstrated that suppression of endogenous TC45 expression via RNA interference enhanced phosphorylation of the β-subunit of the insulin receptor and signaling in response to insulin, consistent with removal of a signaling component that exerts an inhibitory constraint on the system. Consistent with this, we observed that substrate-trapping mutant forms of TC45 formed a complex with the β-subunit of the insulin receptor. Our study not only demonstrates that TC45 may function as a regulator of insulin signaling but also illustrates the potential for applying this strategy to the identification and characterization of PTPs that control a broad array of signal transduction pathways.

MATERIALS AND METHODS

Preparation of Small Interfering RNA (siRNA) Oligonucleotides—To design specific siRNA duplexes, we scanned through the open reading frame of TC45 mRNA and selected sequences of 5′-AA(N_{19})-3′ (N = any nucleotide) for further characterization. Two oligonucleotides, 5′-AACAGAUACAGAGAUGUAAGC-3′ (termed TCPTP1) and 5′-AGCCCAUAUGAUCACAGUCG-3′ (TCPTP2), were chosen. These sequences were submitted to a BLAST search against human, rat, and mouse genome databases to ensure specificity for TC-PTP. The 21-nucleotide siRNA duplexes were purchased from Dharmacon Research in a deprotected and desalted form. At 50–100 nM, both siRNA oligonucleotides suppressed expression of endogenous TC45 in HepG2 cells (human hepatocellular carcinoma) and Rat-1 fibroblasts. Data are presented mostly from the use of the TCPTP1 siRNA, but were confirmed with the TCPTP2 siRNA, to illustrate that the observed effects were target-specific rather than siRNA-specific.

Cell Culture and Transient Transfection with Plasmids and siRNA—Rat-1 and HepG2 cells were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. For stimulation with insulin, cells were plated in medium containing 10% fetal bovine serum for 48 h and then were serum-starved for 16 h before treatment. For transient transfection, cells were plated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for 16 h and then in OptiMEM (Invitrogen) without serum, after which the plasmid (5 μg/dish for Rat-1, 30 μg/dish for HepG2) was introduced by LipofectAMINE and PLUS reagents (Invitrogen), according to the manufacturer's recommendations. For RNA interference (RNAi) experiments, cells were plated as above, and the TC-PTP siRNA duplexes were introduced by Oligofectamine (Invitrogen) according to the guidelines provided by Dharmacon Research Inc.

Substrate-trapping, Immunoprecipitation, and Immunoblotting—For substrate-trapping experiments, HepG2 cells ectopically expressing wild-type or substrate-trapping mutant (DA) forms of PTPs were rinsed with ice-cold PBS and then lysed in trapping lysis buffer (20 mM Tris (pH7.4), 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 10 mM iodoacetic acid (IAA), and protease inhibitors (25 μg/ml of aprotinin and leupeptin)). For immunoprecipitation, cells were lysed in lysis buffer containing 20 mM Hepes (pH 7.5), 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 200 μM Na_3VO_4 and protease inhibitors (25 μg/ml of aprotinin and leupeptin). Lysate (1 mg for substrate-trapping experiments, 400 μg for immunoprecipitation, or as indicated in the figure legend for the individual experiment) was incubated with 5 μg of antibody conjugated to protein A/G-Sepharose (Amersham Biosciences) for 2 h at 4 °C. For immunoblotting, aliquots of total lysates (30 μg/sample) or immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose filters, which were incubated with appropriate primary and secondary antibodies, and the specific signals were visualized by the ECL detection system (Amersham Biosciences).

Modified In-gel PTP Activity Assay—After stimulation, cells were lysed under anaerobic conditions in an argon chamber. The lysis buffer comprised 25 mM CH_3COONa, 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, pH 5.5, which had been degassed prior to addition of catalase and superoxide dismutase (both 100 μg/ml), protease inhibitors, and 10 mM IAA. Lysates (25 μg) were subjected to an "in-gel" phosphatase assay (26) using SDS-PAGE gels containing ^{32}P-labeled reduced, carboxamidomethylated and maleylated lysozyme (RCML) as substrate (1.5×10^6 cpm/20 ml of gel solution, ~2 μM p-Tyr). The details of this method are described by Meng and Tonks (27).

RESULTS

Hydrogen Peroxide Mediates Insulin Signaling in Rat-1 Cells—We tested the hypothesis that production of ROS is important for an optimal signal transduction response to insulin. Rat-1 cells were preloaded with a ROS indicator, H_2DCFDA, which, upon encountering ROS, such as H_2O_2, in cells is oxidized, thereby converting it to the fluorescent derivative 2′,7′-dichlorofluorescein (DCF). The data shown in Fig. 1*A* illustrate that treatment of Rat-1 cells with insulin triggered production of ROS, as observed by the increased fluorescence resulting from the generation of DCF. Furthermore, ectopic expression of catalase, to suppress the production of intracellular H_2O_2, abrogated the generation of DCF fluorescence in response to insulin. This observation indicates that H_2O_2 is a major constituent of the ROS produced in the cells in response to insulin stimulation. In conjunction with this approach, we assessed the importance of insulin-mediated ROS production on phosphorylation of tyrosyl residues in the activation loop of the β-subunit of the insulin receptor and downstream signaling to the protein kinase PKB/AKT. Transfectants expressing different levels of exogenous catalase (Fig. 1*B*) were exposed to insulin for 10 min, after which the extent of phosphorylation of the tandem tyrosine residues ($pYpY^{1162/1163}$) in the activation loop of the insulin receptor β-subunit and the phosphorylation of PKB/AKT were analyzed by immunoblotting with appropriate phospho-specific antibodies. Both the tyrosine phosphorylation of the insulin receptor and the activation of PKB/AKT were inhibited in the presence of exogenous catalase in a dose-dependent fashion (Fig. 1*B*). These data suggest that H_2O_2 production in response to insulin is important for an optimal signaling response to the hormone.

