



Published in final edited form as:

J Cell Biochem. 2013 August ; 114(8): 1810–1818. doi:10.1002/jcb.24525.

Methylation of the PTPRO Gene in Human Hepatocellular Carcinoma and Identification of VCP as Its Substrate

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Abstract

We have previously reported that the gene encoding protein tyrosine phosphatase receptor type-O (*PTPRO*) is suppressed by promoter methylation in a rat model of hepatocellular carcinoma (HCC) and it functions as tumor suppressor in leukemia and lung cancer. Here, we explored the methylation and expression of *PTPRO* as well as its function in human HCC. MassARRAY analysis of primary human HCC and matching liver samples ($n = 24$) revealed significantly higher ($P = 0.004$) methylation density at the promoter CGI in tumors. Combined bisulfite restriction analysis (COBRA) of another set of human HCC samples ($n = 17$) demonstrated that the CGI was methylated in 29% of tumors where expression of *PTPRO* was lower than that in corresponding matching livers. A substrate-trapping mutant of *PTPRO* that stabilizes the bound substrates was used to identify its novel substrate(s). *VCP/p97* was found to be a *PTPRO* substrate by mass spectrometry of the peptides pulled down by the substrate-trapping mutant of *PTPRO*. Tyrosyl dephosphorylation of *VCP* following ectopic expression of wild-type *PTPRO* in H293T and HepG2 cells confirmed that it is a *bona fide* substrate of *PTPRO*. Treatment of *PTPRO* overexpressing HepG2 cells with Doxorubicin, a DNA damaging drug commonly used in therapy of primary HCC, sensitized these cells to this potent anticancer drug that correlated with dephosphorylation of *VCP*. Taken together, these results demonstrate methylation and

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The authors declare no conflict of interest.

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downregulation of PTPRO in a subset of primary human HCC and establish VCP as a novel functionally important substrate of this tyrosine phosphatase that could be a potential molecular target for HCC therapy.

Keywords

TYROSINE PHOSPHATASE; PTPRO; P97/VCP; HCC; METHYLATION

Protein tyrosine phosphatases (PTPs) have been shown to play a pivotal role in the control of numerous physiological processes, including cell proliferation and differentiation (for reviews, see [Jacob and Motiwala, 2005; Motiwala and Jacob, 2006; Ostman et al., 2006; Julien et al., 2011]). The family of PTPs is composed of over 100 members, which are structurally diverse and include non-transmembrane and receptor-like enzymes [Wang et al., 2003; Alonso et al., 2004]. PTPs dephosphorylate tyrosine residues thereby modulating the activities of its substrates and subsequent signaling pathway(s). PTPs have been shown to exhibit the characteristics of oncogenes and tumor suppressors by modulating the intensity and duration of the signaling pathway regulated by the cascade of kinases [Motiwala and Jacob, 2006; Sastry and Elferink, 2011]. Recent studies have developed novel therapeutic strategies to target PTPs that are either over-expressed or hyperactive in different types of cancers [Easty et al., 2006; Jiang and Zhang, 2008]. Targeting these deregulated PTPs by small molecule inhibitors has been used in clinical trials [Blaskovich, 2009]. These recent advances highlight the importance of elucidating the biological function of PTPs and their role in tumorigenesis.

Several investigations, including ours, have explored the role of protein tyrosine phosphatase receptor-type O (PTPRO) in tumor suppression and in apoptosis [Motiwala et al., 2004; Motiwala and Jacob, 2006; Juszczynski et al., 2009; Motiwala et al., 2009]. In a rat model of hepatocarcinogenesis induced by folate-methyl deficient diet, PTPRO was identified as a candidate tumor suppressor by genome wide screening for hypermethylated genes [Motiwala et al., 2003]. This study showed significant down-regulation of the full-length variant of *PTPRO* (*PTPRO*-FL) in diet-induced hepatocellular carcinoma (HCC). Further, we found an inverse correlation between *PTPRO* expression and methylation of its CpG island (CGI) in this animal model of HCC. Similar observations concerning methylation and suppression of PTPRO were made in other types of cancer, including lung cancer [Motiwala et al., 2004], leukemia [Motiwala et al., 2007, 2009, 2011; Juszczynski et al., 2009] and breast cancer [Ramaswamy et al., 2009]. A few oncogenic kinases such as Bcr-abl, Lyn, Zap70, and Syk were identified as substrates of PTPRO_t, the truncated form of PTPRO, in leukemia/lymphoma [Chen et al., 2006; Motiwala et al., 2009, 2010]. PTPRO_t can function as a tumor suppressor by inactivating these oncogenic kinases. In contrast to extensive studies on PTPRO_t over the past few years, the role of PTPRO-FL in malignant transformation is only being explored now. A recent study has demonstrated that PTPRO is suppressed in hepatocellular carcinoma and that its expression is important in regulating oncogenic STAT3 signaling [Hou et al., 2012]. Another report showing upregulation of PTPRO during mammary epithelial cell morphogenesis and a direct correlation between PTPRO expression and breast cancer patient survival suggests tumor suppressor function of

PTPRO in breast cancer [Yu et al., 2012]. The present study was undertaken to determine whether PTPRO was also methylated in primary human liver tumors and identify its substrate(s) in this tumor.

Here, we show that the CpG island (CGI) of *PTPRO* is significantly hypermethylated in a subset of primary human hepatocellular carcinomas relative to their matching normal tissues. To investigate its biological function further, we combined substrate-trapping assay with Mass Spectrometry (MS) to identify its substrates in HCC cell lines. After several substrate-trapping assays, we confirmed that VCP (Valosin containing protein), an ATP-binding protein implicated in multiple cellular events [Sauer et al., 2004; Jentsch and Rumpf, 2007; Stolz et al., 2011], is a *bona fide* substrate of PTPRO in HCC cells. This enzyme-substrate relationship was confirmed in vivo in HepG2 liver cancer cells. Further, ectopic expression of PTPRO sensitized HCC cells to Doxorubicin, a major anticancer drug used in HCC therapy. These data, taken together, indicate that suppression of *PTPRO* and enhanced phosphorylation of its substrate VCP could contribute to hepatocarcinogenesis.

