The Putative Tumor Suppressor Gene PTPN13/PTPL1 Induces Apoptosis through Insulin Receptor Substrate-1 Dephosphorylation

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Abstract
The protein tyrosine phosphatase (PTP) PTPL1/PTPN13 is a candidate tumor suppressor gene. Indeed, PTPL1 activity has been reported recently to be decreased through somatic mutations, allelic loss, or promoter methylation in some tumors. We showed previously that its expression was necessary for inhibition of Akt activation and induction of apoptosis by antiestrogens in breast cancer cells. Implications of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway in cancer progression are now well established, and our study was therefore designed to define whether PTPL1 is sufficient to inhibit this pathway and, if so, to identify a direct substrate of this PTP, which may trigger a proapoptotic effect. We first show by complementary approaches that PTPL1 specifically dephosphorylates insulin receptor substrate-1 (IRS-1) in vitro and in cellulo. Next, our experiments using a dominant-negative mutant and RNA interference confirm the crucial role of PTPL1 in IRS-1 dephosphorylation. Finally, we report that PTPL1 expression is sufficient to block the IRS-1/PI3K/Akt signaling pathway, to inhibit the insulin-like growth factor-I effect on cell survival, and to induce apoptosis. Altogether, these data provide the first evidence for a direct positive role of the putative tumor suppressor gene PTPL1/PTPN13 in apoptosis and identify its target in the IRS-1/PI3K/Akt signaling pathway. [Cancer Res 2007;67(14):6806–13]

Introduction
Protein tyrosine phosphorylation plays a major role in many cellular functions, including cell survival, proliferation, differentiation, and motility. It is now well established that deregulation of intracellular signaling pathways triggering these processes is implicated in cancer development and progression. Tyrosine phosphorylation of proteins is a dynamic process controlled by two opposing biochemical reactions implicating protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP), whereas much progress has been made over the last 20 years in elucidating the significance of the numerous PTKs in signal transduction (1). However, less is known about PTPs. It is only recently that the restricted specificity of these enzymes and the complexity of the PTP family became evident (2, 3). Furthermore, studies using the so-called substrate trapping method (4) clearly showed an intrinsic specificity of some PTP catalytic domains in their substrate recognition (5). Altogether, those studies successfully pinpointed specific roles for PTPs in intracellular signal transduction (6, 7).

We have shown that the inhibition of growth factor action by antiestrogens was accompanied by an increase of PTP activity in breast cancer cells. Furthermore, vanadate, a PTP-specific inhibitor, abrogated the antiestrogen effect, thus emphasizing the major role of PTP in this inhibition (8). Later, we showed that PTPL1, the largest intracellular PTP (also known as hPTP1E, PTP-BAS, FAP-1, or PTPN13; refs. 9, 10), was up-regulated by these antagonists and had a prominent role in the 4-hydroxy-tamoxifen (OH-Tam) negative effect on growth factor signaling in these cells. Indeed, although PTPL1 expression had no effect on OH-Tam antiestrogenic activity, abolition of its expression completely abrogated OH-Tam anti-growth factor action (11). In addition, we have more recently shown that PTPL1 was necessary for OH-Tam inhibition of the PI3K/Akt antiapoptotic pathway (12). These results altogether suggest a proapoptotic role for PTPL1.

It is interesting to note that PTPL1/PTPN13 gene presents the characteristics of a tumor suppressor gene (6, 7). It is located on chromosome 4q21, a region frequently deleted in ovarian and liver cancers (13), and its expression was frequently down-regulated or silenced through promoter hypermethylation within several tumor types (14, 15). Furthermore, a mutational analysis of colorectal cancers identified different somatic mutations in PTPL1 (16).

In addition to its catalytic unit, PTPL1 has two major structural domains: a FERM domain involved in plasma membrane and cytoskeleton binding and five PDZ domains (Fig. 1). Those latter suggest the interaction of PTPL1 with several partners and consequently that PTPL1 may act as an integrator between different signaling pathways. About the FERM domain of PTPL1, we showed recently that it was necessary and sufficient to target PTPL1 at the plasma membrane. This membrane localization is consistent with an involvement of PTPL1 in the insulin-like growth factor-I receptor (IGFI-R)/insulin receptor substrate-1 (IRS-1)/phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway (17).