Insulin Stimulation Leads to Reversible Oxidation and Inactivation of PTP1B and TC45—We examined the effect of insulin-induced H_2O_2 production on PTP oxidation using a modified in-gel PTP assay (19, 27). Control and insulin-stimulated Rat-1 cells were lysed under anaerobic conditions in the presence of IAA, which led to irreversible alkylation of the catalytic Cys residue of any PTPs in the lysate that were in the reduced, active form. In contrast, any PTPs that had been oxidized in response to insulin-induced production of H_2O_2 were protected from alkylation by IAA. In the assay, an aliquot of lysate was subjected to SDS-PAGE in a gel that was cast to contain a radioactively labeled substrate, and the proteins in the gel were sequentially denatured and then renatured in the presence of reducing agent. Under these conditions, only the activity of those PTPs that were susceptible to insulin-induced oxidation was recovered and visualized by the appearance of a clear, white area of dephosphorylation, surrounding the position of the PTP in the gel, on the black background of radioactively labeled substrate. We observed that two PTPs, of M_r

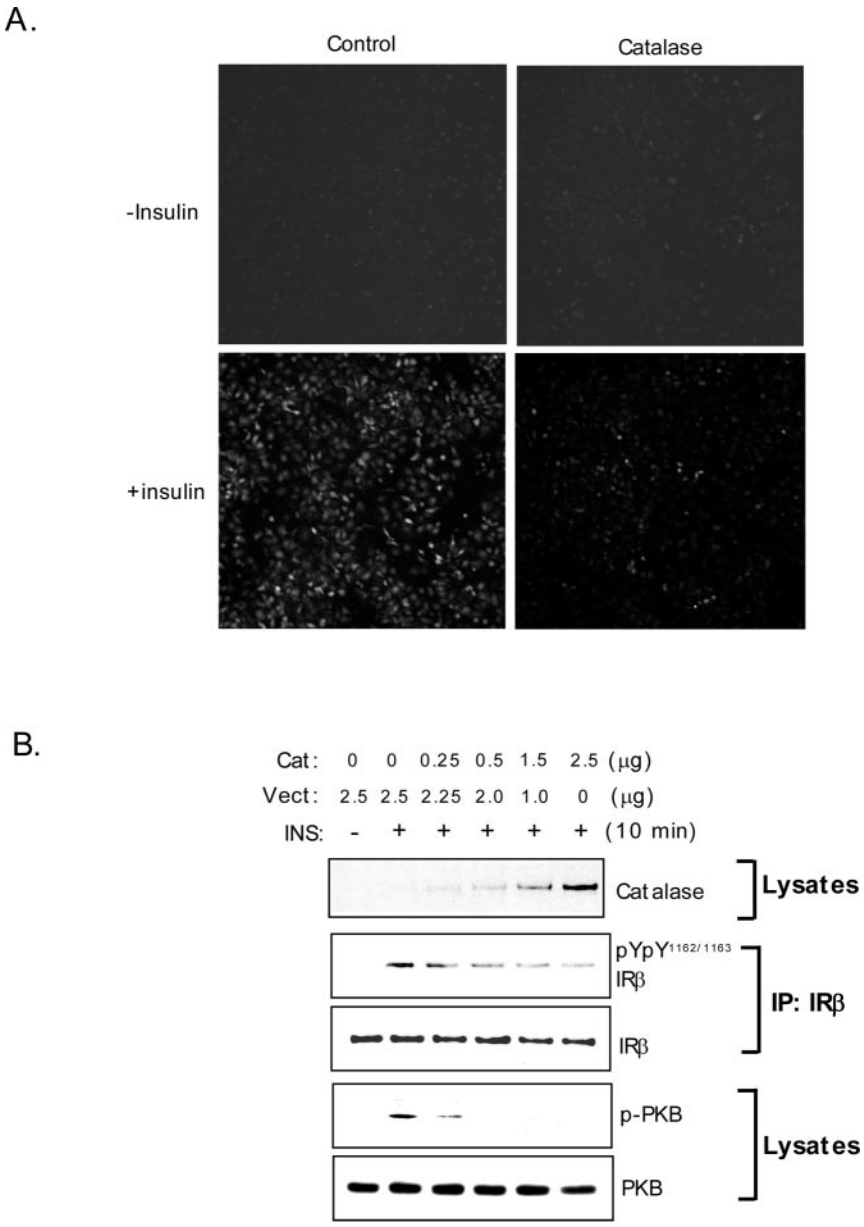

FIG. 1. **Hydrogen peroxide is an important mediator of insulin signaling.** *A*, serum-starved Rat-1 cells, either control (5 μg of vector DNA) or ectopically expressing human catalase (5 μg of plasmid DNA, a gift from Dr. Toren Finkle, National Institutes of Health, Bethesda, MD), were preloaded with 5 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Molecular Probes) in the dark for 15 min and then exposed to insulin (50 nM). Images of ROS-induced DCF fluorescence were captured by fluorescence microscopy by using a Zeiss Axiovert 405M inverted microscope equipped with a fluorescence attachment and digital camera and are shown at ×50. The data are representative of three independent experiments. *B*, Rat-1 cells were transiently transfected with different quantities of plasmid encoding human catalase. The empty vector (*Vect*) was included together with the catalase expression plasmid (*Cat*) to normalize the total amount of DNA added to cells. Two days after transfection, cells were serum-deprived and then stimulated with 50 nM insulin (*INS*) for 10 min. The cells were lysed, and catalase expression was verified by immunoblotting with anti-catalase antibody (Calbiochem) (*top panel*). The insulin receptor β-subunit was immunoprecipitated (*IP*) with antibody 29B4 (Santa Cruz) and then immunoblotted with anti-pYpY$^{1162/1163}$ antibody (Biosource) to examine the phosphorylation status and subsequently with the anti-β-subunit antibody clone C-19 (Santa Cruz) as a loading control (*middle panel*). An aliquot of lysate (30 μg) was subjected to immunoblotting with anti-phospho-PKB antibody (Cell Signaling). The same filter was then stripped and reprobed with anti-PKB antibody (Cell Signaling) as a loading control (*bottom panel*).

50,000 and 45,000, were rapidly and reversibly oxidized, reaching a maximum at 5–10 min, after insulin stimulation (Fig. 2). Ectopic expression of catalase, which impaired the signaling response to insulin, also inhibited the oxidation of these PTPs (Fig. 2). On the basis of the apparent M_r of the 50K and 45K PTPs, we predicted their identities as PTP1B and TC45, the 45-kDa splice variant of TC-PTP, respectively. This was confirmed by immunodepletion and immunoblot analyses. The anti-PTP1B antibody FG6 immunoprecipitated the 50-kDa PTP from cell lysates, leaving the 45-kDa PTP in the immunosupernatant (Fig. 3*A*). Conversely, antibodies to TC45 depleted the 45-kDa PTP specifically, leaving the 50-kDa enzyme in the supernatant (Fig. 3*B*).

Ablation of Endogenous TC45 Expression by RNAi Results in Enhanced PKB/AKT Activation in Response to Insulin—A substantial body of literature already links PTP1B to the regulation of signaling in response to insulin. To explore the potential regulatory role of TC45 in insulin signaling, we examined the phosphorylation status of PKB/AKT, which is a critical effector in the phosphatidylinositol 3-kinase pathway that mediates various intracellular responses to insulin (28), following ablation of the PTP by RNAi. Transfection of Rat-1 cells with siRNA specific for TC45 led to ablation of expression of the endogenous enzyme (Fig. 4). In contrast, we detected no effect on the level of expression of PTP1B, chosen as a control because it is the closest homolog of TC-PTP. Furthermore, there was no effect on the level of PKB/AKT. Rat-1 transfectants were stimulated with insulin, and the phosphorylation of PKB/AKT was monitored over the indicated time course by immunoblotting with phospho-PKB/AKT-specific antibodies. We observed that the ablation of TC45 by RNA interference enhanced and prolonged the activation of PKB/AKT in response to insulin, compared with the signaling response in the control cells (Fig. 4).