MATERIALS AND METHODS

ANTIBODIES

Anti-VCP antibody was generously provided by Dr. Nicholas K. Tonks [Zhang et al., 1999]. Other reagents/antibodies and sources (in parentheses) are as follows: Anti-Syk (Santa Cruz, sc-1077), anti-Ku70 (Santa Cruz, sc-56129), anti-Histidine (Abgent, AM1010a), anti-Villin-1 (Cell signaling, 2369), anti-Spectrin (Abcam, ab11755), anti-Flag (Sigma, F1804).

MASSARRAY ANALYSIS OF THE METHYLATION LEVEL OF *PTPRO* CGI

To quantify DNA methylation level of PTPRO CGI, MassARRAY analysis was performed as described [Ehrich et al., 2005; Ghoshal et al., 2010]. Briefly, genomic DNA was treated with sodium bisulfite and the PTPRO CGI was PCR amplified followed by in vitro transcription and RNase A cleavage. The molecular weight of the resulting RNA fragment was further analyzed by MALDI-TOF to determine the methylation level.

CLONING, EXPRESSION AND PURIFICATION OF GST-TAGGED SUBSTRATE-TRAPPING MUTANTS OF PTPRO_t

These analyses were performed essentially as described [Motiwala et al., 2010].

REAL-TIME RT-PCR ANALYSIS

Real-time RT-PCR analysis of mRNAs was performed using SYBR Green chemistry. Relative expression was calculated using C_T method [Livak and Schmittgen, 2001].

IN VITRO SUBSTRATE-TRAPPING ASSAY

The assay was performed as described [Motiwala et al., 2010] with the following modifications. HepG2 cells (ATCC) were treated with 100 μ M pervanadate for 20 min at 37°C. Whole cell extract from the pervanadate-treated HepG2 cells (10 mg) was incubated overnight at 4°C with either GST alone or GST-tagged PTPRO_t (WT, CS/DA) bound to GSH sepharose beads. The washed beads were boiled in 2× SDS-PAGE loading buffer and

protein supernatant was separated on 8% SDS-PAGE followed by Coomassie Brilliant Blue staining. The protein bands of interest were excised from the stained gel and subjected to mass spectrometry (see below). This experiment was repeated with reduced amount of whole cell extract (1 mg) for validation of the substrates by Western blot analysis.

IDENTIFICATION OF SUBSTRATES BY MASS SPECTROMETRY (MS)

The protein bands enriched in CS/DA group as well as the corresponding bands in WT group were selectively cut from the Coomassie brilliant blue stained gels and subjected to in-gel trypsin digestion [Zhang et al., 2007]. Briefly, the gel bands were soaked in wash buffer (50% Methanol, 5% acetic acid, and 45% dH₂O) twice for 1 h, dried in acetonitrile and incubated with 10mM DTT and 50mM iodoacetamide for 30 min. After washing twice with 100mM ammonium bicarbonate and drying in a speedvac, the gel pieces were incubated for 10 min in 50mM ammonium bicarbonate and 300 ng trypsin/gel band. Another 20 μ l of 50mM ammonium bicarbonate was then added and the mixture was incubated at 37°C for 2 h. The peptides were extracted from the gel three times with total 90 μ l extraction solution (acetonitrile: formic acid: water = 50:5:45), concentrated to 15–20 μ l aliquot in a speedvac, and the extracted proteins were subjected to liquid chromatography followed by tandem MS. The MS scan data was further analyzed by MassMatrix algorithm as described [Xu and Freitas, 2007].

IN VIVO DEPHOSPHORYLATION OF POTENTIAL SUBSTRATES IN H293T CELLS EXPRESSING PTPRO

H293T cells (ATCC) were transiently transfected with flag-tagged PTPRO-FL and treated with 10 μ M pervanadate for 10 min prior to harvest. Whole cell extracts were prepared in RIPA buffer (50mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1mM sodium orthovanadate, 1mM NaF, and protease inhibitors) and any insoluble debris was removed by centrifugation. Equal volumes of protein extracts (500 μ g) were incubated with anti-phosphotyrosine antibodies [a 1:1 mixture of 4G10 (Millipore, 05-321) and PY20 (Santa Cruz, sc-508)]. Protein G agarose beads (Invitrogen 15920010) were then added to precipitate the antibody-bound peptides harboring phospho-tyrosine residue(s). The immunoprecipitated proteins were separated on SDS-PAGE and immunoblotted with the indicated antibodies.

GENERATION OF PTPRO^t EXPRESSING HepG2 CELLS

HepG2 cells stably expressing PTPRO^t-Flag were generated as described [Motiwala et al., 2007] using pRetro-On system. Briefly, HepG2 cells were infected with viral particles from either pRetro-On (vector control) or PTPRO^t (WT or CS mutant) transfected phoenix cells. HepG2 cells expressing PTPRO^t were selected with puromycin (0.5 μ g/ml) for 10 days and maintained in medium containing puromycin (0.5 μ g/ml). PTPRO^t expression was confirmed by Western blot analysis.

IN VIVO DEPHOSPHORYLATION OF VCP IN HepG2 CELLS EXPRESSING PTPRO^t

The selected HepG2 cells expressing PTPRO^t were subsequently split in medium containing Doxorubicin (5 μ g/ml) for 24 h. The phosphorylation levels of VCP in PTPRO^t expressing

HepG2 cells were analyzed by similar experiments described above for in vivo dephosphorylation of VCP in H293T cells.

CLONING OF VCP (WT AND Y → F MUTANT)

VCP(wt)-EGFP was a gift of Dr. Nico Dantuma (Addgene plasmid #23971) [Tresse et al., 2010]. HA-tagged WT and mutant VCP were generated by PCR amplification of VCP from VCP(wt)-EGFP using a common forward primer (hVCP-F-BamHI: 5'-CTGGATCCTCATGGCTTCTGGAG-3') and reverse primers with single base pair change to introduce the Y → F mutation at Y805 and Y796 (hVCP-RXbaI: 5'-ATTCTAGATTAGCCATACAGGTCATC-3'; hVCP-Y805F-RXbaI: 5'-ATTCTAGATTAGCCAAACAGGTCATC-3'; hVCP-Y796FR-XbaI: 5'-ATTCTAGATTAGCCATACAGGTCATCATCATTGTCTTC TGTGAATACA-3'). The mutant bases are indicated in bold and restriction sites are underlined. The amplified VCP was sub-cloned into the *Bam*HI and *Xba*I sites of HA-pcDNA3.