Our study was therefore designed to define whether PTPL1, which is necessary to antiestrogen inhibition of the IGFI-R/IRS-1/PI3K/Akt signaling pathway, is sufficient to inhibit this pathway and, if so, to identify a direct substrate of this PTP, which may trigger its proapoptotic effect. Using complementary substrate trapping, colocalization, and in cellulo dephosphorylation methods, herein we show that PTPL1 directly and specifically dephosphorylates IRS-1 and induces apoptosis at least by inhibition of this survival pathway.
IGFI-R and IRS-1 expression vectors were a gift of Dr. E. Surmacz (Kimmel de la Recherche Scientifique UMR5237, Montpellier, France). A single mutation TGC (amino acid 2389) to TCC. The constitutively active PTPL1-CS and PTPL1-YF/DA, which were used in GST ''pool-down'' experiments. The expression construct PTPL1 wild-type (Wt) was described previously as pHM6-PTPL1 (17). Mutants were generated from Dharmacon. All glutathione-S-transferase (GST) fusion proteins were constructed in BL21 as described by Teyssier et al. (18). After expression level and integrity of GST fusion proteins were verified by SDS-PAGE and Coomassie blue staining (data not shown).

Materials and Methods

Expression plasmids. The expression construct PTPL1 wild-type (Wt) was described previously as pHM6-PTPL1 (17). Mutants were generated with the QuikChange site-directed mutagenesis kit (Strategene). Mutant PTPL1-YF/DA was obtained by the double mutation TAT (amino acid 2224) to TTT and GAC (amino acid 2359) to GCC and mutant PTPL1-CS by the single mutation TGC (amino acid 2389) to TCC. The constitutively active Akt mutant (308D/473D) was a generous gift of Morin N. (Centre National de la Recherche Scientifique UMR2237, Montpellier, France).

All glutathione-S-transferase (GST) fusion proteins were constructed in pGEX-4T1 (Pharmacia Biotech) by insertion of the Wt catalytic domain (amino acids 2106–2459) or catalytic domain carrying the DA mutation, IGFI-R and IRS-1 expression vectors were a gift of Dr. E. Surmacz (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA).

Cell culture and transient transfection. HeLa and HEK293 cells were cultured in DMEM and MCF7 cells in Ham’s F12/DMEM (50%/50%), all cultured in DMEM.

Preparation of GST fusion proteins. pGEX–FT1 Wt or DA were transfected with IGFI-R and Induces Apoptosis

PTPL1 Dephosphorylates IRS-1 and Induces Apoptosis

Figure 1. PTPL1 expression constructs. A schematic representation of the PTPL1 expression constructs. Protein segments produced by transient expression are within parentheses. Numbers correspond to the first and last amino acid positions according to the PTPL1 sequence. PTPL1 WT corresponds to the HA-tagged whole enzyme. PTPL1-CS and PTPL1-YF/DA correspond to PTPL1 Wt carrying mutation that abolishes catalytic activity or creates an enzyme unable to dissociate from its substrates, respectively. The catalytic domain of these three proteins were fused to GST to create GST-Wt, GST-CS, and GST-DA, which were used in GST "pool-down" experiments.

Cell culture and transient transfection. HeLa and HEK293 cells were cultured in DMEM and MCF7 cells in Ham’s F12/DMEM (50%/50%), all supplemented with 10% FCS.

Transient transfections were carried out using the jet PEI Cationic Polymer Transfection Reagent method according to the manufacturer’s instructions with a ratio 1:1.5 of IRS-1/IGFI-R/PTPL1 Wt or mutant or empty pHM6 vector. Small interfering RNA (siRNA) transfections were carried out using the Oligofectamine reagent (Invitrogen) method according to the manufacturer’s instructions. The PTPL1-specific siRNA (6896-GGAAAAGAAGAGUUCGUUUA-6914) and the control nontargeting siRNA were from Dharmacon.

Preparation of GST fusion proteins. pGEX–FT1 Wt or DA were produced in Escherichia coli BL21 as described by Teyssier et al. (18). After one wash with ST buffer [20 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 10% glycerol] containing 10 mmol/L DTT expression level and integrity of GST fusion proteins were verified by SDS-PAGE and Coomassie blue staining (data not shown).

Immunoblotting analysis. Equal amounts of lysate or immunoprecipitate were separated on a SDS/polyacrylamide gel and electrotransferred onto polyvinylidene difluoride membrane. Blots were stained with Coomassie blue (R250, Sigma) to ensure that protein amounts were comparable. Membranes were blocked with TBST [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.1% Tween 20] containing 5% nonfat milk or [5% bovine serum albumin (BSA) when anti-phosphotyroine (pTyr) was used as primary antibody] and immunoblotted with anti-HA (12CA5, Roche), anti-IRS-1 (Upstate Cell Signaling), anti-Akt or anti-Akt phospho-Ser473 (Cell Signaling Technology), anti-pTyr (4G10 and P20, Sigma), anti-PTPL1 (H300, Santa Cruz Biotechnology), or anti-actin (Sigma) monoclonal antibodies (mAb) or polyclonal antibodies. Primary antibodies were detected using horseradish peroxidase–conjugated goat anti-mouse IgG or anti-rabbit IgG, along with the Western Lighting enhanced chemiluminescence detection kit (Perkin-Elmer Life Sciences).