To explore additional cell models that are used more extensively to study insulin signaling, we chose the human hepatoma cell line HepG2 (29). HepG2 cells expressed higher levels of insulin receptor than Rat-1 cells and displayed a more robust response to insulin stimulation, in terms of overall tyrosine phosphorylation of the insulin receptor β-subunit and autophosphorylation of the activation loop tyrosines 1162 and 1163 (Fig. 5*A*). Furthermore, and of particular importance to our study, it has been shown that treatment with insulin triggers H_2O_2 production in HepG2 cells (21). As shown in Fig. 5*B*, upon transfection of HepG2 cells with siRNA specific for TC-PTP, we

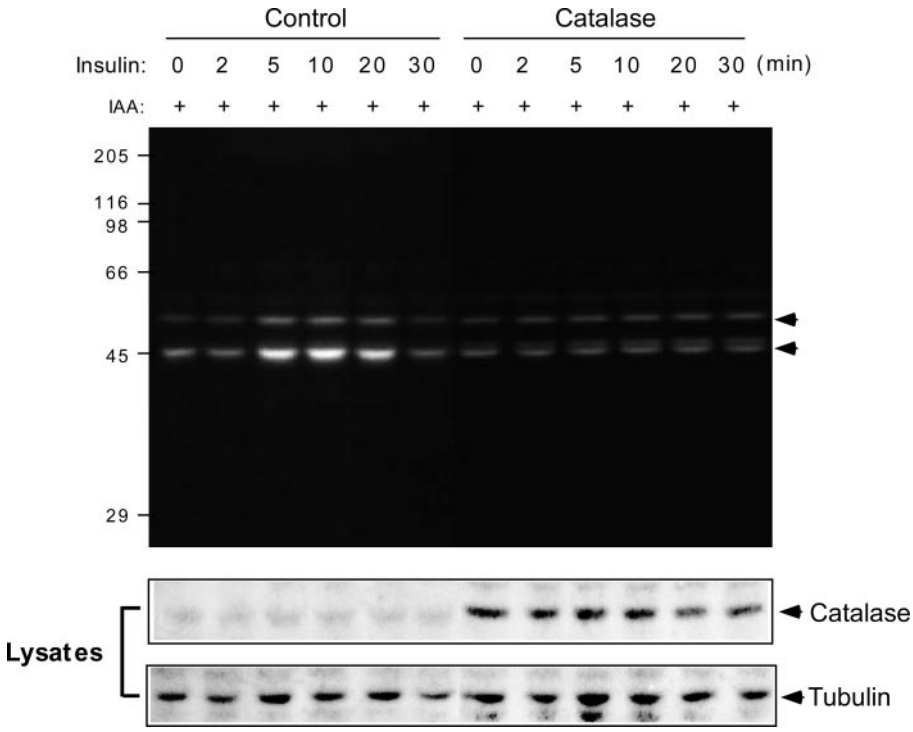

FIG. 2. **Insulin induced the transient oxidation of two PTPs in Rat-1 cells.** Serum-starved Rat-1 cells, either control (5 μg of vector DNA) or ectopically expressing catalase (5 μg of plasmid DNA), were exposed to 50 nM insulin for the indicated times. Lysates were prepared under anaerobic conditions in the presence of 10 mM IAA and then subjected to in-gel PTP assays (*upper panel*). The *arrowheads* indicate the 50-kDa and 45-kDa PTPs that were transiently oxidized in response to insulin. The *lower panels* are immunoblots to illustrate the expression of catalase and tubulin; the latter were included as loading controls.

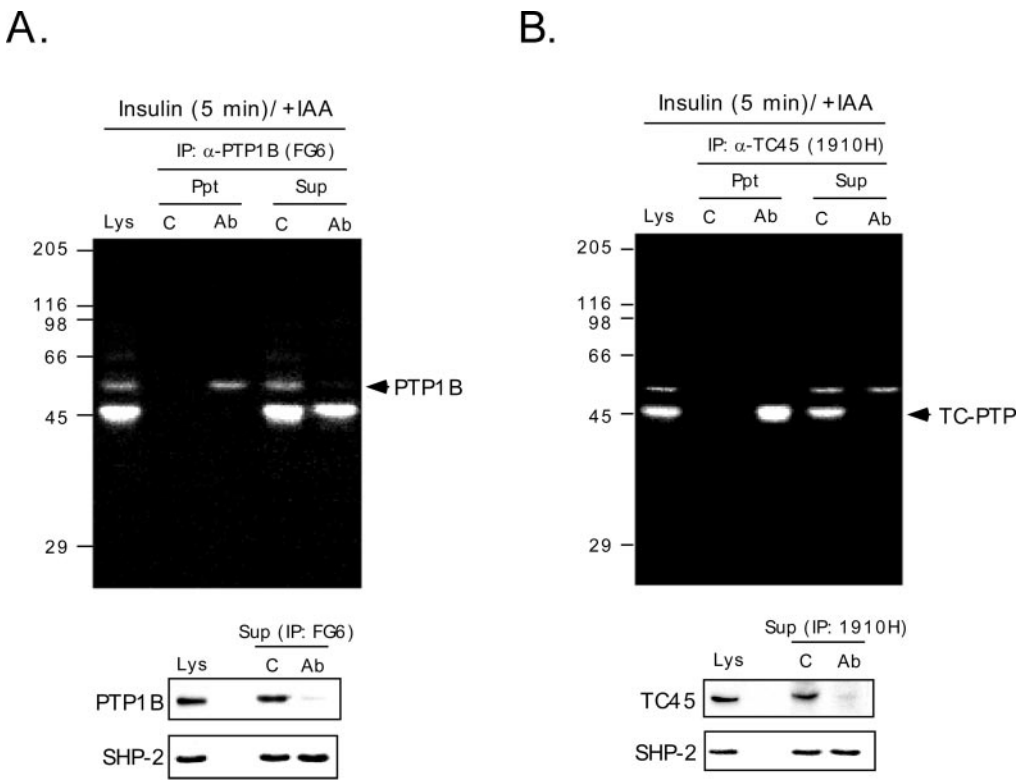

FIG. 3. **Insulin induced the transient oxidation of PTP1B and TC45.** *A* and *B*, total lysate (400 μg) was incubated with either normal IgG (*C*), anti-PTP1B antibody (*FG6* in *A*), or anti-TC45 antibody (*1910H* (59) in *B*) coupled to protein G-Sepharose beads. After immunoprecipitation, the immunocomplexes and supernatants (*Sup*) were collected and then subjected to in-gel PTP assays. *A*, immunodepletion of the M_r 50K PTP from the lysate with anti-PTP1B antibody. *B*, immunodepletion of the M_r 45K PTP with antibody specific for TC45. The lane marked *Lys* represents cell lysate before immunodepletion. The *lower panels* illustrate immunoblots of total lysate and the supernatants after immunodepletion, using either anti-PTP1B (*A*) or anti-TC45 (*B*) antibody (*Ab*). The data show complete depletion of the PTP protein after immunoprecipitation (*IP*) with the specific antibody. The same blot was subsequently reprobed with anti-SHP-2 antibody (C-18, Santa Cruz) to ensure equal loading.

