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Running title

PTPL1 dephosphorylates IRS-1 and induces apoptosis

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Footnotes

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ABSTRACT

The protein tyrosine phosphatase PTPL1/PTPN13 is a candidate tumour suppressor gene. Indeed, PTPL1 activity has been recently reported to be decreased through somatic mutations, allelic loss or promoter methylation in some tumours. We previously demonstrated that its expression was necessary for inhibition of Akt activation and induction of apoptosis by antioestrogens in breast cancer cells. Implications of the PI3K/Akt signalling 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 pro-apoptotic effect. We first show by complementary approaches that PTPL1 specifically dephosphorylates 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 signalling pathway, to inhibit the IGF-1 effect on cell survival and to induce apoptosis.

Altogether, these data provide the first evidence for a direct positive role of the putative tumour suppressor gene PTPL1/PTPN13 on apoptosis and identify its target in the IRS-1/PI3K/Akt signalling pathway.

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 signalling 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 (PTKs) and protein tyrosine phosphatases (PTPs). Whereas much progress has been made over the last 20 years in elucidating the significance of the numerous PTKs in signal transduction (1), 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 demonstrated that the inhibition of growth factor action by antioestrogens was accompanied by an increase of PTP activity in breast cancer cells. Furthermore, vanadate, a PTP-specific inhibitor, abrogated the antioestrogen 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) (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 signalling in these cells. Indeed, while PTPL1 expression had no impact on OH-Tam antioestrogenic activity, abolition of its expression completely abrogated OH-Tam anti-growth factor action (11). In addition, we have more recently demonstrated that PTPL1 was necessary for OH-Tam inhibition of the PI3-kinase/Akt anti-apoptotic pathway (12). These results altogether suggest a pro-apoptotic role for PTPL1.

It is interesting to note that PTPL1/PTPN13 gene presents the characteristics of a tumour 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 tumour 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 5 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 signalling pathways. Concerning the FERM domain of PTPL1, we recently demonstrated that it was necessary and sufficient to target PTPL1 at the plasma membrane. This membrane localisation is consistent with an involvement of PTPL1 in the IGF1-R/IRS-1/PI3K/Akt signalling pathway (17).

Our study was therefore designed to define whether PTPL1, which is necessary to antioestrogen inhibition of the IGF1-R/IRS-1/PI3K/Akt signalling pathway, is sufficient to inhibit this pathway, and, if so, to identify a direct substrate of this PTP which may trigger its pro-apoptotic effect. Using complementary substrate trapping, co-localisation, and *in cellulo* dephosphorylation methods, herein we demonstrate that PTPL1 directly and specifically dephosphorylates IRS-1 and induces apoptosis at least by inhibition of this survival pathway.

MATERIALS AND METHODS

Expression plasmids

The expression construct PTPL1 Wt was described previously as pHM6-PTPL1 (17). Mutants were generated with the QuikChange site-directed mutagenesis kit (Stratagene). Mutant PTPL1-YF/DA was obtained by the double mutation TAT (aa 2224) to TTT and GAC (aa 2359) to GCC and mutant PTPL1-CS by the single mutation TGC (aa 2389) to TCC. The constitutively active Akt mutant (308D/473D) was a generous gift of Morin N. (CNRS UMR5237, Montpellier).

All GST fusion proteins were constructed in pGEX-4T1 (Pharmacia Biotech) by insertion of the wild type catalytic domain (aa 2106 - 2459) or catalytic domain carrying the DA mutation. IGF-1-R and IRS-1 expression vectors were a gift of Dr E. Surmacz **(**Kimmel Cancer Center, Thomas Jefferson University, Philadelphia).

Cell culture and transient transfection

HeLa and HEK 293 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) and MCF7 cells in Ham's F12/DMEM (50%/50%), all supplemented with 10% *F*œtal *C*alf *S*erum (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/IGF1-R/PTPL1 wild type or mutant or empty pHM6 vector. SiRNA transfections were carried out using the Oligofectamine reagent (Invitrogen) method according to the manufacturer's instructions. The PTPL1-specific siRNA (6896-G G A A A G A A G A G U U C G U U U A-6914) and the control non-targeting siRNA were from Dharmacon.