Before reprobing with different primary antibodies, blots were stripped by incubation in 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 2% SDS, and 100 mmol/L l-mercaptoethanol for 30 min at 50°C.

Substrate trapping in vitro. For trapping experiments 48 h after transfection, the cells were serum deprived for 3 h and unstimulated or stimulated with 10−8 mol/L pervanadate for 30 min or with 10−7 mol/L IGFI for 10 min. The cells were washed in PBS and then lysed in ST buffer protease inhibitors completed [1 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 1/250 aprotinin]. The treated cell lysates were incubated on ice for 30 min in the presence of 5 mmol/L iodoacetic acid to irreversibly inactivate endogenous PTPs. After incubation, DTT was added for 10 min at 4°C to a final concentration of 10 mmol/L to inactivate the iodoacetic acid. The lysates were then centrifuged at 10,000 × g for 15 min at 4°C and the resulting supernatants were incubated overnight at 4°C with 20 μL GST fusion protein–coupled beads (10 μg/mL). The beads were washed four times with 1 mL ST buffer. The affinity complexes were boiled in Laemmli sample buffer (Sigma) at 95°C for 3 min and analyzed by immunoblot.

Immunoprecipitation. Forty-eight hours after transient transfection, the cells were washed twice in ice-cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris (pH7.5), 150 mmol/L NaCl, 0.5% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 10 mmol/L NaF, 1 mmol/L Na3VO4, 1/250 aprotinin, 1/250 AEBSF] by 10 passages through a G25 needle to shear DNA. After centrifugation (15 min, 10,000 × g) at 4°C, equal amounts of each cell lysate were used for immunoprecipitation experiments with 2 μg antibodies for 2 h at 4°C and then incubated further overnight with 40 μL protein A-Sepharose (6%) blocked in 4% BSA in PBS. The immune complexes were washed thrice in RIPa buffer before immunoblot.

In vitro dephosphorylation. Tyrosyl-phosphorylated IRS-1 was immunoprecipitated from the lysate of serum-deprived HEK293 cells cotransfected with IRS-1/IGFI-R, stimulated with 10−8 mol/L IGFI for 10 min and lysed in RIPA buffer using anti-IRS-1 antibodies. The immune complexes were washed twice with RIPA buffer and incubated in phosphatase lysis buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L MgCl2, 0.1% l-mercaptoethanol (pH 7.2), 1/250 aprotinin] for 1 or 10 min with equal concentrations of soluble GST fusion protein in the absence or presence of 0.1 mmol/L Na3VO4 at 4°C. The reaction was terminated by adding an equal volume of Laemmli sample buffer (Sigma), boiling for 3 min, and subjecting to immunoblotting.

Immunofluorescence microscopy analysis. Forty-eight hours after transient transfection, HeLa cells were grown on coverslips and serum deprived for 3 h. Cells were either unstimulated or stimulated with IGFI (10−7 mol/L) for 10 min. Immunolabeling was done as described (17) with FITC or Texas red–conjugated secondary antibodies (Jackson Immunoresearch). Immunofluorescence microscopy analysis was carried out with a Bio-Rad 1024 CLSM system using a 60× (1.4 NA) planapochromatic objective (Nikon). Series of optical sections were collected and projected onto a single image plane using the laser sharp 1024 software and deconvolution system. Colocalization was estimated with the laser sharp software on 10 representative cells and expressed as percentage of IRS-1 colocalized with PTPL1.

Immunoelectron microscopy. Forty-eight hours after transient transfection, ultrathin Lowicryl HM20 sections of cells prepared as described (19) were first preincubated in PBS containing 0.1% cold water fish gelatin, 1% BSA, and 0.05% Tween 20 (incubation buffer) for 2 h at room temperature.
The sections were then incubated overnight at 4°C with rabbit anti-IRS-1 (33 μg/mL) and mouse anti-HA (40 μg/mL) in the incubation buffer. The primary antibodies were visualized using a goat anti-mouse IgG conjugated to 6-nm colloidal gold particles and a goat anti-rabbit IgG conjugated to 15-nm colloidal gold particles (Aurion) both diluted 1:20 in the same buffer. The sections were observed using a Hitachi 7100 transmission electron microscope.

**Apoptosis assays.** Forty-eight hours after transient transfection, HeLa cells growing on glass coverslips were stimulated for 6 h by IGFI (10 nmol/L) or unstimulated after 18 h of serum deprivation. They were irradiated using a UV Stratalinker 1800 (Stratagene) with 300 J/m² and then cultivated in DMEM serum-free medium supplemented or not with 10⁻⁸ mol/L IGFI for an additional 24 h. Cells were subjected to indirect immunofluorescence with anti-HA antibody and Alexa Fluor 568 goat anti-mouse IgG antibody (1:200; Invitrogen) for 30 min. Nuclei were stained with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI; Sigma). Images were acquired with a microscope immunofluorescent Leica objective 63×.

