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Insulin-Like Growth Factor Type-I Receptor-Dependent Phosphorylation of Extracellular Signal-Regulated Kinase 1/2 but not Akt (Protein Kinase B) Can Be Induced by Picropodophyllin

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ABSTRACT

The initial event upon binding of insulin-like growth factor 1 to the insulin-like growth factor type-I receptor (IGF-1R) is autophosphorylation of tyrosine residues within the activation loop of the kinase domain followed by phosphorylation of other receptor tyrosine residues and the subsequent activation of the intracellular signaling cascades. We found recently that the cyclolignan picropodophyllin (PPP) inhibits phosphorylation of IGF-1R and phosphatidyl-3 kinase/Akt (protein kinase B) signaling molecules without interfering with the highly homologous insulin receptor. Furthermore, PPP causes regression of tumor grafts and substantially prolongs the survival of animals with systemic tumor disease. It is of interest that we show here that short treatments with PPP activate the intracellular extracellular signal-regulated kinase (ERK) signaling. Our data suggest that PPP induces IGF-1R ubiquitination and in turn activates ERK1/2. The PPP-induced ERK activation requires IGF-1R because PPP is not able to induce ERK phosphorylation in IGF-1R-negative cells or in cells in which the receptor is knocked down by small interfering RNA. Moreover, in the absence of Mdm2, an E3 ligase that has been shown previously to be involved in IGF-1R ubiquitination, the phosphorylation of ERK did not occur. Thus, apart from inhibiting the receptor activity, PPP can induce IGF-1R ubiquitination and stimulate ERK in an Mdm2-dependent manner. This response could contribute to the apoptotic effect of PPP.

The insulin-like growth factor type-I receptor (IGF-1R) is important for transformation and proliferation of malignant cells (Baserga, 1995, 1999, 2000; Girnita et al., 2000a,b; Yu and Rohan, 2000; Larsson et al., 2005) and is crucial for preventing apoptosis (Baserga, 1995, 1999, 2000; Yu and Rohan, 2000). On the other hand, IGF-1R is not critical for growth of normal cells (LeRoith et al., 1995; Yu and Rohan, 2000).

Ligand (IGF-1) induced tyrosine phosphorylation of IGF-1R leads to activation of the phosphatidyl inositol-3 kinase (PI3K), the mitogen-activated protein kinase (MAPK)/ERK and the 14-3-3 pathways (Baserga, 1995, 2000; LeRoith et al., 1995; Yu and Rohan, 2000). Recent data have shown that IGF-1R is also a substrate for ubiquitination (Girnita et al., 2003, 2005, 2007; Vecchione et al., 2003). We identified Mdm2 to be an E3 ligase involved in the covalent attachment of ubiquitin moieties to lysine residues in IGF-1R (Girnita et al., 2003). Furthermore, we showed that Mdm2-mediated IGF-1R ubiquitination requires β-arrestin1 as a molecular scaffold in bridging the ligase to the receptor (Girnita et al., 2005, 2007). The interaction of Mdm2 and β-arrestin1 seems to have two functions in regulation of IGF-1R, one being down-regulation (desensitization) of IGF-1R (Girnita et al., 2005), and the other one to control ERK1/2 activation and G_1-S progression of the cell cycle (Girnita et al., 2007). Cells with a dominant-negative *MDM2* con-

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ABBREVIATIONS: IGF-1R, insulin-like growth factor type-I receptor; PPP, picropodophyllin; PI3K, phosphatidyl inositol-3 kinase; siRNA, small interfering RNA; SBS, substrate binding site; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; WT, wild type; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DN, double negative; IRS, insulin receptor substrate.

struct or siRNA to β-arrestin1 do not exhibit IGF-1R-dependent ERK phosphorylation and are arrested in the G_1 phase (Girnita et al., 2007). These data suggest that ubiquitination plays important roles in biology of IGF-1R.

Recently we identified an inhibitor (picropodophyllin or PPP) of IGF-1R that caused tumor regression in animal models (Girnita et al., 2004; Menu et al., 2006). PPP inhibits activity of IGF-1R (Girnita et al., 2004, 2006; Vasilcanu et al., 2004, 2006; Ulfarsson et al., 2005; Strömberg et al., 2006; Colón et al., 2007; Conti et al., 2007; Guha et al., 2007; Razuvaev et al., 2007; Shields et al., 2007) but also down-regulates the receptor (Vasilcanu et al., 2008). Even though PPP may have other effects, we conclude that it does not cross-target the highly homologous insulin receptor (Girnita et al., 2004). A recent study shows that PPP drastically prolongs the survival in an animal model of multiple myeloma. The animals were treated daily up to 150 days, and survival was prolonged with almost 3 months compared with the control group (Menu et al., 2007). Despite having a high antitumor efficacy, PPP is apparently also well-tolerated in vivo.