RESULTS

PTPRO IS METHYLATED AND SUPPRESSED IN PRIMARY HUMAN HCC

We have previously demonstrated methylation of *PTPRO* encoding the receptor-type protein tyrosine phosphatase and its suppression in the liver tumors induced in rodents fed folate-methyl deficient diet [Motiwala et al., 2003]. Here, we extended this study to human hepatocellular carcinoma to determine whether it was similarly methylated and suppressed in the human disease. For this purpose, we analyzed a panel of liver tumor tissue and its adjacent normal tissue by MassARRAY that can quantitatively measure the methylation level of each CpG dinucleotide within the CGI (Fig. 1A). Unsupervised clustering of the data shows that a subset of tumor tissues with hypermethylated CGI segregate from the unmethylated normal tissues and unmethylated tumor tissues (Fig. 1B). The overall methylation density at the promoter CGIs was significantly higher ($P = 0.004$) in tumors compared to the matching livers from the same patients (Fig. 1B,C). Similarly, MassARRAY analysis of HCC cell lines, including HepG2, Huh7, and Hep3B also revealed hypermethylation at the promoter CGI (Fig. 1C). Because a majority of samples analyzed for methylation were available as formalin fixed tissues it was technically difficult to obtain good quality RNA from them for RT-PCR analysis and, therefore, to make quantitative correlation between the methylation status and *PTPRO* expression. We were, however, able to secure a new set of freshly frozen primary HCC samples, prepare intact RNA and measure *PTPRO* expression quantitatively by real-time RT-PCR. Of the 17 pairs of normal and tumor tissues analyzed, seven exhibited either undetectable ($n = 3$, data not shown) or unaltered ($n = 4$, blue labeled data in Fig. 1D) expression of *PTPRO*. Lack of *PTPRO* expression in certain samples is consistent with the recently published report that also observed negative immunohistochemical staining for *PTPRO* in ~44% of cases analyzed [Hou et al., 2012]. Among the 14 pairs where *PTPRO* mRNA was detectable at different levels in normal and tumor samples, 29% ($n = 4$) exhibited lower expression of *PTPRO* mRNA in the tumors relative to the matching normal tissue (Fig. 1D). Combined bisulfite restriction analysis (COBRA) of a few representative samples from each group demonstrated methylation of CGI in the tumors where expression was below that of

matching liver samples (Fig. 1E, top panel). On the contrary, no significant methylation was observed in the tumors with higher levels of *PTPRO* expression (Fig. 1E, bottom panel). These data demonstrate that downregulation of *PTPRO* in primary human HCC samples is associated with methylation of its CGI.

VCP IS A *BONA FIDE* SUBSTRATE OF PTPRO

Due to the very fast dephosphorylation reaction that *PTPRO* catalyzes, we used substrate-trapping mutants as described previously [Motiwala et al., 2010] to identify its potential substrates in hepatocellular carcinoma (see Materials and Methods for details). The full-length *PTPRO* (*PTPRO*-FL) shares identical intracellular domains with its truncated form (*PTPRO*t), which is expressed exclusively in hematopoietic cells. We, therefore, used both WT and CS/DA double mutants of *PTPRO*t for binding to potential tyrosyl phosphorylated (pY) substrates (see Materials and Methods for detailed description of this assay). Whole cell lysates from HepG2 cells treated with pervanadate (100 μ M), a potent phosphatase inhibitor, to stabilize tyrosyl phosphorylated proteins, were incubated with GST, GST-wild type, and GST-substrate-trapping CS/DA mutant of *PTPRO*t (Fig. 2A). Compared to the wild type *PTPRO*, CS/DA mutant efficiently trapped several proteins including p97 and p200 (Fig. 2B). The bands corresponding to proteins pulled down by CS/DA mutant and the corresponding band/gel region from the pull-down with WT protein were both excised from the gel, digested with trypsin, and subjected to mass spectrometry.

Analysis of the peptide fragments by mass spectrometry revealed two high-scoring proteins in the p97 band. The top-ranked protein corresponded to valosin containing protein (VCP) (Fig. 2C), a member of the ATPase family. Twenty peptides matched the VCP protein sequence, spanning 31% of the full-length protein (Fig. 2D, grayed text). Supplementary Figure 1 shows the list of 20 peptide sequences of VCP detected by mass spectrometry. The second protein in the p97 band corresponded to Villin-1 (Fig. 2C). Validity of the mass spectrometry data was assessed by immunoblot analysis of the pulled-down proteins. Consistent with the high hit score for VCP in the CS/DA pull-down (score = 478) and lack of any hits in the WT pull-down, VCP was only detected in the CS/DA pull-down (Fig. 3A). This data confirmed the authenticity of this assay showing specific interaction between VCP and *PTPRO*. On the other hand, immunoblot analysis with antibody against Villin-1 showed higher levels of association with WT than CS/DA mutant of *PTPRO*t (Fig. 3A). This is in agreement with the mass spectrometry data that detected this protein in both CS/DA (hit score = 391) and WT (hit score = 262) groups. These data suggest that Villin-1 interacts nonspecifically with *PTPRO* without functioning as an authentic substrate.

The p200 band was identified as α -Spectrin by mass spectrometry analysis (Fig. 2C). Although the hit score of α -Spectrin in the p200 band was higher in the CS/DA group, subsequent Western blot with anti-Spectrin antibody showed that similar levels of this protein were pulled-down by both WT and CS/DA (Fig. 3A). Because this protein is frequently observed in other mass spectrometry-related studies as well, it probably interacts with *PTPRO* nonspecifically, and is, therefore, not considered a biologically relevant substrate of this protein tyrosine phosphatase. Taken together, these data indicate that VCP is a candidate substrate of *PTPRO* in HepG2 cells.