observed ablation of expression of the endogenous enzyme. As in Rat-1 cells, we detected no effect on the level of expression of PTP1B, the closest homolog of TC-PTP, or PKB/AKT. A comparison of insulin-induced phosphorylation of PKB/AKT in control and siRNA-transfected HepG2 cells illustrated that depletion of TC45 enhanced both the intensity and duration of the signaling response. It is important to note that these effects were observed with two distinct TC-PTP-directed siRNA oligonucleotides (Fig. 5*C*).

PKB/AKT is phosphorylated and activated in response to

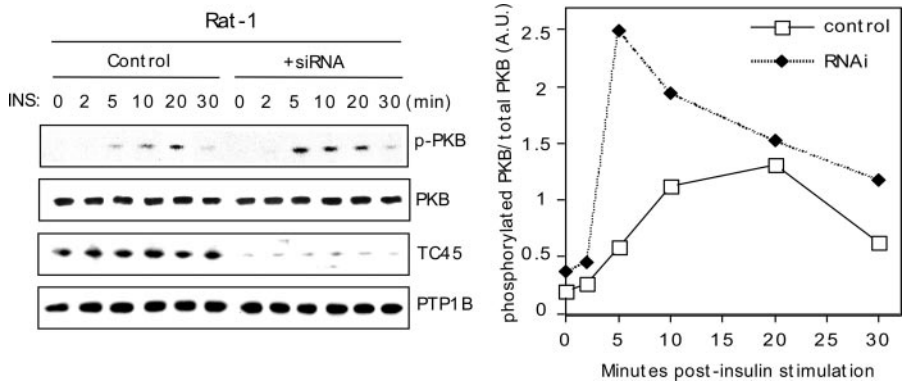

FIG. 4. **Ablation of TC45 by RNAi enhanced insulin-induced activation of PKB in Rat-1 cells.** Rat-1 cells were left untransfected (*Control*) or transfected (+*siRNA*) with 100 nM siRNA oligonucleotide to TC45 (TCPTP1). Two days after transfection, cells were deprived of serum for 16 h and then stimulated with 10 nM insulin for the indicated times. Total lysates (30 μg) were immunoblotted with antibodies to phospho-PKB, PKB, TC45 (*1910H*), or PTP1B (*FG6*) (*left panel*). The *right panel* illustrates densitometric analyses of the gel images to show the ratio of phosphorylated PKB to total PKB (*A.U.*, arbitrary unit). Similar results were observed in two independent experiments.

multiple stimuli (30). Therefore, we compared the effects of ablation of TC-PTP expression on activation of PKB/AKT in response to different stimuli to provide insights into the potential for selectivity in the effects of the phosphatase on signaling events. Both Rat-1 control cells and siRNA transfectants were stimulated with platelet-derived growth factor BB (PDGF-BB). The tyrosine phosphorylation of the β-form of PDGF receptor (PDGFR) was analyzed by immunoblotting. As shown in Fig. 6, the ablation of endogenous TC45 expression via RNAi did not alter PDGF-induced autophosphorylation of PDGFR-β. Furthermore, suppression of TC45, which augmented insulin-induced activation of PKB/AKT (Figs. 4 and 5), did not affect the activation of PKB/AKT in response to PDGF stimulation (Fig. 6). These results reveal specificity in the signaling function of TC-PTP and illustrate that it did not play a regulatory role in PKB/AKT signaling induced by PDGF.

TC45 Recognizes Tyrosine Phosphorylated Insulin Receptor-β-Subunit as a Substrate in Vivo—To address the mechanism by which TC45 inhibited insulin-mediated signaling, we used a substrate-trapping mutant form of the enzyme. We had shown previously that in any PTP mutation of the invariant aspartate residue, which functions as a general acid in catalysis, to alanine creates a substrate-trapping mutant that can be used to identify physiological substrates of the enzyme (31). We expressed wild-type and substrate-trapping mutant (DA) forms of TC45 and PTP1B in HepG2 cells and tested whether a complex was detected between the PTP and the insulin receptor β-subunit. As expected, an association between the DA mutant, but not the wild-type form of PTP1B, and the β-subunit was observed in immunoprecipitates of the PTP from insulin-treated HepG2 cells (Fig. 7*A*), consistent with other reports (32). Furthermore, we also observed association between the DA mutant form of TC45 and the insulin receptor β-subunit (Fig. 7*A*). These data show that TC45 can recognize the insulin receptor directly as a substrate and suggest that it may function in a coordinated manner with PTP1B to regulate phosphorylation of the insulin receptor.

We pursued this matter further by direct examination of the phosphorylation status of the insulin receptor β-subunit in TC45 siRNA transfected HepG2 cells, including the use of antibodies to particular phosphorylation sites in the receptor. We observed, by immunoblotting with antibodies to phosphotyrosine, that the overall tyrosine phosphorylation of the β-subunit was enhanced and remained elevated for a prolonged period after ablation of TC45 by RNAi (Fig. 7*B*). Interestingly, at these early time points after insulin stimulation, there was little discernible effect of TC45 siRNA on autophosphorylation of the activation loop, whereas the phosphorylation of Tyr-972 was enhanced in the absence of the PTP (Fig. 7*B*). Tyr-972 of the β-subunit, which is located in the juxtamembrane segment of the insulin receptor, is important for the recruitment of IRS-1 and Shc and for the activation of phosphatidylinositol 3-kinase (33). Thus, its dephosphorylation by TC45 would be consistent with a mechanism by which the PTP may influence insulin-induced signaling via PKB/AKT. The data also suggest that TC45 influences the phosphorylation status of the insulin receptor β-subunit at later points in the time course of insulin stimulation, although the identity of the phosphorylation sites remains to be determined. Interestingly, there are additional sites of tyrosine phosphorylation in the C-terminal portion of the insulin receptor that have been implicated in the regulation of receptor function (34). Although these sites may also be substrates for TC-PTP, generation of appropriate phospho-specific antibodies will be required to address this issue further. Nonetheless, these observations highlight the important point that PTPs not only have the potential to display specificity for particular substrate proteins but also to show a preference for particular sites within those proteins.