The PPP mechanisms of action are not yet completely understood. We and others demonstrated that PPP is an inhibitor of the IGF-1R tyrosine phosphorylation (Girnita et al., 2004; Colón et al., 2007; Conti et al., 2007; Shields et al., 2007). In contrast, PPP did not inhibit the tyrosine kinase activity of insulin receptor or other major cancer-relevant growth factor receptors (Girnita et al., 2004). PPP did not interfere with the IGF-1R tyrosine kinase at the level of ATP binding site (Girnita et al., 2004), suggesting other mechanisms of action (e.g., inhibition at the level of receptor substrate) (Vasilcanu et al., 2004). In addition, we have demonstrated recently that PPP induces ubiquitination and down-regulation of the IGF-1R (Vasilcanu et al., 2008). However, PPP does not affect signaling or degradation of the highly homologous insulin receptor (Girnita et al., 2004; Fulzele et al., 2007; Vasilcanu et al., 2008).

The apoptotic effect of PPP has been associated with substantial inhibition of the PI3K/Akt pathway (Vasilcanu et al., 2004), whereas in many studied cell systems, the inhibitory effects on the ERK pathway were lower or absent (Girnita et al., 2004; Conti et al., 2007). From a therapeutic point of view, this imbalance in effects on the two major pathways could be favorable. Phosphorylation of ERK1/2 is namely important for the G_1-S transition, and inhibition of this reaction leads to G_1 arrest (Weber et al., 1997; Hoshino et al., 2001; Johnson and Lapadat, 2002). Therefore, a comparably weak inhibition of ERKs may maintain cells in the cell cycle. Because cycling cells are more prone to apoptotic cell death compared with G_1-arrested ones (Baserga, 1994), an agent attenuating Akt phosphorylation but more or less preserving the ERK activity should increase apoptotic cell death.

In this study, we aim to investigate the effects of PPP on ERK phosphorylation in closer detail. An interesting question is whether PPP just causes a weak inhibition of phospho-ERK or even induces an activation of ERK phosphorylation.

Materials and Methods

Reagents. PPP was synthesized as described previously (Buchardt et al., 1986), and after recrystallization, its purity was 99.7%. For experimental purposes, PPP was dissolved in dimethyl sulfoxide before addition to cell cultures. Polyclonal IGF-1R antibodies (N-20, C-20, and H-60), a monoclonal antibody to phosphotyrosine (PY99), a monoclonal antibody to Mdm2 (SMP14), monoclonal antibodies to p-p38 (D-8), p38 (A-12), JNK (FL), p-JNK (G-7), and a monoclonal antibody to ubiquitin (p4D1) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-pErk1/2, anti-Erk1/2, anti-pAkt (serine 473), and anti-Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA). All other reagents unless stated otherwise were from Sigma (St. Louis, MO).

Cell Cultures. The human glioblastoma cell line U343MG was given to us by Dr. Monica Nistér (CCK, Karolinska Institutet, Stockholm, Sweden). The human melanoma cell line BE has been described elsewhere (Kanter-Lewensohn et al., 2000). The R−, R+, P6, 46, 56, and 96 mouse cell lines were from Dr. Renato Baserga (Thomas Jefferson University, Philadelphia, PA). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. P6, R−, R+, 46, 56, and 96 cell lines were cultured in the presence of G418 (Promega, Madison, WI).

Small Interfering RNA. Chemically synthesized, double-strand small interfering RNAs (siRNAs), with 19-nucleotide duplex RNA and 2-nucleotide 3′-dTdT overhangs, were purchased from Dharmacon (Lafayette, CO). The siRNA targeting the human IGF-1R sequence 5′-GCAGACACCUACAAC AUCAUU-3′ was used to deplete endogenous IGF-1R levels in the BE cell line (Rosengren et al., 2006). The cells were transfected using Dharmafect siRNA Transfection reagent 1 according to manufacturer's protocol. Mdm2 expression was lowered using siRNA targeting human MDM2 mRNA (5′-AAG CCA UUG CUU UUG AAG UUA-3′) supplied by Dharmacon (Lafayette, CO). siRNA (200 pmol) was transfected into cells using oligofectamine reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. A nonsilencing RNA duplex (SmartPool; Dharmacon) was used as a control as described previously (Girnita et al., 2005).

Transfections. The BE melanoma cell line plated at subconfluent density in 6-cm dishes was transiently transfected with 2 μg/ml DNA plasmids containing Mdm2 constructs WT-MDM2 ($MDM2_{1–491}$) or DN-MDM2 ($MDM2_{1–400}$) as described elsewhere (Girnita et al., 2003) using Lipofectamine 2000 (Invitrogen). After 24 h, the transfected cells were split into six-well plates and cultured for an additional 24 h. During the last 12 h, cells were starved and then stimulated for with 50 ng/ml IGF-1 and/or treated with PPP. Protein extracts were prepared for immunoprecipitation or Western blot analyses.