Next, we repeated the substrate-trapping assay using extracts from pervanadate treated Hep3B cells followed by immunoblotting with antibodies against proteins identified by mass spectrometry. Since Hep3B cells express Syk [Yuan et al., 2006], an established substrate of PTPROt in B-cells [Chen et al., 2006], we also examined the potential association of Syk with the CS/DA mutant of PTPRO. Indeed, Syk was associated with the CS/DA mutant of PTPROt but not with WT PTPROt (Fig. 3B). Similarly, VCP was only associated with the CS/DA mutant of PTPROt (Fig. 3B). On the contrary, α -Spectrin and Villin-1 were not associated with either WT or the CS/DA mutant of PTPROt (data not shown). This data again suggests that VCP, but not Villin-1 or α -Spectrin, is a physiological substrate of PTPRO.

PTPRO SUPPRESSES GROWTH OF HCC CELLS AND INCREASES THEIR SUSCEPTIBILITY TO DOXORUBICIN BY DEPHOSPHORYLATING ITS SUBSTRATES

Although it would be ideal to generate stable HCC cells expressing the full-length PTPRO, the predominant form expressed in the liver [Motiwala et al., 2003], we were unable to detect its ectopic expression in HCC cell lines despite several attempts. It is likely that overexpression of PTPRO-FL suppresses the tumor cell proliferation as demonstrated by us earlier [Motiwala et al., 2004]. We, therefore, stably expressed the wild type (WT) and catalytic site mutant (CS) of the truncated form of PTPRO (PTPROt) in HepG2 cells and used these cell lines to study the role of PTPRO as a tumor suppressor and in the regulation of VCP phosphorylation. Assessment of growth by MTT assay over a period of 72 h demonstrated a significant decrease in growth of PTPROt-WT expressing cells compared to the vector transfected and PTPROt-CS expressing cells (Fig. 4A) suggesting that phosphatase activity of PTPRO was necessary for its growth suppressive function. To determine whether PTPROt can sensitize HCC cells to commonly used chemotherapeutic drugs, HepG2 cells expressing PTPROt were treated with Doxorubicin. As expected, PTPROt-WT expressing HepG2 cells exhibited increased sensitivity to the drug after 72 h of treatment compared to vector control and PTPROt-CS expressing cells (Fig. 4B) reinforcing the significance of phosphatase activity of this enzyme. Reduced level of pY-VCP in HepG2 cells overexpressing PTPROt-WT, compared to the vector transfected and PTPROt-CS expressing cells, was confirmed by immunoprecipitating tyrosine phosphorylated proteins followed by Western blot analysis with anti-VCP antibody (Fig. 4C). These observations strongly suggest that PTPROt expression enhances the susceptibility of HCC cells to Doxorubicin, at least, in part, due to hypophosphorylation of VCP.

To confirm that the full-length form of PTPRO can dephosphorylate VCP, H293T cells were transiently transfected with PTPRO-FL. pY-proteins were immunoprecipitated from vector control and PTPRO expressing H293T cells treated with pervanadate. The extracts and immunoprecipitated proteins were immunoblotted with anti-VCP, anti-Syk (a previously identified substrate of PTPROt) and anti-Ku-70 (normalizer). As anticipated, both VCP and Syk were hypophosphorylated in H293T cells expressing PTPRO (Fig. 4D). This observation confirms that the substrates identified and verified by in vitro and in vivo assays performed with PTPROt are indeed substrates of PTPRO-FL.

PTPRO TARGETS Y805 OF VCP

To identify the tyrosine residue(s) of VCP that is(are) dephosphorylated by PTPRO, we investigated whether mutation of a specific tyrosine of VCP to phenylalanine (Y → F) would disrupt its interaction with the CS/DA mutant of PTPROt. Previous studies suggest that Y805 is the major phosphorylation site of VCP with some phosphorylation occurring at Y796 [Ferlay et al., 2010]. We, therefore, initially focused on these two sites to determine if they are the targets of PTPROt. The HA-tagged VCP, WT, or Y → F mutants, were transfected into H293T cells and cell lysates from pervanadate treated cells were used for substrate-trapping with bacterially expressed and purified 6X-His-PTPROt-CS/DA. The data demonstrated that binding of both Y805F and Y796F/Y805F was dramatically reduced (>90%) suggesting that Y805 is the major target site of PTPRO (Fig. 5). Further, binding of Y796F was also reduced by ~50% suggesting that this site may also partially contribute to the interaction of VCP with PTPRO (Fig. 5).

DISCUSSION

The present study has demonstrated high levels of *PTPRO* methylation in a subset of primary human HCC relative to matching normal liver tissues. It is noteworthy that methylation in the promoter region correlated inversely with expression of *PTPRO* in a few samples, which is consistent with its growth suppressor function in lung cancer [Motiwala et al., 2004] and leukemia [Motiwala et al., 2007, 2009, 2010]. However, *PTPRO* expression in a few HCC samples was not altered or was higher than that observed in matching liver tissue from the same patient. Interestingly, the *PTPRO* CpG island was not methylated in these specimens. It is likely that some tumors expressing relatively high level of PTPRO are at early stage of tumorigenesis or that PTPRO expression may vary depending on the tumor etiology. Interestingly, a large cohort study of human primary HCC that included 120 male and 60 female patients, has shown that PTPRO expression is higher in the adjacent tissues compared to the tumor tissues [Hou et al., 2012]. The same study also demonstrated that PTPRO functions as a tumor suppressor in HCC by downregulating the activity of STAT3, a strong tumor activator [Hou et al., 2012].