DISCUSSION

Although initially viewed as a harmful by-product of life in an aerobic environment, it is now apparent that there are beneficial effects of the controlled production of ROS in the regulation of cellular homeostasis. The production of ROS by phagocytic leukocytes plays a critical role in the innate immune response to pathogens (12). Detailed analysis of the multi-component NADPH oxidase in leukocytes has illustrated how the production of ROS may be tightly regulated. The core of the NADPH oxidase enzymes is a two-subunit flavocytochrome b_{558} (cyt b) comprising $gp91^{phox}$ (Nox 2) and $p22^{phox}$, which catalyzes the single-electron reduction of oxygen from NADPH as an electron donor, to generate superoxide, which is then converted to H_2O_2, either spontaneously or by the action of superoxide dismutase (11, 12). The activity of the Nox enzyme in cell membranes is tightly controlled by additional, cytosolic regulatory proteins. The small GTPase Rac, an activator protein $p67^{phox}$ and an organizer protein $p47^{phox}$, together with an additional subunit $p40^{phox}$, form a complex with cyt b after cell stimulation that induces the reduction of molecular oxygen (12, 35). This process is regulated by phosphorylation of $p47^{phox}$, which together with Rac, links signaling events to the generation of ROS (36). Importantly, a family of Nox homologs has now been identified in nonphagocytic cells. The production of ROS by these enzymes and the resulting post-translational modification of proteins by reversible oxidation have been implicated in the regula-

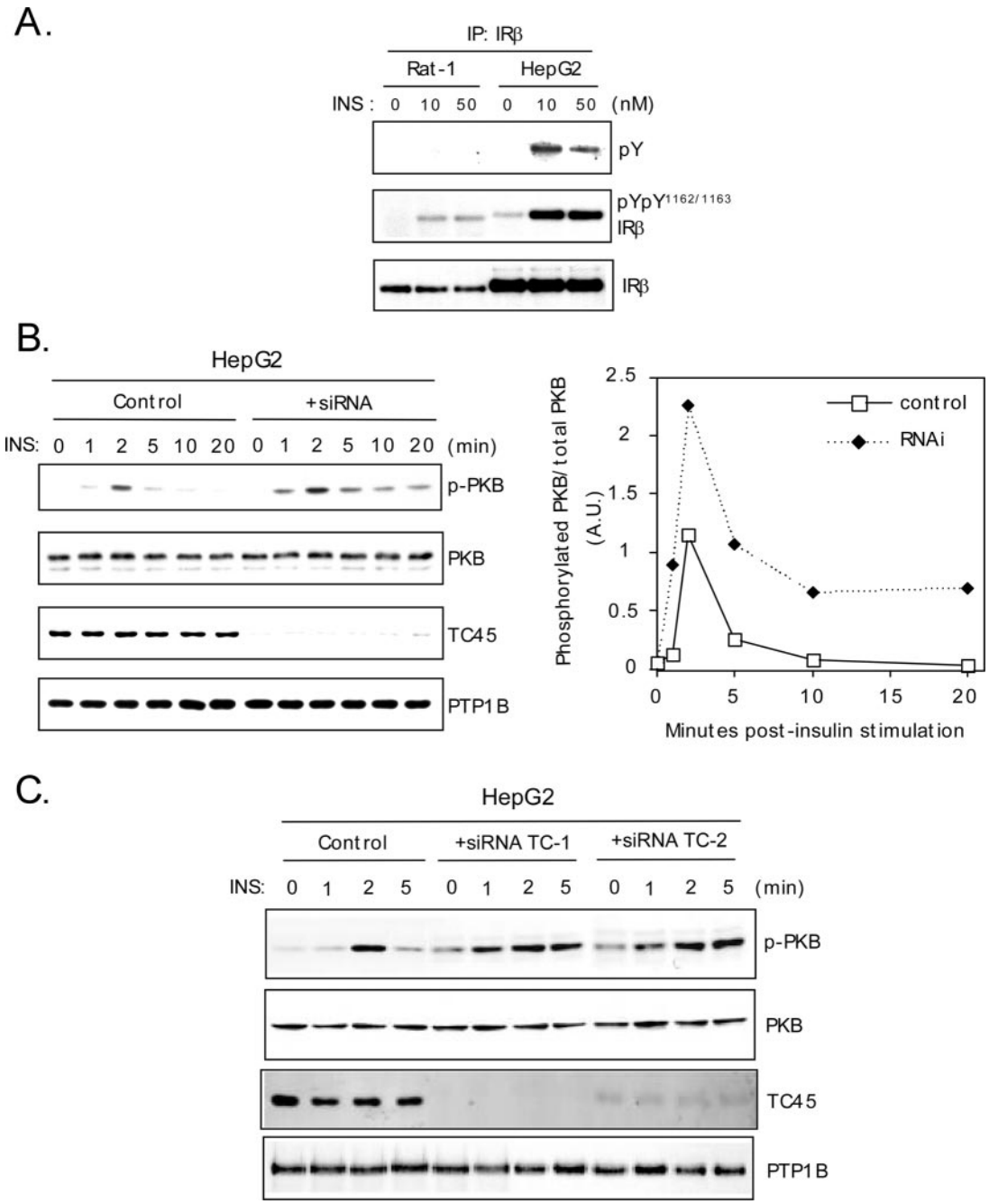

FIG. 5. **Ablation of TC45 enhanced insulin-induced activation of PKB in HepG2 cells.** *A*, serum-deprived Rat-1 and HepG2 cells were exposed to 10 or 50 nM insulin for 5 min and lysed. The insulin receptor was immunoprecipitated with anti-β-subunit antibody 29B4 and then immunoblotted with anti-phosphotyrosine, anti-pY$Y^{1162/1162}$-IR-β, and anti-IR-β (C-19) antibodies. *B*, HepG2 cells were left untransfected (*Control*) or transfected (+*siRNA*) with 100 nM siRNA oligonucleotide to TC45 (TCPTP1). Two days after transfection, cells were serum-starved for 16 h and then stimulated with 10 nM insulin for the indicated times. Total lysates (30 μg) were immunoblotted with anti-phospho-PKB, anti-PKB, anti-TC45, and anti-PTP1B antibodies (*left panel*). The *right panel* illustrates a densitometric analysis of the gel image to show the ratio of phosphorylated PKB relative to total PKB (*A.U.*, arbitrary unit). Similar results were observed in three independent experiments. *C*, the experiment described in *B* was repeated using two distinct TC45 siRNAs.

tion of tyrosine phosphorylation-dependent signaling pathways initiated by a wide array of stimuli, including hormones, growth factors, cytokines, and cellular stresses (12).