**Results**

PTPL1 catalytic domain has intrinsic specificity for IRS-1. The substrate specificity of PTPs depends on a combination between subcellular targeting and catalytic domain intrinsic selectivity (2). To evaluate the intrinsic specificity of the PTPL1 catalytic domain, we did an in vitro substrate trapping assay on pervanadate-treated HeLa cell lysate. Pervanadate induces tyrosine phosphorylation of a large number of proteins as judged by immunoblotting with an anti-pTyr antibody (Fig. 2, bottom). Lysates were then incubated with either recombinant PTPL1 catalytic domain fused to GST (GST-Wt) or the substrate trapping fusion protein GST-DA (Fig. 1), which forms stable complexes with substrates. Tyrosine-phosphorylated proteins specifically retained by GST-Wt or GST-DA were immunoblotted for pTyr. Whereas no protein retained by GST-Wt was detectable (except an unspecific signal corresponding to GST fusion protein), GST-DA trapping fusion protein bound a restricted set of tyrosyl-phosphorylated proteins (Fig. 2A, bottom). This result suggests that the phosphatase catalytic domain displays striking substrate specificity. Indeed, four tyrosine-phosphorylated proteins among more than 20 were affinity precipitated by GST-DA (Fig. 2A, bottom, arrows 1–4). Two of them (Fig. 2A, bottom, arrows 3 and 4) were mainly represented on lysate, whereas the other two (Fig. 2A, bottom, arrows 1 and 2) were obviously enriched. Interestingly, the slowest migrating band corresponds to a 170-kDa protein and was further identified as IRS-1 by immunoblotting using a specific antibody (Fig. 2A, top).

Using nonphysiologic pervanadate stimulation, we identified highly phosphorylated IRS-1 as a potential PTPL1 substrate. To validate this result with IRS-1 phosphorylated by IGFI-R, similar experiments were done using lysates from HeLa cells overexpressing IRS-1 and IGFI-R and stimulated with IGFI as a source of tyrosyl-phosphorylated proteins. IGFI mainly induced the tyrosine phosphorylation of two proteins (Fig. 2B, bottom), one slightly phosphorylated protein of 170 kDa, which corresponds to IRS-1 as shown by immunoblot analysis (Fig. 2B, top), and one highly phosphorylated protein with a molecular weight corresponding to IGFI-R. It is important to note that the two phosphorylated proteins were unequally retained by the GST-DA mutant protein. Indeed, the signal of phosphorylated IGFI-R precipitated from 150 μL lysate was lower than signal obtain from direct analysis of 40 μL lysate, whereas phosphorylated IRS-1 was strongly precipitated by the trapping mutant. These data further support the notion that IRS-1, more likely than IGFI-R, is a potential physiologically relevant substrate for PTPL1.

To further characterize IRS-1 as a substrate for PTPL1, we next asked whether PTPL1 directly dephosphorylates IRS-1 in vitro. Tyrosyl-phosphorylated IRS-1, immunoprecipitated from IGFI-stimulated HEK293 cells overexpressing IRS-1 and IGFI-R, was incubated with equal amounts of GST, GST-Wt, or GST-CS (Fig. 2C). In contrast to the lack of GST effect on the IRS-1 phosphorylation level, GST-Wt dephosphorylated IRS-1 by ~80% after 10 min of incubation. Importantly, the ability of GST-Wt to dephosphorylate IRS-1 was inhibited by vanadate or mutation of the catalytic domain (GST-CS; Fig. 2C, lanes 7 and 8). These data provide strong evidence that the catalytic activity of PTPL1 can directly regulate IRS-1 tyrosyl phosphorylation.

**PTPL1 dephosphorylates IRS-1 in cellulo.** To test whether PTPL1 has any effects on IGFI-stimulated tyrosine phosphorylations in vivo, we assessed PTPL1-induced phosphorylation changes on HEK293 cells overexpressing IGFI-R, IRS-1, and PTPL1 Wt or PTPL1-CS. Total lysates were analyzed by direct immunoblotting using an anti-pTyr antibody. In the absence of PTPL1, three proteins were highly tyrosine phosphorylated after IGFI stimulation. Their
molecular weights correspond to transfected IRS-1 (p170), IGFI-R β chain (p95), and endogenously hyperexpressed IRS-4 (p160; Fig. 3A; ref. 20). In the presence of PTPL1 Wt, the phosphorylation of p170 was abolished, whereas p95 tyrosine phosphorylation was only partially inhibited and p160 phosphorylation remained unaffected (Fig. 3A, bottom). On the contrary, in similar conditions, p170 phosphorylation was unaffected on expression of the catalytic inactive PTPL1-CS mutant, suggesting that phosphatase activity of PTPL1 is essential for the hypophosphorylation of p170. To further characterize p170 as IRS-1, IRS-1 was immunoprecipitated from cells treated as above and its phosphorylation status was analyzed by immunoblotting using anti-pTyr. Similarly to p170, IGFI-induced tyrosine phosphorylation of IRS-1 was strongly impaired by PTPL1 Wt expression but not by the phosphatase-dead mutant PTPL1-CS (Fig. 3B). These experiments show that IRS-1 is a preferential substrate for PTPL1 in cellulo.