Preparation of GST- fusion proteins

pGEX-4T1 Wt or DA were produced in E. coli BL21 as described in (18) . After one wash with ST buffer (20 mM Tris pH7.5, 100mM NaCl, 1 mM EDTA, 1% Triton X-100, 10%

glycerol) containing 10 mM 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 an SDS/polyacrylamide gel and electrotransferred onto PVDF membrane. Blots were stained with Coomassie blue (R250, Sigma) to ensure that protein amounts were comparable. Membranes were blocked with TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk (or 5% bovine serum albumin when anti-phosphotyrosine was used as primary antibody) and immunoblotted with anti-HA (12CA5, Roche), anti-IRS-1 (Upstate Cell signaling), anti-Akt or anti-Akt phospho-Ser 473 (Cell Signaling Technology), anti-phosphotyrosine (4G10 and PY20, Sigma), anti-PTPL1 (H300; Santa Cruz Biotechnology) or anti-Actin (Sigma) monoclonal or polyclonal antibodies. Primary antibodies were detected using horseradish peroxydase-conjugated goat anti-mouse IgG or anti-rabbit IgG, along with the Western Lighting ECL detection kit (PerkinElmer Life Sciences).

Before reprobing with different primary antibodies, blots were stripped by incubation in 50 mM Tris pH 7.5, 150 mM NaCl, 2,5% SDS 20% and 100 mM β-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^{-4} M pervanadate for 30 min, or with 10^{-8} M IGF-1 for 10 min. The cells were washed in PBS, and then lysed in ST buffer protease inhibitors completed (1mM AEBSF, 1/250 Aprotinin). The treated cell lysates were incubated on ice for 30 min in the presence of 5 mM iodoacetic acid to irreversibly inactivate endogenous PTPs. After incubation, dithiothreitol was added for 10 min at 4°C to a final concentration of 10mM 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 20µl of GST fusion proteins-coupled beads (10µg/µl). The beads were washed four times with 1 ml of ST buffer. The affinity complexes were boiled in Laemmli sample buffer (Sigma) at 95°C for 3 min and analysed by immunoblot.

Immunoprecipitation

48 h after transient transfection, the cells were washed twice in ice cold PBS and lysed in RIPA buffer (50 mM Tris pH7.5, 150mM NaCl, 0.5% NP-40, 0.1% SDS, 0.5% DOC, 10mM NaF, 1mM Na_3VO_4, 1/250 Aprotinin, 1/250 AEBSF) by 10 passages through a G25 needle to shear DNA. After centrifugation (15 minutes, 10 000*g*) at 4°C, equal amounts of each cell lysate were used for immunoprecipitation experiments with 2µg antibodies for 2 h at 4 °C and then incubated further overnight with 40µl of protein A-Sepharose (6%) blocked in 4% bovine serum albumin in PBS. The immune complexes were washed three times in RIPA buffer before immunoblot.

***In Vitro* dephosphorylation**

Tyrosyl-phosphorylated IRS-1 was immunoprecipitated from the lysate of serum-deprived HEK 293 cells cotransfected with IRS-1/IGF1-R, stimulated with 10^{-8} M IGF-1 for 10 min and lysed in RIPA buffer using anti-IRS-1 antibodies. The immune complexes were washed three times with RIPA buffer and incubated in phosphatase lysis buffer (50 mM tris pH 7.5, 5mM $MgCl_2$, 0.1% β-mercaptoethanol pH7.2,1/250 aprotinin) for 1 or 10 min with equal concentrations of soluble GST-fusion protein in the absence or presence of 0.1mM Na_3VO_4 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

48 h after transient transfection, HeLa cells were grown on coverslips and serum-deprived for 3 h. Cells were either unstimulated or stimulated with IGF-1 (10^{-8} M) for 10 min.

Immunolabelling was performed as described (17) with fluorescein isothiocynate (FITC), or Texas-red-conjugated secondary antibodies (Jackson ImmunoResearch). Immunofluorescence microscopy analysis was carried out with a Biorad 1024 CLSM system using a 60X (1.4NA) planapochromatic objective (Nikon). Series of optical sections were collected and projected onto a single image plane using the laser sharp 1024 software and processing system. Co-localisation was estimated with the lasersharp software on ten representative cells and expressed as percent of IRS-1 co-localised with PTPL1.