The architecture of the active site of members of the PTP family and the critical role of the invariant, low-pK_a, catalytic cysteine residue highlight the potential of the PTPs as targets for such a regulatory mechanism (3, 37). Work from several laboratories has now established that multiple members of the PTP family are susceptible to reversible oxidation, both *in vitro* and in cell culture (9, 25). In the classical PTPs, such as PTP1B, oxidation of this cysteine to sulfenic acid, with subsequent conversion into a sulfenamide species (38), abrogates the nucleophilic properties of this residue, thereby inhibiting PTP activity. This is a reversible modification. Conversion of the oxidized sulfenic acid to the sulfenamide form of the active-site cysteine induces profound conformational changes at the PTP active site, which both disrupt the interaction with substrate and expose the oxidized cysteine to the environment of the cell. This serves the dual purpose of preventing irreversible oxidation to higher oxidized forms of the active site cysteine (sulfinic and sulfonic acid) and facilitating the reduction of the sulfenamide to restore the active form of the PTP (38). The dual

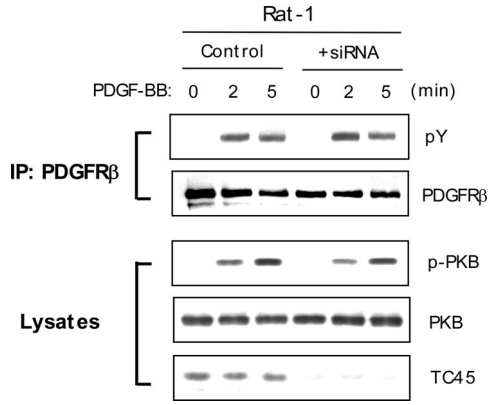

FIG. 6. **Ablation of TC45 by RNAi did not enhance the activation of PKB in response to PDGF in Rat-1 cells.** Rat-1 cells were left untransfected (*Control*) or transfected (+*siRNA*) with 100 nM siRNA oligonucleotide to TC45 (TCPTP1). Two days after transfection, cells were deprived of serum for 16 h and then stimulated with 50 ng/ml of PDGF for the indicated times. The immunoprecipitated (*IP*) PDGFR-β and total lysates (30 μg) were immunoblotted with antibodies to phosphotyrosine (G104 (60)), PDGFR-β (958, Santa Cruz), phospho-PKB, PKB, or TC45 (1910H) as indicated. Similar results were observed in two independent experiments.

specificity phosphatases cdc25C (20) and PTEN (18), as well as the low M_r PTP (22), are also sensitive to oxidation. Unlike the classical PTPs, these enzymes contain a second cysteine residue within the active site. After oxidation of the nucleophilic cysteine within the signature motif, a disulfide bond is formed with the vicinal cysteine within the active site, which protects the enzymes from the irreversible inactivation that would result from the formation of higher oxidized species. The S—S bond can be readily reduced, for example mediated by thioredoxin in the case of PTEN (18), which ensures the transient nature of the modification and returns the enzymes to their active form. Interestingly, oxidation may also underlie a mechanism for regulation of receptor PTPs. In RPTPα, the cysteine of the signature motif from the second, membrane-distal PTP domain (RPTPα-D2) is more susceptible to oxidation than the membrane-proximal catalytic domain (39). Oxidation of RPTPα-D2 inside the cell leads to a change in the conformation of the extracellular segment of RPTPα (40), suggesting that the membrane-distal PTP domain may serve as an oxygen sensor and may underlie inside-out signaling through RPTPα.

With the completion of the sequence of the human genome, we are now in a position to define the composition and diversity within families of enzymes. In the case of the PTPs, we have identified ~100 PTP genes in humans (3, 6). This represents the minimal level of complexity in the family, with additional diversity introduced through use of alternative promoters, alternative mRNA splicing, and post-translational modification. Broadly speaking, PTPs are known to play either inhibitory or permissive roles in regulating the physiological response to particular ligands. For example, SHP-2 promotes signaling through the epidermal growth factor receptor but down-regulates signaling in response to PDGF (41). Nevertheless, the majority of the PTPs are known primarily by their sequence, there being little information on their physiological function. The current challenge is to develop methods for assigning function to each of the PTPs. The generation of substrate-trapping mutant PTPs allows exploration of the physiological substrate specificity of these enzymes, which provides insights into function (31). The limitation is the requirement first to identify an appropriate system in which to express these mutant PTPs. Ideally, one would like to start with a particular signaling pathway and find a method to tag and identify the critical PTPs that are the regulators of that pathway. The reversible oxidation of PTPs that is induced by various physiological stimuli offers such a strategy. We formulated the hypothesis that stimulus-induced oxidation could be used as a means of "tagging" the specific PTPs that are integral to the regulation of signal transduction pathways initiated by that stimulus. In this study, we have focused on signaling in response to insulin and have shown that oxidation can serve as a molecular tag, which allowed us to identify PTPs that play regulatory roles in the insulin signaling pathway.

The importance of understanding the regulation of insulin signaling is emphasized by the current prevalence of diabetes and obesity in western society (42). A defect in post-insulin receptor signaling is thought to be the basis of insulin resistance in type II diabetes, and abnormal function of PTPs that control the phosphorylation of the insulin receptor β-subunit and/or its substrates may contribute to the disease (43). Consequently, there is excitement in the pharmaceutical industry regarding the potential for development of PTP inhibitors as a novel therapeutic strategy for treatment of type II diabetes. For such a strategy to succeed, it is important to understand which PTPs are the relevant regulators of insulin signaling. A variety of studies have indicated that the phosphorylation of the insulin receptor β-subunit may be regulated by multiple PTPs, depending upon the cellular context (43). Most prominent among these is PTP1B, which has been implicated in the down-regulation of insulin signaling by a variety of approaches, including the phenotype of the PTP1B knock-out mouse (44, 45). This and the identification of this phosphatase as a regulator of signaling in response to cytokines such as leptin (46–48) have led to considerable attention being focused on PTP1B as a target for development of novel therapeutics for treatment of both diabetes and obesity (49, 50). In our analysis, we observed that insulin stimulation induced the rapid and transient oxidation and inactivation of PTP1B. This observation, which identified a PTP that is a known regulator of insulin signaling as a target for reversible oxidation, provides validation for our strategy. A similar observation has been reported by Mahadev *et al.* (21), who went on to show that the Nox homolog, Nox 4, is a regulator of PTP1B in this context (51).