IGFI stimulation induces a rapid IRS-1 delocalization from cytoplasm to plasma membrane where it recruits PI3K (21). To confirm that PTPL1 interferes with IRS-1 signaling, we studied the subcellular localization of IRS-1 by indirect immunofluorescence and confocal microscopy after IGFI stimulation in the presence of PTPL1. In HeLa cells overexpressing IGFI-R and IRS-1, IGFI stimulation induced marked IRS-1 relocalization from the cytoplasm to the plasma membrane (Fig. 3C, compare 1 with 2). On the contrary, in the presence of PTPL1, a large proportion of IRS-1 remained in the cytoplasm on growth factor stimulation (Fig. 3C, 3). This inhibition of IRS-1 translocation by PTPL1 required its catalytic activity because IRS-1 localization was unaffected in cells overexpressing the phosphatase-dead mutant PTPL1-CS (Fig. 3C, 4). This indicates that in intact cells, PTPL1 dephosphorylates IRS-1 and inhibits its recruitment to the plasma membrane, which leads to a block of the IGFI-R/IRS-1/PI3K pathway.

To test the direct action of PTPL1 on IRS-1 in intact cells, in cellulo substrate trapping was done. Although the GST-DA fusion protein trapped IRS-1 in vitro, such an interaction could not be detected with the full-length PTPL1-DA construct in cells. We noted that the PTPL1-DA mutant incorporated a significant level of pTyr when expressed in mammalian cells (data not shown). A similar observation has been reported with the DA mutant of PTPH1 (22). In this case, a conserved tyrosine within the catalytic domain seemed to serve as a phosphate acceptor, thereby impeding access to any additional substrate and blocking the trapping activity. Mutation of this residue to phenylalanine in the DA context restored trapping ability. We generated such a double PTPL1 mutant (Y2224F/DA) and observed a drastic reduction of tyrosine phosphorylation as noted for the PTPH1 mutant (data not shown).

IRS-1 and IGFI-R were transfected with either PTPL1 Wt or PTPL1-YF/DA expression vectors. Proteins associated with IRS-1 were immunoprecipitated with anti-IRS-1 antibodies from lysates prepared from IGFI-treated HEK293 cells and immunoblotted for the presence of PTPL1 using anti-HA antibodies. Ectopically expressed IRS-1 complexed with the substrate trapping double mutant; however, complex formation was very low with PTPL1 Wt (Fig. 4A, bottom). Under control conditions, detected PTPL1-YF/DA-IRS-1 complex was likely to be formed before serum deprivation of the cells. IRS-1 binding to PTPL1 was also studied after PTPL1 immunoprecipitation. Immunoblot analysis using anti-pTyr antibodies revealed the presence of only one 170-kDa protein specifically retained from the IGFI-stimulated cells with anti-pTyr antibodies revealed the presence of only one 170-kDa IRS-1 (170 kDa), endogenous IRS-4 (160 kDa), and transfected IGFI-R (95 kDa), respectively. Equivalent amounts of IRS-1 and PTPL1 were confirmed by reprobing the blots with anti-IRS-1 (top) and anti-HA antibodies (middle). B, cell lysates analyzed above by immunoblot were used for immunoprecipitation experiments with anti-IRS-1 polyclonal antibodies. The immune complexes were then immunoblotted with anti-pTyr antibodies (bottom). Equivalent amounts of IRS-1 and PTPL1 were confirmed by reprobing the blots with anti-IRS-1 antibody (top). C, transiently transfected HeLa cells, unstimulated (1) or stimulated (2, 3, and 4), were treated for indirect TRITC localization of IRS-1. Images represent horizontal confocal sections. In cells cotransfected with an empty vector, IGFI stimulation induces a marked IRS-1 delocalization from cytoplasm to plasma membrane (white arrow, 2). In cells overexpressing PTPL1 Wt, IRS-1 remains partially localized in the cytoplasm (3), whereas expression of IRS-1 was localized at the plasma membrane in cells overexpressing the catalytically inactive PTPL1-CS (white arrow, 4).