This study used Mass Spectrometry based technique to identify the tyrosyl phosphorylated substrates of PTPRO in hepatocellular carcinoma. To our knowledge, this is the first report on the application of this non-candidate approach to identify the less prominent substrate(s) of PTPRO. These analyses have led to the identification of VCP as a substrate of PTPRO in HCC cells. VCP, an ATPase, is a ubiquitously expressed enzyme with a wide variety of cellular functions [Frohlich et al., 1991; Wang et al., 2004]. It is involved in cell division, membrane fusion, inhibition of apoptosis, DNA damage repair, and ubiquitin-dependent protein degradation. VCP was initially identified as a tyrosyl phosphorylated protein in antibody stimulated B- and T-cells [Schulte et al., 1994]. It was later demonstrated that its phosphorylation did not affect ATPase activity but altered its association with clathrin, thus regulating clathrin coated vesicular transport [Egerton and Samelson, 1994]. Cell cycle dependent tyrosyl phosphorylation of cdc48, the yeast homolog of VCP, also results in its translocation from ER to nucleus where it localizes at the centrosome during mitosis. Mutation of the tyrosine results in loss of nuclear translocation and inhibition of cell growth

[Madeo et al., 1998]. Consistent with this observation, protein tyrosine phosphatase (PTPH1) has been shown to affect growth of NIH3T3 cells through tyrosyl dephosphorylation of VCP [Zhang et al., 1999]. Similarly, our studies have shown that growth of HCC cell line HepG2 is inhibited following ectopic expression of PTPRO that targets VCP. These findings strengthen the notion that tyrosyl phosphorylation of VCP is closely associated with the regulation of cell growth and that the growth inhibitory function of PTPRO could possibly involve dephosphorylation of VCP. An important aspect of this study is that tyrosine dephosphorylation of VCP by PTPRO sensitizes HepG2 cells to Doxorubicin, a chemotherapeutic drug commonly used for a variety of cancers. Further, we have identified Y805 of VCP as the major target of PTPRO. Since phosphorylated Y834 of cdc48 (corresponding to Y805 of VCP) is important for cell cycle progression [Madeo et al., 1998], our data suggests that PTPRO functions to inhibit cell growth through hypophosphorylation of VCP. It is also noteworthy that elevated expression of VCP in HCC is known to be associated with increased incidence of tumor recurrence [Yamamoto et al., 2003]. Thus it appears that reduced expression of PTPRO in a subset of HCC probably facilitates the function of over-expressed VCP.

Another key observation in this study is that Syk, a previously identified substrate of PTPRO in B-cells [Chen et al., 2006], is also a substrate in Syk-expressing Hep3B cells (Fig. 3B). Contrary to its function as an oncogene in B-cell malignancies, Syk has been reported to function as a tumor suppressor in solid tumors like breast cancer and HCC [Coopman et al., 2000; Yuan et al., 2006; Inubushi et al., 2008]. It is possible that PTPRO functions as an oncogene in a subset of HCC patients exhibiting relatively higher level of PTPRO, potentially by inactivating the tumor suppressor Syk.

In summary, the data generated here have led to the following conclusions: (i) PTPRO CpG island is methylated in a subset of primary HCC relative to the matching normal tissue, (ii) methylation of CpG island in the tumor correlates with lower expression of PTPRO, (iii) VCP and Syk are among the substrates of PTPRO in the liver, (iv) expression of PTPRO in non-expressing HCC cell line sensitizes these cells to the commonly used anticancer drug Doxorubicin, (v) the tumor suppressor function of PTPRO in HCC is, at least in part, mediated by reduced phosphorylation of VCP, and (vi) VCP Y805 is the major target site of PTPRO. These results thus support a tumor suppressor function of PTPRO in a subset of HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to acknowledge Drs. Liwen Wang, Kelly DiRienzo, and Jia You from Dr. Michael Freitas' team for help with the mass spectrometry.

Grant sponsor: NIH; Grant numbers: CA086978, DK088076.

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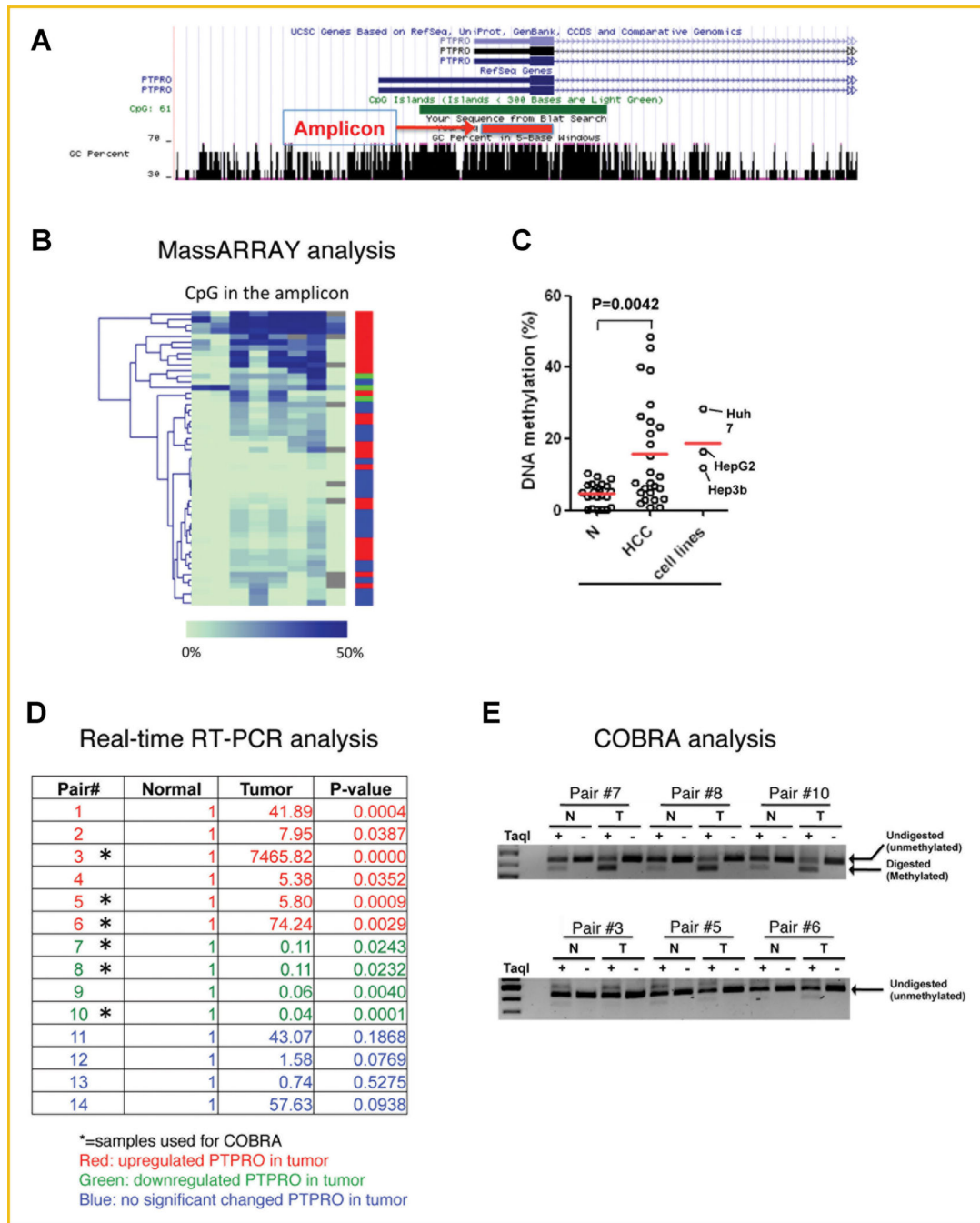