Immunoelectron Microscopy

48 h 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% tween20 (incubation buffer) for 2 hours at room temperature. The sections were then incubated overnight at 4°C with rabbit anti-IRS-1 (33µg/ml) and mouse anti-HA (40µg/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

48 h after transient transfection, HeLa cells growing on glass coverslips were stimulated for 6h by IGF-1 (10nM) or unstimulated after 18h serum-deprivation. They were irradiated using a UV Stratalinker 1800 (Stratagene) with 300 J/m^2, and then cultivated in DMEM serum-free medium supplemented or not with 10^{-8} M IGF-1 for an additional 24h. Cells were subjected to indirect immunofluorescence with anti-HA antibody and Alexa Fluor 568 goat anti-mouse IgG antibody (Invitrogen, 1:200) for 30 min. Nuclei were stained with 1 µg/ml DAPI (Sigma). Images were acquired with a microscope immunofluorescent Leica obj X 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 performed 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-phosphotyrosine antibody (Fig. 2A, lower panel). 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 phosphotyrosine. 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, lower panel). This result suggests that the phosphatase catalytic domain displays striking substrate specificity. Indeed, four tyrosine-phosphorylated proteins among more than twenty were affinity precipitated by GST-DA (arrows 1 to 4). Two of them (arrows 3 and 4) were mainly represented on lysate, while the other two (arrows 1 and 2) were obviously enriched. Interestingly, the slowest migrating band corresponds to a 170kDa protein and was further identified as IRS-1 by immunoblotting using a specific antibody (Fig. 2A upper panel).

Using non-physiological pervanadate stimulation, we identified highly phosphorylated IRS-1 as a potential PTPL1 substrate. To validate this result with IRS-1 phosphorylated by IGF1-R, similar experiments were performed using lysates from HeLa cells overexpressing IRS-1 and IGF1-R and stimulated with IGF-1 as a source of tyrosyl-phosphorylated proteins. IGF-1 mainly induced the tyrosine phosphorylation of two proteins (Fig. 2B lower panel), one slightly phosphorylated protein of 170 kDa, which corresponds to IRS-1 as demonstrated by

Immunoblot analysis (Fig. 2B, upper panel) and one highly phosphorylated protein with a molecular weight corresponding to IGF1-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 IGF1-R precipitated from 150µl of lysate was lower than signal obtain from direct analysis of 40µl of lysate, whereas phosphorylated IRS-1 was strongly precipitated by the trapping mutant. These data further support the notion that IRS-1, more likely than IGF1-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 IGF-1-stimulated HEK 293 cells overexpressing IRS-1 and IGF1-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 about 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, two last tracks). 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 IGF-1-stimulated tyrosine phosphorylations *in vivo*, we assessed PTPL1-induced phosphorylation changes on HEK 293 cells overexpressing IGF1-R, IRS-1 and PTPL1 Wt or PTPL1-CS. Total lysates were analysed by direct immunoblotting using an anti-phosphotyrosine antibody. In the absence of PTPL1, three proteins were highly tyrosine-phosphorylated after IGF-1 stimulation. Their molecular weights correspond to transfected IRS-1 (p170), IGF1-R β chain (p95) and endogenously hyper-expressed IRS-4 (p160) (20) (Fig. 3A). 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, lower panel). On the contrary, in similar conditions, p170 phosphorylation was unaffected upon expression of the catalytic inactive PTPL1-CS mutant, suggesting that phosphatase activity of PTPL1 is essential for the hypo-phosphorylation of p170. To further characterize p170 as IRS-1, IRS-1 was immunoprecipitated from cells treated as above and its phosphorylation status was analysed by immunoblot using anti-phosphotyrosine. Similarly to p170, IGF-1-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 demonstrate that IRS-1 is a preferential substrate for PTPL1 in cellulo.