To further substantiate the interpretation that PTPL1 interacts directly with IRS-1, we studied the localization of PTPL1 and IRS-1 on IGFI stimulation by confocal microscopy in HeLa cells overexpressing IGFI-R, IRS-1, and PTPL1 Wt or PTPL1-YF/DA. PTPL1 and PTPL1-YF/DA both localized at the plasma membrane as described previously (Fig. 4C; ref. 17). In cells overexpressing PTPL1 Wt, IRS-1 was detected in the cytoplasm and at the plasma membrane, leading to a partial colocalization with PTPL1 Wt (54 ± 8%; Fig. 4C). In cells overexpressing PTPL1-YF/DA, IRS-1 was totally delocalized to the plasma membrane and a complete
colocalization with PTPL1 was observed (92 ± 3%; Fig. 4C). Immunoelectron microscopy study further confirmed this colocalization in microvilli-like protrusions (Fig. 4D, left) and in the apical submembrane region (Fig. 4D, right) in agreement with reported mouse PTPL1 localization (23).

Altogether, these data clearly show that PTPL1 directly regulates IRS-1 tyrosyl phosphorylation in intact cells.

**Endogenous PTPL1 was responsible for IRS-1 dephosphorylation.** We next attempted to evaluate the role of endogenous PTPL1 on IRS-1 dephosphorylation. For this purpose, IRS-1 and IGFI-R, alone or in combination with PTPL1-CS, were overexpressed in HEK293 cells expressing endogenous PTPL1. IRS-1 tyrosine phosphorylation induced by IGFI was then analyzed during a time course after growth factor deprivation. In the absence of exogenously expressed PTPL1, IRS-1 tyrosine phosphorylation decreased immediately after IGFI deprivation (Fig. 5A, left). On the contrary, in cells overexpressing the phosphatase-dead mutant of PTPL1, IRS-1 phosphorylation remained unchanged after 60 min (Fig. 5A, right). This result indicates that PTPL1-CS efficiently acts as a dominant-negative mutant, which prevents IRS-1 dephosphorylation by endogenous PTPL1.

Poor transfection efficiency in human breast cancer MCF7 cells, where endogenous IRS-1 phosphorylation was detectable after IGFI stimulation, prevents experiments using overexpression of PTPL1 or phosphatase-dead mutant. So, we used a siRNA approach to determine the role of endogenous PTPL1 on IRS-1 dephosphorylation. siRNA transfection resulted in a 70% to 90% inhibition of PTPL1 transcription and expression, quantified by reverse transcription-PCR (data not shown) and immunoblot analysis (Fig. 5B), respectively. In MCF7 cells, IRS-1 tyrosine phosphorylation decreased rapidly after IGFI deprivation (Fig. 5C, left). PTPL1 extinction resulted in an inhibition of IRS-1 dephosphorylation, which was detectable 30 and 120 min after depletion (Fig. 5C, right). This result shows that PTPL1 regulates IRS-1 phosphorylation in the more physiologic cellular model, in which we originally evidenced the importance of PTPL1 in anti-growth-factor activity of antiestrogens.

**PTPL1 inhibits IGFI-induced Akt activation and blocks this survival pathway.** We have shown previously that PTPL1 was necessary for antiestrogen inhibition of the IGFI-R/IRS-1/Pik3/Akt pathway in MCF7 cells (12) and showed, in the present study, the ability of PTPL1 to inhibit IRS-1 phosphorylation. Therefore, we next tested whether endogenous PTPL1 extinction potentiated this survival pathway and conversely whether PTPL1 overexpression was sufficient to inhibit it. For this purpose, Akt activation was studied in HEK293 cells overexpressing both IRS-1 and IGFI-R either with or without PTPL1 Wt and in MCF7 cells after PTPL1 extinction. In HEK293 cells, PTPL1 addition drastically decreased basal and IGFI-induced Akt phosphorylation (Supplementary Fig. S1A). In MCF7 cells, PTPL1 extinction increased the IGFI-induced Akt phosphorylation and prolonged Akt activation after IGFI deprivation (Fig. 5D).

The ability of PTPL1 to induce apoptosis through the inhibition of the IGFI-R/IRS-1/Akt survival effect was therefore evaluated in HeLa and HEK293 cells, which overexpressed IGFI-R/IRS-1 alone or in association with PTPL1 Wt, and in MCF7 cells transfected with control or PTPL1-specific siRNA. The effects of UV exposure and IGFI treatments, on the percentage of apoptotic (Fig. 6) or living cells (Supplementary Fig. S1), were evaluated by direct DAPI staining of nuclei or trypan blue exclusion, respectively. IGFI-R/IRS-1 overexpression was sufficient to protect HeLa cells from apoptosis even after UV exposure (Fig. 6B) in agreement with high Akt activation level observed in HEK293, in which IRS-1 and IGFI-R alone were introduced (Supplementary Fig. S1A).