Fig. 1. Methylation and expression of PTPRO in primary human HCC. A: Schematic representation of the amplicon where CpG methylation was measured by Mass Array. B: Heatmap displaying quantitative DNA methylation level of single CpG units (columns). Rows represent samples (red = tumor samples; blue = normal controls, green = cell lines). Dark blue encodes high methylation values (>50%), bright green stands for low methylation levels and gray for missing data. Unsupervised clustering was performed using Euclidian distance. C: Scatter plot displaying the average DNA methylation values for the CpG island

at the PTPRO transcriptional start site (TSS) in matching benign livers (N), HCC specimens (HCC) and cell lines. Red bars represent the median. Significance was assessed by non-parametric two-sided Mann–Whitney test. D: Real-time RT-PCR analysis of PTPRO in 14 pairs of primary HCCs and matching liver tissues. The data is represented as fold change in tumor over normal. The samples labeled with asterisks were used for COBRA assay. E: COBRA assay for methylation in a few representative pairs where PTPRO expression was lower in tumors (top panel) and where PTPRO expression was higher in tumors (bottom panel).

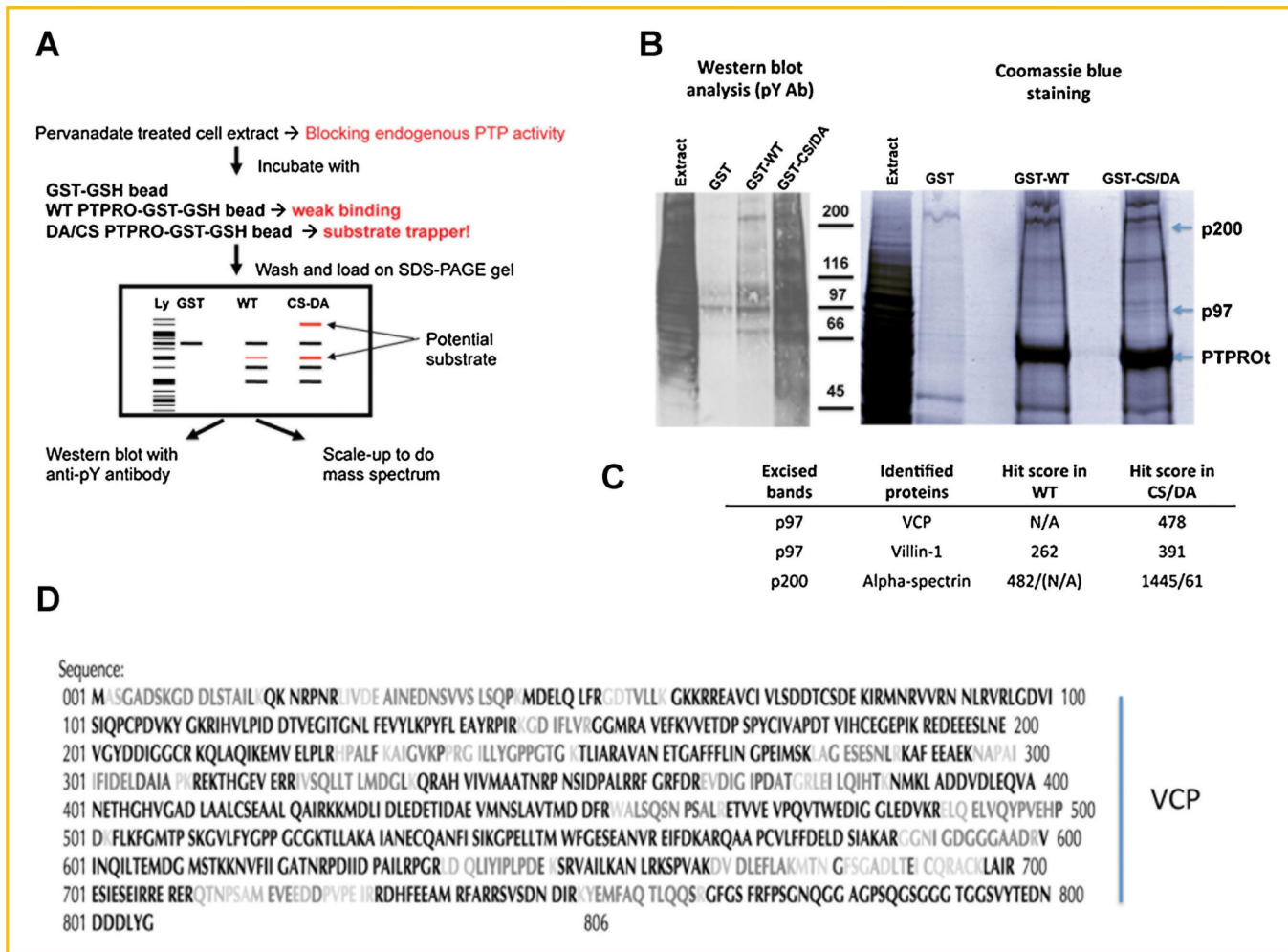


Fig. 2. Identification of PTPRO substrate(s) in the HCC cell line by mass spectrometry. **A:** Schematic view of the substrate trapping method. **B:** Total lysate prepared from pervanadate treated HepG2 cells was incubated with GSH-beads conjugated to GST-tagged WT form or CS/DA mutant form of PTPRO. After washing, the associated proteins and beads were boiled in SDS-PAGE sample buffer, resolved on a SDS-PAGE gel followed by Western blot analysis with anti-pY Ab (left panel) or Coomassie blue staining (right panel). Arrows indicate the position of protein bands excised from gel for LC/MS-MS analysis. **C:** Tabular list of proteins identified from the excised band by mass spectrometry. Hit scores were generated by MassMatrix database [Xu and Freitas, 2007]. **D:** Twenty peptides matched the VCP protein sequence (grayed), spanning 31% of the full-length protein.