Our study also identified TC45, a spliced variant of TC-PTP, as a second PTP that is subject to insulin-induced, reversible oxidation and inactivation. The two alternatively spliced forms of TC-PTP share the same catalytic domain but differ at their extreme C termini (52). The C terminus of TC48, similar to PTP1B, is hydrophobic in nature and directs the protein to the endoplasmic reticulum. Our data do not rule out the possible involvement of TC48 in regulating insulin signal transduction; however, this spliced variant displays a more restricted expression pattern than TC45 (52). TC45, which is characterized by the presence of a bipartite nuclear localization signal, is rapidly translocated from the nucleus to the cytoplasm after epidermal growth factor stimulation, where it dephosphorylates the epidermal growth factor receptor, as well as downstream adapters including $p52^{Shc}$, at the plasma membrane, thereby regulating growth factor signaling (53, 54). Our observation of insulin-induced oxidation and inactivation of TC45 suggests that this PTP may also function as a negative regulator of insulin signaling. Ablation of TC45 expression by RNAi led to enhanced and sustained activation of PKB/AKT in response to insulin, consistent with removal of an inhibitory constraint on the signaling pathway. This suggests that, at least in Rat-1 and HepG2 cells, both PTP1B and TC45 function in down-regulation of the signaling response to insulin. Furthermore, in a parallel study, it was shown that in immortalized fibroblasts derived from TC-PTP −/− mice the signaling response to insulin was enhanced compared with control TC-PTP +/+ fibro-

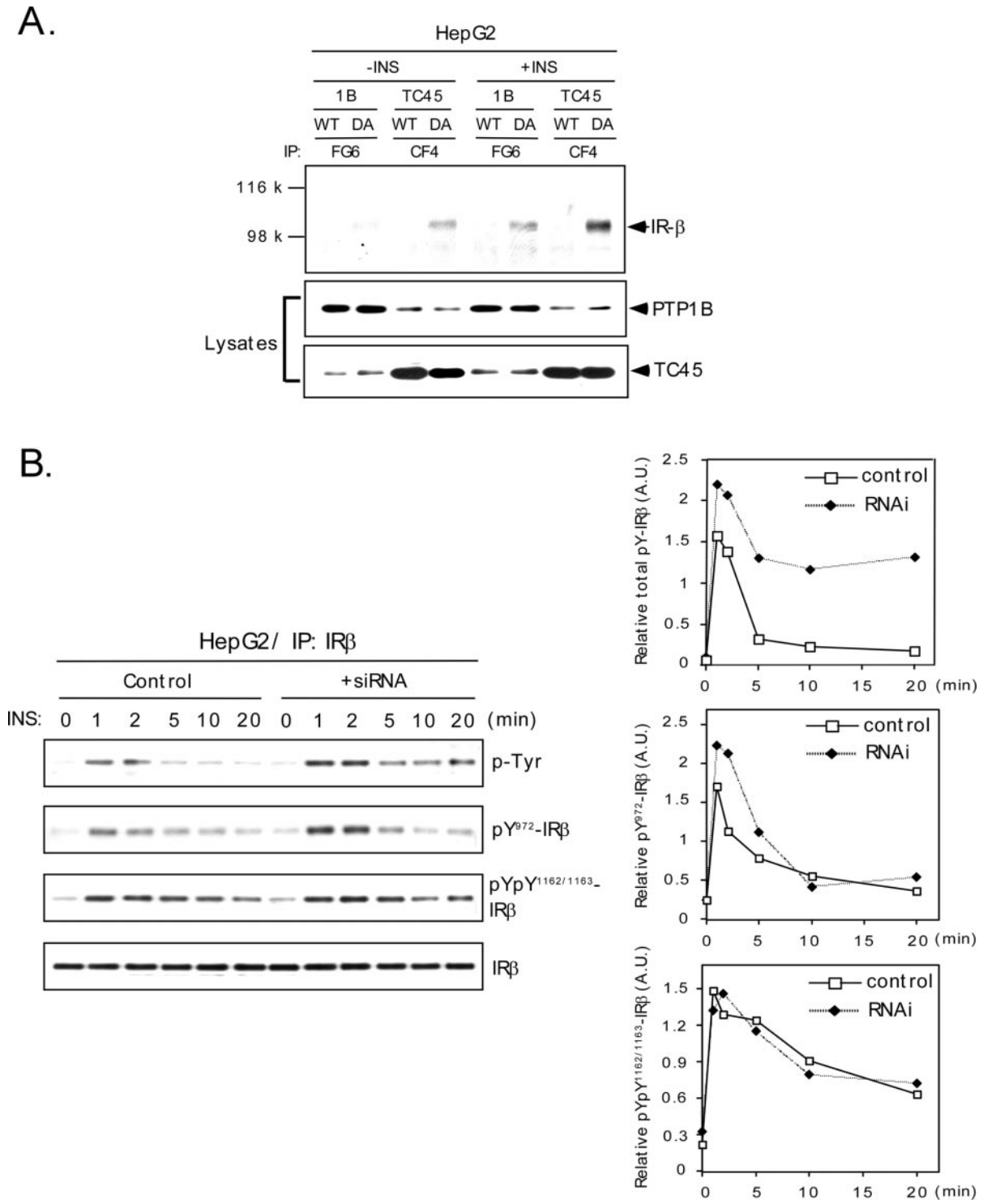

FIG. 7. **Tyrosine phosphorylated IR-β-subunit is a substrate of TC45 *in vivo*.** *A*, HepG2 cells overexpressing wild-type (*WT*) or trapping mutant (*DA*) forms of PTP1B and TC45 were either left untreated (−*INS*) or stimulated with 10 nM insulin for 5 min (+*INS*) and then lysed in substrate-trapping lysis buffer. Aliquots (1 mg) of cell lysate were incubated with anti-PTP1B antibody (*FG6*) or anti-TC45 antibody (*CF4*), as indicated. The immunocomplexes were washed with lysis buffer, subjected to SDS-PAGE, and then immunoblotted with anti-IR-β (C-19) antibody (*top panel*). An aliquot of lysate (30 μg) was immunoblotted with anti-PTP1B antibody (*FG6*) or anti-TC-PTP antibody (*CF4*) to verify PTP expression (*bottom panel*). Data shown are representative of three independent experiments. *B*, serum starved, untransfected (*Control*), or TC45 siRNA (100 nM) transfected (+*siRNA*) HepG2 cells were stimulated with 10 nM insulin for the indicated times. The insulin receptor was immunoprecipitated from 750 μg of cell lysate with anti-IR-β antibody 29B4 and immunoblotted with anti-phosphotyrosine, anti-pY^{972}-IR-β (BIOSOURCE), anti-$pYpY^{1162/1163}$-IR-β, and anti-IR-β (C-19) antibodies (*left panel*). The *right panel* illustrates densitometric analyses of the gel image to show the ratio of phosphorylated IR-β relative to total IR-β for total phosphotyrosine (*upper*), phosphorylation of Tyr-972 (*middle*), and phosphorylation of the activation loop tyrosines 1162 and 1163 (*lower*). Similar results were observed in two independent experiments. *A.U.*, arbitrary unit.

blasts, consistent with our data and the conclusion that, indeed, insulin receptor signaling is regulated by TC-PTP (55).