The addition of PTPL1 totally inhibited the IRS-1/IGFI-R protective effect regardless of the presence or absence of IGFI (Fig. 6B; Supplementary Fig. S1B). In addition, it weakly induced apoptosis in the absence of UV and strongly potentiated UV proapoptotic effect (Fig. 6B).
Moreover, the failure of PTPL1 to induce apoptosis in HeLa cells overexpressing constitutively active Akt confirmed a PTPL1 action upstream of Akt (Fig. 6B). Beside, in MCF7 cells, PTPL1 extinction decreased cell susceptibility to apoptosis even after UV exposure and drastically increased IGF1 survival effect (Fig. 6D). These experiments clearly show that PTPL1 expression is sufficient to modulate Akt activation and to induce apoptotic cell death by inhibition of the IGF1-R/IRS-1/Akt pathway.

Discussion

Regulation of apoptosis is a fundamental feature both in the control of normal development and in the progression of cancer. The role of PTP in the progression of breast cancer is poorly documented (24), and the relation between PTPL1 and apoptosis is still a matter of debate in the literature. In the past, we have addressed the role of PTPL1 in the model of antiestrogen-induced apoptosis in hormone-responsive human breast cancer cells positive or defective for this particular tyrosine phosphatase. We have shown that PTPL1 is necessary for the early inhibition of the IGF1-R/PI3K/Akt pathway, which leads to increased apoptosis and inhibition of the IGF1 survival effect. Here, we show that PTPL1 is sufficient to induce apoptosis and to inhibit the IGF1-induced IGF1-R/IRS-1/PI3K/Akt pathway and we show that its action targets the adaptor protein IRS-1.

**PTPL1 acts directly on the IGF1-R/IRS-1/PI3K/Akt pathway through IRS-1 dephosphorylation.** Although several studies have identified potential substrates for PTPL1, among which ephrin B is the most documented (25), our study is the first that associates in vivo dephosphorylation and functional assays with in vitro and in vivo substrate trapping experiments. A crystal structure study of the PTPL1 catalytic domain shows that this enzyme, like PTP1B, interacts with and preferentially dephosphorylates biphosphorylated insulin receptor peptides (26). However, specificity of PTPL1 for peptides from other multiphosphorylated proteins implicated in this transduction pathway, like IRS-1, has not yet been tested. Here, by in vitro substrate trapping, we show, for the first time, its specificity for full-length IRS-1 among more than 20 tyrosine-phosphorylated proteins (vanadate stimulation) or compared with full-length IGF1-R (IGF1 stimulation). Furthermore, after PTPL1 overexpression, we observe a total inhibition of IRS-1 phosphorylation, whereas IGF1-R phosphorylation is only partially decreased. This result agrees with recent data that indirectly pointed to PTPL1 involvement in IRS-1 signaling. In fact, Toretsky et al. (27) have shown that EWS-FI11 inhibits basal IRS-1 phosphorylation without inhibition of IGF1-R phosphorylation and, more recently, that PTPL1 is a direct transcriptional target of EWS-FI11 and mediates its effects (28).

The PTPL1 proapoptotic effect corroborates its function as tumor suppressor gene. Several studies have addressed the role of PTPL1 in apoptosis regulation, focusing particularly on its interaction with Fas. Sato et al. (29) introduced the first evidence of such a relationship in their demonstration of the inhibitory interaction of PTPL1 with the COOH-terminal tail of the proapoptotic receptor Fas. On the other hand, Cuppen et al. (30) could not generate an inhibition of Fas-induced apoptosis by overexpressing PTP-BL in mouse T-cell lymphoma overexpressing human Fas, which interacted with PTP-BL, indicating that this interaction was not sufficient to inhibit Fas transduction in this cell type. More recently, silencing of PTPL1 was shown to abolished squamous cell carcinomas of the head and neck resistance to Fas-mediated apoptosis (31). At the opposite, PTPL1 overexpression in Fas-resistant colon cancer cells restores susceptibility to Fas-mediated apoptosis (32), starting again the debate on the role of PTPL1-Fas interaction. The last study was realized in cells in which IGF1 was inefficient to induce Akt phosphorylation, and consequently, PTPL1 positive effect on apoptosis was not correlated with suppression of the Akt activation. The authors suggest a positive effect of PTPL1 on Fas-mediated apoptosis through up-regulation of p21 by undetermined pathway (32).