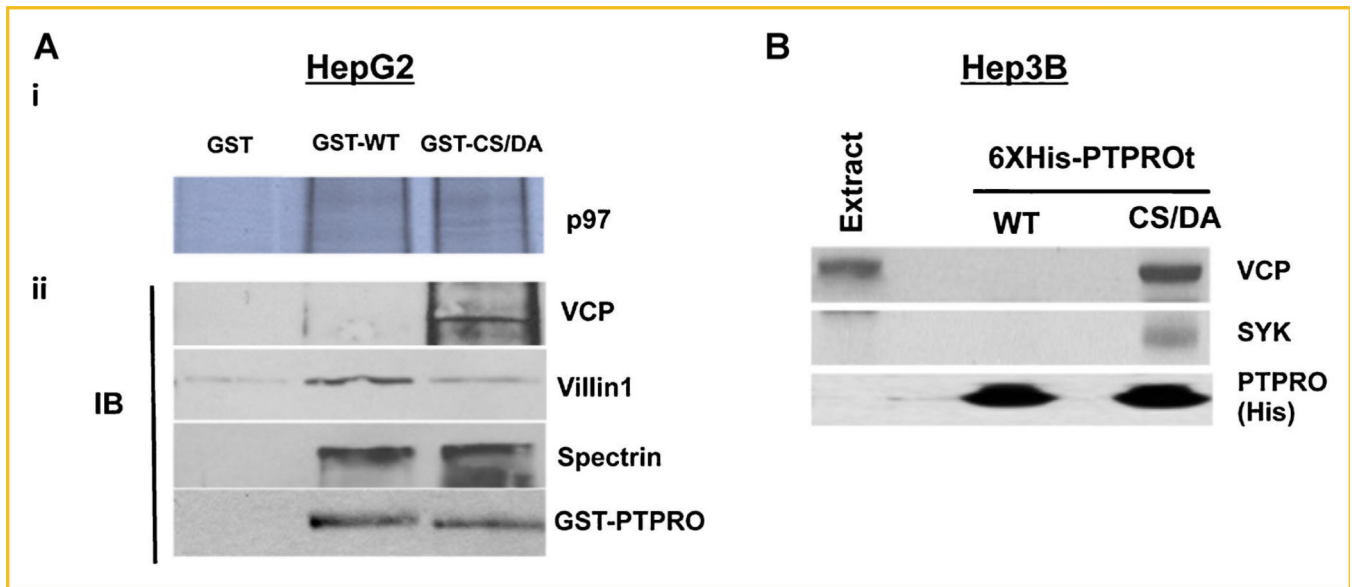


Fig. 3. Identification of PTPRO substrate(s) in the HCC cell lines by Western blot analysis. A: (i) Enlarged middle section at p97 position of Figure 2B, right panel. (ii) Immunoblot analysis with anti-VCP, anti-Villin-1, anti- α -Spectrin, anti-GST antibodies was performed on a duplicate blot. B: Substrate-trapping followed by Western blot analysis was performed using whole cell extracts from pervanadate treated Hep3B cells. Since Hep3B cells express Syk, a previously identified substrate of PTPRO, immunoblotting with anti-Syk was used as a positive control.