IGF-1 stimulation induces a rapid IRS-1 delocalisation from cytoplasm to plasma membrane where it recruits PI3K (21). In order to confirm that PTPL1 interferes with IRS-1 signalling, we studied the subcellular localisation of IRS-1 by indirect immunofluorescence and confocal microscopy after IGF-1 stimulation in the presence of PTPL1. In HeLa cells overexpressing IGF1-R and IRS-1, IGF-1 stimulation induced marked IRS-1 relocalisation from the cytoplasm to the plasma membrane (Fig. 3C compare 1 to 2). On the contrary, in the presence of PTPL1 a large proportion of IRS-1 remained in the cytoplasm upon growth factor stimulation (Fig. 3C panel 3). This inhibition of IRS-1 translocation by PTPL1 required its catalytic activity since IRS-1 localisation was unaffected in cells overexpressing the phosphatase-dead mutant PTPL1-CS (Fig. 3C panel 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 IGF1-R/IRS-1/PI3K pathway.

To test the direct action of PTPL1 on IRS-1 in intact cells, *in cellulo* substrate trapping was performed. 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 phosphotyrosine 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 IGF-1-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 IGF-1-treated HEK 293 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 lower panel). 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-phosphotyrosine antibodies revealed the presence of only one 170kDa protein specifically retained from the IGF-1 stimulated cells with the PTPL1-YF/DA (Fig. 4B lower panel). Re-probing of the blot with anti-IRS-1 confirmed that phospho-p170 was indeed IRS-1 (Fig. 4B median panel). The presence of a low amount of IRS-1 complexed with PTPL1 Wt, independent of IGF-1 stimulation, suggests a lower interaction of IRS-1 with PTPL1 independent of the PTP catalytic domain.

To further substantiate the interpretation that PTPL1 interacts directly with IRS-1, we studied the localisation of PTPL1 and IRS-1 upon IGF-1 stimulation by confocal microscopy in HeLa cells overexpressing IGF1-R, IRS-1 and PTPL1 Wt or PTPL1-YF/DA. PTPL1 and PTPL1-YF/DA both localised at the plasma membrane as previously described (17) (Fig. 4C). In cells

overexpressing PTPL1 Wt, IRS-1 was detected in the cytoplasm and at the plasma membrane, leading to a partial co-localisation with PTPL1 Wt (54% +/- 8%) (Fig. 4C). In cells overexpressing PTPL1-YF/DA, IRS-1 was totally delocalised to the plasma membrane and a complete co-localisation with PTPL1 was observed (92% +/- 3%) (Fig. 4C). Immunoelectron microscopy study further confirmed this co-localisation in microvilli-like protrusions (Fig. 4D, left panel) and in the apical submembrane region (Fig. 4D, right panel) in agreement with reported mouse PTPL1 localisation (23).

Altogether, these data clearly demonstrate 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 IGF1-R, alone or in combination with PTPL1-CS, were overexpressed in HEK 293 cells expressing endogenous PTPL1. IRS-1 tyrosine phosphorylation induced by IGF-1 was then analysed during a time course after growth factor deprivation. In the absence of exogenously-expressed PTPL1, IRS-1 tyrosine phosphorylation decreased immediately after IGF-1 deprivation (Fig. 5A, left panel). On the contrary, in cells overexpressing the phosphatase-dead mutant of PTPL1, IRS-1 phosphorylation remained unchanged after 60 min (Fig. 5A, right panel). 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 IGF-1 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 70-90% inhibition of PTPL1 transcription and expression, quantified by RT-PCR

(data not shown) and immunoblot analysis (Fig. 5B) respectively. In MCF7 cells, IRS-1 tyrosine phosphorylation decreased rapidly after IGF-1 deprivation (Fig. 5C, left panels). PTPL1 extinction resulted in an inhibition of IRS-1 dephosphorylation which was detectable 30 and 120 minutes after depletion (Fig. 5C, right panels). This result demonstrates that PTPL1 regulates IRS-1 phosphorylation in the more physiological cellular model in which we originally evidenced the importance of PTPL1 in anti growth-factor activity of antioestrogens.

PTPL1 inhibits IGF-1 induced Akt activation and block this survival pathway.

We have previously shown that PTPL1 was necessary for antioestrogen inhibition of the IGF1-R/IRS-1/PI3K/Akt pathway in MCF7 cells (12) and demonstrated, 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 HEK 293 cells overexpressing both IRS-1 and IGF1-R either with or without PTPL1 Wt and in MCF7 cells after PTPL1 extinction. In HEK293 cells, PTPL1 addition drastically decreased basal and IGF-1-induced Akt phosphorylation (Fig. S1A). In MCF7 cells, PTPL1 extinction increased the IGF-1-induced Akt phosphorylation and prolonged Akt activation after IGF-1 deprivation (Fig. 5D).