Our data also address the issue of specificity in the role of PTPs as regulators of signal transduction. Ablation of TC45 by RNAi led to enhanced tyrosine phosphorylation of the insulin receptor β-subunit. Furthermore, the use of antibodies that

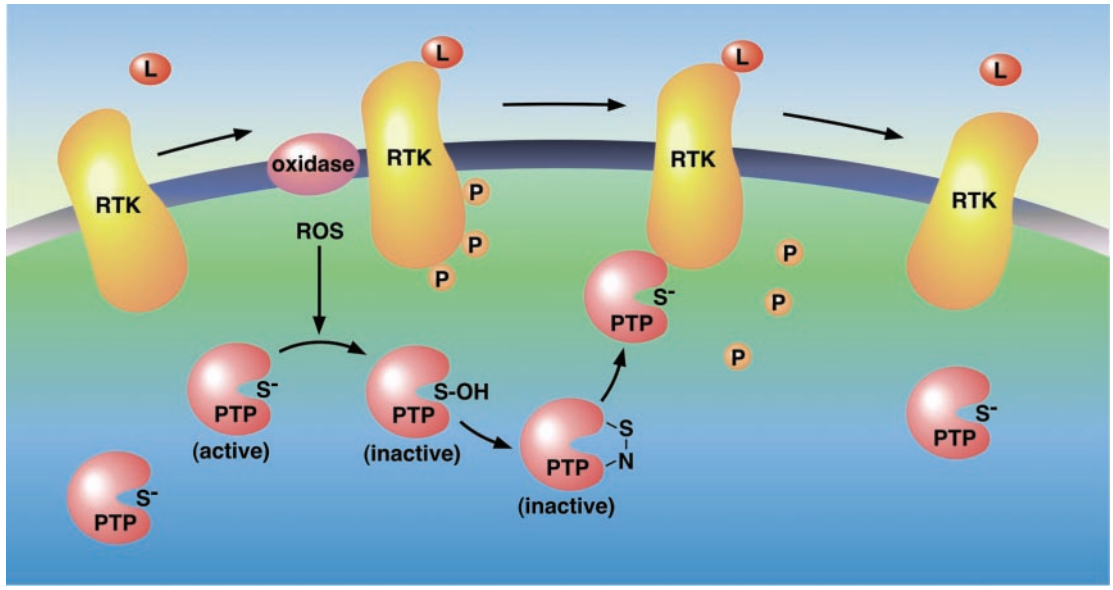

FIG. 8. **A general model for the regulation of PTP activity by reversible oxidation.** In response to stimulation of a protein tyrosine kinase, such as a transmembrane receptor tyrosine kinase (*RTK*), a Rac-dependent multiprotein NADPH oxidase complex is assembled and activated, leading to production of ROS. Although the cartoon illustrates activation of Nox in the plasma membrane, the precise intracellular location and identity of the oxidase remain to be determined. The sulfur atom of the Cys residue at the active site of members of the PTP family is normally present as a thiolate ion, which promotes its nucleophilic function but renders it exquisitely sensitive to oxidation. Upon encountering ROS, this Cys residue is oxidized to sulfenic acid and then rapidly converted to a cyclic sulfenamide. This results in inhibition of PTP activity, thereby fine tuning the tyrosine phosphorylation response. However, oxidation of the PTPs is transient. Restoration of PTP activity after reduction back to the thiolate form of the active site Cys terminates the tyrosine phosphorylation-dependent signal.

recognize specific phosphorylation sites within the insulin receptor β-subunit revealed that TC45 has the potential to show preferential recognition of particular phosphorylation sites. This is an important point for the PTP field, adding to the change in the perception of these enzymes from that of playing a housekeeping role to the current view of PTPs as specific regulators of signaling, displaying specificity not only for particular proteins as substrates but also showing preferential recognition of particular sites within those proteins. We report in this study that ablation of TC45 led to enhanced phosphorylation of Tyr-972 in the insulin receptor β-subunit, with little or no effect on Tyr-1162 and Tyr-1163 of the activation loop during the time course that was examined. Tyr-972 is an important residue for the recruitment of IRS-1 and Shc and the activation of phosphatidylinositol 3-kinase (33), consistent with the observation that dephosphorylation of the insulin receptor β-subunit by TC45 leads to inactivation of PKB/AKT after insulin stimulation. It has been shown that PKB/AKT is an important downstream effector of various receptor protein tyrosine kinases through the activation of phosphatidylinositol 3-kinases (56). Therefore, we addressed further the issue of specificity of TC45 in regulating signal transduction pathways by testing its effects on PDGF signaling. Our results showed that the ablation of endogenous TC45 by RNAi, conditions that augmented tyrosine phosphorylation of the insulin receptor β-subunit as well as PKB/AKT signaling in response to insulin, did not alter PDGF-induced autophosphorylation of PDGFR-β or activation of PKB/AKT. Interestingly, PDGF stimulation does not lead to oxidation of TC-PTP. In contrast, PDGF induced the transient oxidation and inactivation of a distinct PTP, SHP-2 (19). Our observation is consistent with reports that the ablation of TC-PTP by gene knock-out did not affect either activation of extracellular signal-regulated kinase/mitogen-activated protein kinase or activation of PKB/AKT in response to PDGF stimulation (57, 58). These data provide evidence that TC45 neither recognizes all receptor protein tyrosine kinases as substrates *in vivo* nor regulates all PKB/AKT-dependent signaling but rather exerts specificity in its effects as a regulator of signal transduction.

Now that the composition of enzyme families can be defined, based upon the sequence of the human genome, the task at hand is to establish the physiological function of the constituents of the human proteome. Although the importance of PTPs in the regulation of signal transduction is becoming apparent, the regulatory links between particular PTPs and specific signaling pathways largely remain to be defined. This study illustrates that ligand-induced oxidation, which is required for optimal tyrosine phosphorylation, can be harnessed as a strategy for "tagging" the PTPs that are critical regulators of the signaling response to that ligand. The operating principle is that the agonist (hormone, growth factor, and others) may enhance tyrosine phosphorylation directly, by activation of a protein tyrosine kinase, and/or indirectly, by inactivation of a PTP. Thus, one function of ROS produced after agonist stimulation is to inactivate transiently the PTP that provides the inhibitory constraint upon the system, thus facilitating the initiation of the signaling response (Fig. 8). Treatment of cells with H_2O_2 induced the oxidation of multiple PTPs (19), suggesting that the family as a whole will be susceptible to this mode of regulation. Since many and diverse stimuli have been shown to trigger both tyrosine phosphorylation and ROS production, we anticipate that this strategy may be applied broadly across the PTP family. Upon identification, the use of substrate-trapping and RNAi technologies should generate new insights into the role of the PTP in control of signal transduction under normal and pathophysiological conditions.

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