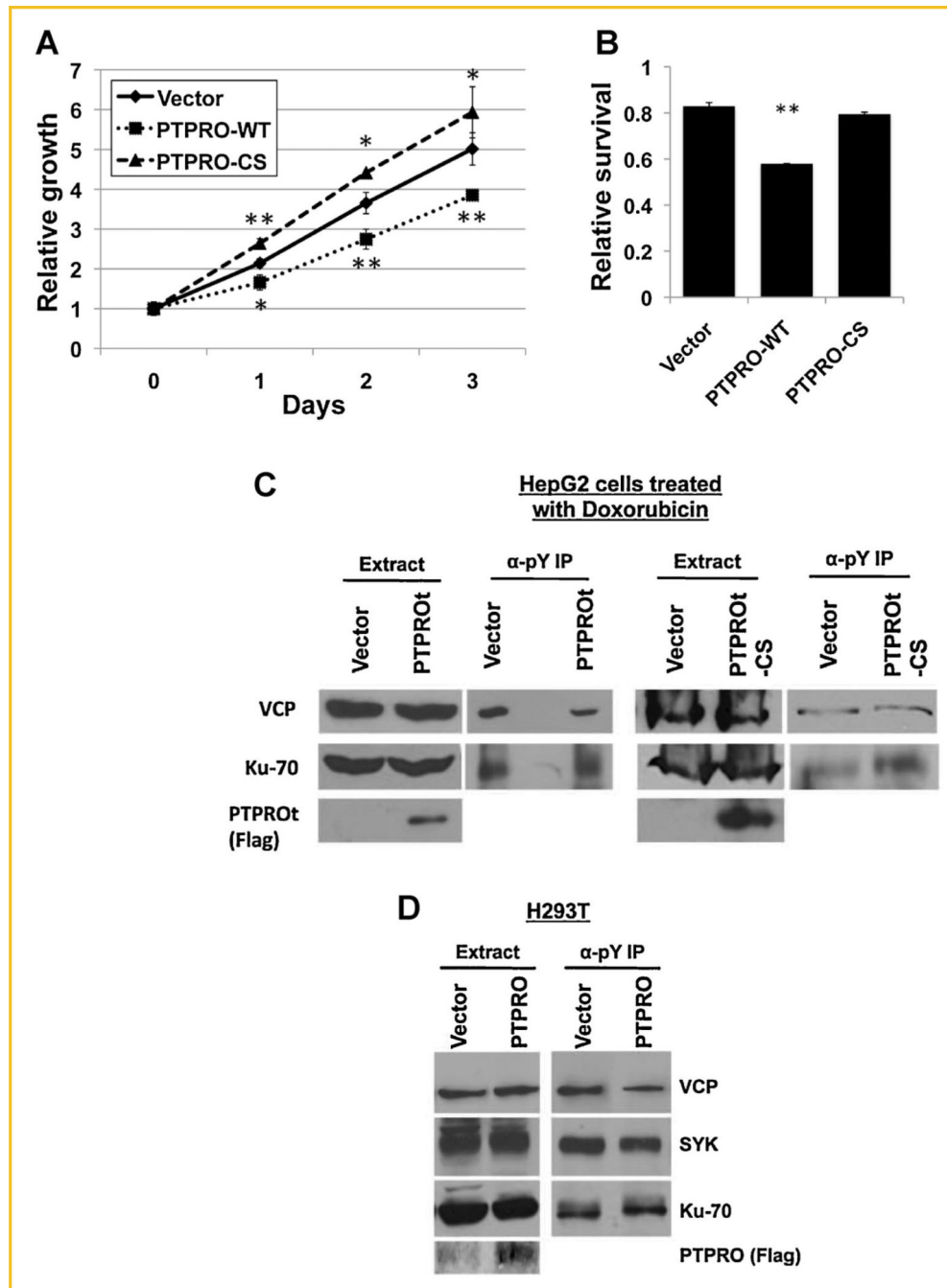


Fig. 4. Ectopic expression of PTPROt sensitizes HCC cells to Doxorubicin treatment. **A:** Growth of vector-transfected and PTPROt expressing HepG2 cells seeded in a 96-well plate was followed by MTT assay over a period of 72 h. **B:** Vector-transfected or PTPROt expressing HepG2 cells were treated with 2.5 μ M Doxorubicin for 72 h and cell survival was measured by MTT assay. Survival of untreated cells was taken as 1. **C:** Phosphotyrosine-containing proteins were immunoprecipitated from cell lysates of pervanadate-treated HepG2 cells (vector control and PTPROt stable transfectants) or, **D,** PTPRO-FL expressing H293T cells

using anti-phosphotyrosine antibody 4G10 + Y20 followed by pull down with protein G agarose beads. The bound proteins were then analyzed by immunoblotting with antibodies as indicated in the figure. *P*-value: * $P < 0.05$; ** $P < 0.01$.

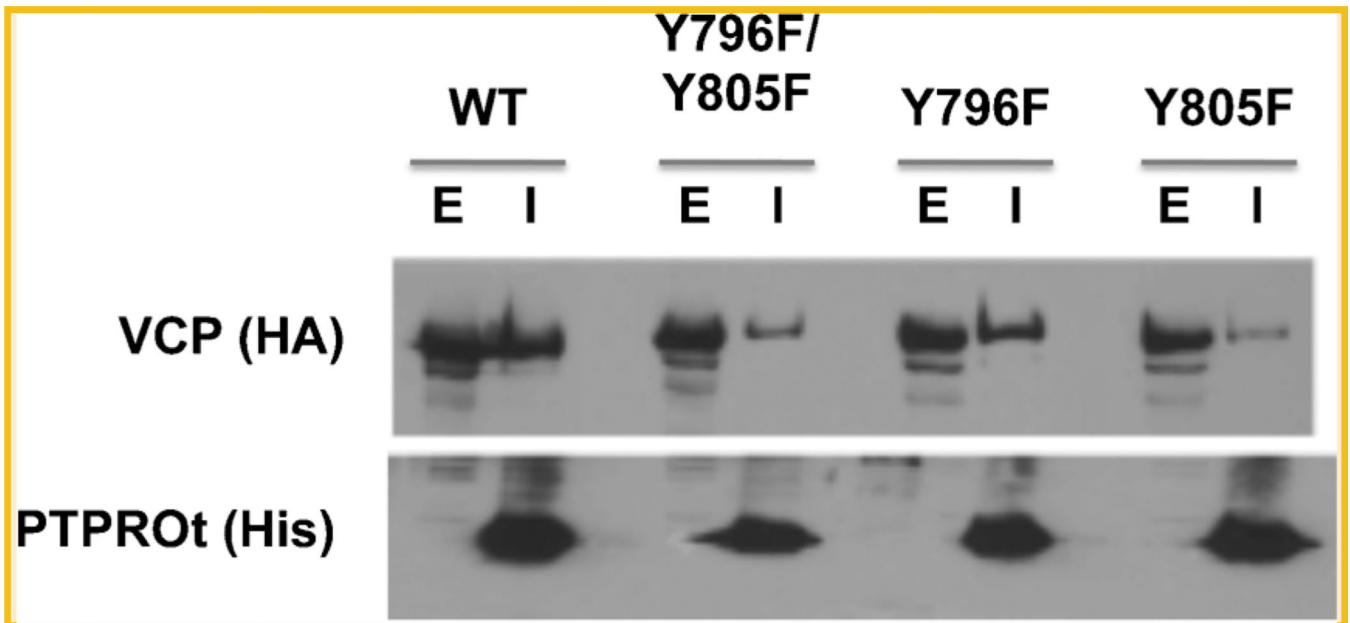


Fig. 5. PTPRO targets Y805 of VCP. HA-tagged VCP (WT and mutants) were transfected into H293T cells and the cells were treated with 100 μ M pervanadate to enhance tyrosine phosphorylation. Equal amount of cell extracts were incubated with bacterially expressed and purified 6X-His-PTPROt-CS/DA. Extracts (E) and proteins bound to PTPROt-CS/DA (I) were separated on SDS-PAGE and immunoblotted with anti-HA (for VCP) and anti-His (for PTPROt).