The ability of PTPL1 to induce apoptosis through the inhibition of the IGF1-R/IRS-1/Akt survival effect was therefore evaluated in HeLa and HEK 293 cells which overexpressed IGF1-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 IGF-1 treatments, on the percentage of apoptotic (Fig. 6) or living cells (Fig. S1), were evaluated by direct DAPI staining of nuclei or trypan blue exclusion, respectively. IGF1-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 HEK 293 in which IRS-1 and IGF1-R alone were

introduced (Fig.S1A). The addition of PTPL1 totally inhibited the IRS-1/IGF1-R protective effect regardless of the presence or absence of IGF-1 (Fig.6B, Fig.S1B). In addition, it weakly induced apoptosis in the absence of UV and strongly potentiated UV pro-apoptotic 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 IGF-1 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 antioestrogen-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 IRS-1/PI3K/Akt pathway, which leads to increased apoptosis and inhibition of the IGF-1 survival effect. Here we show that PTPL1 is sufficient to induce apoptosis and to inhibit the IGF-1-induced IGF1-R/IRS-1/PI3K/Akt pathway and we demonstrate 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 which 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 bis-phosphorylated insulin receptor peptides (26). However, specificity of PTPL1 for peptides from other multi-phosphorylated 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 twenty tyrosine phosphorylated proteins (vanadate stimulation) or compared to full length IGF1-R (IGF-1 stimulation). Furthermore, after PTPL1 overexpression we observe a total inhibition of IRS-1 phosphorylation while IGF1-R phosphorylation is only partially decreased. This result is in agreement with recent data that

indirectly pointed to PTPL1 involvement in IRS-1 signaling. In fact, Toretsky et al. have shown that EWS-FlI1 inhibits basal IRS-1 phosphorylation without inhibition of IGF1-R phosphorylation (27), and, more recently, that PTPL1 is a direct transcriptional target of EWS-FLI1 and mediates its effects (28).

The PTPL1 pro-apoptotic effect corroborates its function as tumour suppressor gene.

Several studies have addressed the role of PTPL1 in apoptosis regulation, focusing particularly on its interaction with Fas. Sato *et al*. introduced the first evidence of such a relationship in their demonstration of the inhibitory interaction of PTPL1 with the carboxyl-terminal tail of the pro-apoptotic receptor Fas (29). 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 IGF-I 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 IRS-1/PI3K/Akt pathway, which leads to increased apoptosis and inhibition of the IGF-1 survival effect by antioestrogens (12). PTPL1 impact on this pathway was then suggested by two other research groups. Indeed, Alessi's group (33) has shown that extinction of TAndem-PH-domain-containing Protein-1 (TAPP1), which binds to the first PDZ domain of PTPL1 and

regulates PTPL1 membrane localisation, enhances Akt/PKB activation in response to IGF-1. Also, 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 tumours where PTPL1 is highly expressed as compared to in HEK 293 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 tumour susceptibility. Indeed none phenotypic consequences have been reported about the PTPN13 KO (34) and studies on mice that lack PTPN13 phosphatase activity focalize on haematopoietic cell lineages and peripheral nervous system (35) which were previously shown to express the phosphatase (36;37). Crossbreeding these mice models with mammary tumour model are, however, required to evaluate the role of PTPL1 in tumour progression or susceptibility.

Key roles for the PI3K/Akt signalling 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 demonstrate that PTPL1 inhibits Akt activation through IRS-1 dephosphorylation. Moreover we show that PTPL1 drastically inhibits IGF-1 apoptosis protection and induces apoptotic cell death at least by inhibition of the IGF1-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 eight deleted the entire catalytic phosphatase domain and five point mutations were located within the phosphatase domain. These finding suggest that

PTPL1 activity plays a role in regulating cancer growth and/or survival responses (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 downregulation or silencing was observed in multiple lymphomas and carcinomas through hypermethylation of its promoter (15). Altogether, these clinical studies suggest PTPL1/PTPN13 as a putative tumour suppressor gene in several tissues and our present results provide mechanistic support to this hypothesis.