On the other hand, we have shown that PTPL1 is necessary for the early inhibition of the IGF1-R/PI3K/Akt pathway, which leads to increased apoptosis and inhibition of the IGF1 survival effect by antiestrogens (12). PTPL1 effect on this pathway was then suggested by two other research groups. Indeed, Kimber et al.
have shown that extinction of tandem-PH-domain-containing protein-1, which binds to the first PDZ domain of PTPL1 and regulates PTPL1 membrane localization, enhances Akt/PKB activation in response to IGF1. In addition, EWS-FLI1, which up-regulates PTPL1 expression, inhibits basal IRS-1 phosphorylation. However, PTPL1 protects against etoposide-induced apoptosis and promotes soft agar cell growth (27, 28), in these tumors where PTPL1 is highly expressed compared with that in HEK293 or MCF7 cells. In addition, these effects were not confirmed in pancreatic carcinoma cell lines, which do not express such a PTPL1 level (28). All these studies point to a tissue specificity of PTPL1 role on apoptosis, depending on Fas pathway effectiveness and PTPL1 expression level. Published studies on mutant mice that lack PTPN13 protein product or phosphatase activity do not report an effect on apoptosis or tumor susceptibility. Indeed, none phenotypic consequences have been reported about the PTPN13 KO (34) and studies on mice that lack PTPN13 phosphatase activity focalize on hematopoietic cell lineages and peripheral nervous system (35), which were shown previously to express the phosphatase (36, 37). Crossbreeding these mice models with mammary tumor model is, however, required to evaluate the role of PTPL1 in tumor progression or susceptibility.

Key roles for the P38K/Akt signaling pathway in cellular processes, such as glucose metabolism, cell proliferation, apoptosis, and cell migration, are now well established (38), along with implications for the pathway in cancer progression. Our present study was designed to determine if PTPL1 is sufficient to inhibit this pathway; we show that PTPL1 inhibits Akt activation through IRS-1 dephosphorylation. Moreover, we show that PTPL1 drastically inhibits IGFI apoptosis protection and induces apoptotic cell death at least by inhibition of the IGFI-R/IRS-1/Akt pathway.

On the other hand, recent clinical studies suggest an anti-oncogene role for PTPL1. In fact, a mutational analysis of the PTP family in colorectal cancers identified 19 different somatic mutations in PTPL1, of which 8 deleted the entire catalytic phosphatase domain and 5 point mutations were located within the phosphatase domain (16). Moreover, in hepatocellular carcinomas, significant loss of PTPL1 expression was observed; in most cases, the RNA level was well correlated with the methylation status of promoter CpGs (14). In addition, frequent PTPL1/PTPN13 down-regulation or silencing was observed in multiple lymphomas and carcinomas through hypermethylation of its promoter (15). Altogether, these

Figure 6. PTPL1 induces apoptosis. HeLa cells (A and B) overexpressing IRS-1 and IGFI-R or Akt 308D/473D (Akt*) were stimulated by IGFI (I) or unstimulated (C) after 24-h serum deprivation in the presence or absence of overexpressed PTPL1 Wt. Cells were submitted to UV light (300 J/m²) and then cultivated for 24 h in the absence of serum. To allow identification of transfected cells by indirect immunofluorescence with anti-HA antibody, PTPL1 Wt was HA tagged and when HA-tagged PTPL1 was not overexpressed, HA-tagged IRS-1 was used. For cotransfection, dual indirect immunostaining with anti-HA mAb and anti-IRS-1 polyclonal antibodies showed that >90% of cells expressing PTPL1 also expressed IRS-1 (data not shown). The count of apoptotic nuclei among the cells unstained by anti-HA antibody corresponded to apoptosis in untransfected cells. Nuclei were stained with DAPI. A, IGFI-stimulated cells submitted to UV light. Arrows, condensed chromatin and apoptotic bodies (left), transfected cells (middle), and apoptotic transfected cells (right). B, the proportion of apoptotic cells among the transfected (150–300 cells, depending on transfection efficiency) and untransfected cells (900 cells) was counted by fluorescent microscopy. Columns, mean (IRS-1/IGFI-R transfected, n = 3; Akt 308D/473D transfected, n = 2); bars, SD. *, P < 0.05; **, P < 0.005, Student’s t test. MCF7 (C and D) cells transfected with control or PTPL1-specific siRNA were stimulated by IGFI or unstimulated after 24-h serum deprivation. Cells were submitted to UV light (150 J/m²) and then cultivated for 24 h in the absence of serum. Nuclei were stained with DAPI. C, representative pictures after indicated treatments. D, the proportion of apoptotic cells (among 500 cells) was counted by fluorescent microscopy. Columns, mean (n = 3); bars, SE. *, P < 0.05; **, P < 0.005, paired Student’s t tests.
clinical studies suggest PTPL1/PTPN13 as a putative tumor suppressor gene in several tissues and our present results provide mechanistic support to this hypothesis.

Conclusion. By identification of the PTPL1 target in the IGF1 survival pathway, we presently describe the first mechanism by which the putative tumor suppressor gene PTPL1/PTPN13 can inhibit tumor progression. This proapoptotic effect of PTPL1 suggests new therapeutic routes and points to the necessity of further studies on the mechanisms by which the expression or catalytic activity of this phosphatase is regulated.


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References
The Putative Tumor Suppressor Gene \textit{PTPN13}/\textit{PTPL1} Induces Apoptosis through Insulin Receptor Substrate-1 Dephosphorylation

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