Conclusion

By identification of the PTPL1 target in the IGF-1 survival pathway, we presently describe the first mechanism by which the putative tumour suppressor gene PTPL1/PTPN13 can inhibit tumour progression. This pro-apoptotic 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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FIGURE LEGENDS

Figure 1 : PTPL1 expression constructs. A schematic representation of the PTPL1 expression constructs is shown. Protein segments produced by transient expression are indicated 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 which 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.

Figure 2 : Identification of IRS-1 as a major substrate of PTPL1 *in vitro*. HeLa cells overexpressing IRS-1 and IGF1-R were serum-starved and stimulated with 10mM Pervanadate (P) for 30 min. at 37°C **(A)** or 10 nM IGF-1 (I) for 10 min. at 37°C **(B)**. Cells were lysed in trapping buffer, and proteins were resolved by SDS-PAGE (7.5% gel) (Input) or incubated with the indicated GST fusion proteins immobilized on beads. Bound materials were immunoblotted with anti-pTyr antibodies (lower panel) and then reprobed with the anti-IRS-1 antibody (upper panel). Molecular weight markers are indicated in kilodaltons on the left. Numerated arrows (**A**) or arrows (**B**) point to the major affinity precipitated proteins. **C** Immunoprecipitates (IP) of IRS-1 were prepared from IGF-1 stimulated HEK 293 cells, co-transfected with a combination of IRS-1 and IGF1-R, and incubated for 1 or 10 min with the indicated soluble GST fusion proteins in the presence or absence of 1mM pervanadate (Perv.). The immunoprecipitates were immunoblotted (IB) with the anti-pTyr antibodies (lower panel). Equivalent amounts of IRS-1 were confirmed by reprobing the blots with the anti-IRS-1 antibody (*top panel*). The two first lanes represent direct immunoblotting of control and IGF-1-stimulated cell lysates.

Figure 3 : PTPL1 dephosphorylates IRS-1 in *cellulo*. A. HEK 293 cells transiently co-transfected with IGF1-R, IRS-1 and HA-tagged PTPL1 Wt or PTPL1-CS expression vectors were unstimulated (-) or stimulated (+) with IGF-1 for 10 min. Total lysates were analysed by direct immunoblotting with anti-PTyr antibodies (lower panel). The arrows 1, 2, 3 indicate the molecular weight corresponding to transfected IRS-1 (170 kDa), endogenous IRS-4 (160 kDa) and transfected IGF1-R (95 kDa), respectively. Equivalent amounts of IRS-1 and PTPL1 were confirmed by reprobing the blots with anti-IRS-1 (upper panel) and anti-HA antibodies (median panel). **B**. Cell lysates analysed above by immunoblot were used for immunoprecipitation (IP) experiments with anti-IRS-1 polyclonal antibodies. The immune complexes were then immunoblotted (IB) with anti-pTyr antibodies (top panel). Equivalent amounts of IRS-1 were confirmed by reprobing the blots with anti-IRS-1 antibody (lower panel). **C.** Transiently transfected HeLa cells, unstimulated (**1**) or stimulated with IGF-1 (**2, 3, 4**) were treated for indirect TRITC localisation of IRS-1. Images represent horizontal confocal sections. In cells co-transfected with an empty vector, IGF-1 stimulation induces a marked IRS-1 delocalisation from cytoplasm to plasma membrane (white arrow **2**). In cells overexpressing PTPL1 Wt, IRS-1 remains partially localised in the cytoplasm (**3**), whereas expression of IRS-1 was localised at the plasma membrane in cells overexpressing the catalytically-inactive PTPL1-CS (white arrow **4**).

Figure 4 : PTPL1-DA complexes with IRS-1 *in cellulo*. IRS-1 and IGF1-R were transiently co-transfected with either the HA-tagged full-length PTPL1 Wt or PTPL1-DA expression vectors into HEK 293 or HeLa cells **A.** IRS-1 was immunoprecipitated from lysates prepared from IGF-1 treated (+) or untreated (-) HEK 293 cells and immunoblotted for the presence of PTPL1 using anti-HA antibodies (lower panel). An equal expression level of PTPL1 and mutant was confirmed by direct immunoblotting of lysate (upper panel). **B.** PTPL1 was immunoprecipitated (IP) from the same lysates using anti-HA antibodies and immunoblotted

(IB) for the presence of associated tyrosine-phosphorylated proteins (lower panel). Re-probing of the blot with anti-IRS-1 allows confirmation that the unique phosphoprotein is IRS-1 (median panel). An equal expression level of IRS-1 was confirmed by direct immunoblotting of lysate (upper panel). **C.** HeLa cells were treated for indirect FITC localisation of PTPL1 constructs with anti-HA antibody (left) and indirect TRITC localisation or IRS-1 (middle). Images represent merges of horizontal confocal sections (right). **D.** HEK 293 cells were subjected to double immunogold labeling in scanning electron microscopy. HA PTPL1-DA was visualized by 6-nm gold particles and IRS-1 by 15-nm gold particles. The left panel correspond to enlargement of microvilli-like protrusions and the right panel to the apical submembrane region. Bars are 100 nm.

Figure 5 : Endogenous PTPL1 is responsible for IRS-1 dephosphorylation. A. HEK 293 cells were co-transfected with IRS-1 and IGF1-R alone (empty vector) or in association with PTPL1-CS. The cells were serum-starved for 3h, then stimulated (+) or not (-) for 10 min. with IGF-1. The stimulated cells were IGF-1-deprived for 30 and 60 min. They were lysed, and equal amount of proteins were resolved by two separate SDS-PAGE. One membrane was probed with anti-Ptyr antibodies (lower panel) and the other with the anti-IRS-1 antibody (upper panel). **B.** 5 days after siRNA transfection, MCF7 cells were lysed and the expression of PTPL1 was monitored by Western blot using anti–PTPL1 antibodies (upper panel). Loading control was obtained by reprobing the membrane with anti-Actin (lower panel). The blots were scanned and values for PTPL1 normalized to β-Actin. **C.** 5 days after siRNA transfection, MCF7 cells, serum-starved for 18h, were stimulated (+) or not (-) for 10 min. with IGF-1, then IGF-1-deprived for 30 and 120 min. Constant level of IRS-1 after IGF-1 starvation was confirmed by Western blot using anti-IRS-1 antibodies (lower panel). The same cell lysates were used for immunoprecipitation experiments with anti-IRS-1 polyclonal antibodies. The immune complexes were then immunoblotted with anti-pTyr antibodies

(upper panel). The blots were scanned and values for IRS-1 tyrosine phosphorylation normalized to total IRS-1. **D.** Equal amount of cell lysates (prepared as in C.) were resolved by two separate SDS-PAGE (12% gel). One membrane was probed with anti-phospho Akt (Ser 473) antibody (upper panel) and the other with anti-Akt antibody (lower panel).

Figure 6 : PTPL1 induces apoptosis. HeLa cells (**A** and **B**) overexpressing IRS-1 and IGF1-R or Akt 308D/473D (Akt*) were stimulated by IGF-1 (I) or unstimulated (C) after 24h serum-deprivation in the presence or absence of overexpressed PTPL1 Wt. Cells were submitted to UV light (300J/m^2) and then cultivated for 24h 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. In the case of co-transfection, dual indirect immunostaining with anti-HA monoclonal antibody and anti-IRS-1 polyclonal antibodies showed that more than 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.** IGF-1 stimulated cells submitted to UV light. Arrows indicate condensed chromatin and apoptotic bodies (left), transfected cells (middle), and apoptotic transfected cells (right). **B.** The proportion of apoptotic cells among the transfected (150 to 300 cells, depending on transfection efficiency) and untransfected cells (900 cells) was counted by fluorescent microscopy. Mean +/- S.D (IRS-1/IGF-1-R transfected n=3; Akt* transfected n=2). (Student's *t*-Tests * $p<0.05$; ** $p<0.005$). MCF7 (C and D) cells transfected with control or PTPL1 specific siRNA were stimulated by IGF-1 (I) or unstimulated (C) after 24h serum-deprivation. Cells were submitted to UV light (150J/m^2) and then cultivated for 24h 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. Mean +/- S.E. (n=3). (Paired Student's *t*-Tests * $p<0.05$; ** $p<0.005$).