Immunoprecipitation. The isolated cells were lysed as described elsewhere (Girnita et al., 2000b). To detect IGF-1-stimulated ubiquitination, 10 mM *N*-ethylmaleimide was added to the lysis buffer. Fifteen microliters of Protein G Plus-A/G agarose and 1 μg of antibody were added to 1 mg of protein material. After overnight incubation at 4°C on a rocker platform, the immunoprecipitates were collected by centrifugation in a microcentrifuge at 2500 rpm for 2 min. The supernatant was discarded, whereupon the pellet was washed and then dissolved in a sample buffer for SDS-PAGE.

SDS-PAGE and Western Blotting. Protein samples were dissolved in a sample buffer containing 0.0625 M Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, bromphenol blue, and dithiothreitol. Samples corresponding to 50 to 100 μg of cell protein were analyzed by SDS-PAGE with a 7.5 or 10% separation gel. Molecular weight markers (Bio-Rad Laboratories, Stockholm, Sweden) were run simultaneously. After SDS-PAGE, the proteins were transferred overnight to nitrocellulose membranes (GE Healthcare, Uppsala, Sweden) and then blocked for 1 h at room temperature in a solution of 5% (w/v) skimmed milk powder and 0.02% (w/v) Tween 20 in PBS, pH 7.5. Incubation with appropriate primary antibodies was performed for 1 h at room temperature or overnight at 4°C. This was followed by washes with PBS and incubation with either a horseradish peroxidase-labeled or a biotinylated secondary antibody (Amersham) for 1 h. After the biotinylated secondary antibody, incubation with streptavidin-labeled horse peroxidase was performed. The detection was made with either ECL, Amersham, or by Supersignal West Pico reagents (Pierce, Rockford, IL). The films were scanned by Fluor-S (Bio-Rad).

Immunofluorescence Confocal Microscopy. Immunofluorescence confocal microscopy was performed as described elsewhere (Girnita et al., 2007). After experimental conditions, cells were fixed with 5% formaldehyde diluted in PBS before confocal analyses. For immunostaining of phospho-ERK1/2, an anti-pErk1/2 antibody (Cell Signaling Technology) was used.

Cell Viability Assay. Cell viability was assessed using the Cell Proliferation kit II (XTT) (Roche, Mannheim, Germany) according to the manufacturer's instructions. In brief, cells were cultured in 96-well plates in 100 μl of medium. After the incubation periods, 50 μl of XTT labeling mixture was added to each well and incubated for an additional 1 h. Spectrophotometric absorbance was measured at 492 nm using an enzyme-linked immunosorbent assay reader.

Results

Effect of PPP on ERK and Akt Phosphorylation in Glioblastoma Cells. We observed that the effects of PPP on ERK and Akt phosphorylation differ substantially in IGF-1-stimulated glioblastoma cells (line U343MG): phosphorylation of Akt is reduced, whereas ERK phosphorylation is increased (data not shown). After serum starvation, the cells were stimulated with different doses of PPP and/or IGF-1. Figure 1 shows the kinetic effects of PPP (500 nM) on IGF-1-induced ERK (Fig. 1A) and Akt (s473) phosphorylation (Fig. 1B) in the glioblastoma cell line. As shown, IGF-1 (10 min) induces a clear ERK activation. We were surprised to find that if the cells are treated with PPP for 1 h, phospho-ERK is drastically increased. After longer treatments with PPP (2–6 h), phospho-ERK was then gradually decreased. Akt was markedly phosphorylated by IGF-1 (Fig. 1B) and clearly decreased by PPP in a time-dependent manner.

Next, we investigated whether PPP in itself could induce ERK activation in the glioblastoma cells. In these experiments, we analyzed the effects after shorter periods of treatment (i.e., 10 and 20 min). Two doses of PPP were used, 500 or 2500 nM. As shown, PPP clearly induced ERK activation (Fig. 1C). A 20-min treatment with 500 nM PPP exceeded the effect of 10-min stimulation with IGF-1. The higher dose of PPP did not generate any significantly higher ERK phosphorylation.

Short Treatment with PPP Induces ERK Activation in IGF-1R-Expressing Cells but Not in IGF-1R-Negative Ones. We also investigated the effects of short PPP exposures on phospho-ERK in melanoma cells (line BE). After serum starvation, the cells were stimulated with IGF-1 or different doses of PPP. Figure 2A demonstrates that both 10- and 20-min treatments with PPP at 500 nM caused significant increases in ERK phosphorylation in these cells as well. Further increase of the PPP dose to 2500 or 5000 nM does not induce a significant additional ERK activation, suggesting that a plateau is reached at 500 nM. The levels were comparable with that obtained by 10-min stimulation with IGF-1 (Fig. 2A). Western blot analysis of the IGF-1R activity showed the absence of IGF-1R phosphorylation. Figure 2A also shows that PPP does not cause activation of Akt (Fig. 1B). Two more signaling pathways were investigated in BE cells: JNK and p38. JNK was constitutively phosphorylated in BE cells, and neither IGF-1 nor PPP increased its phosphorylation (data not shown). A slight increase in p38 phosphorylation was observed both after IGF-1 (12% $\pm$ 3.5; p = 0.56) and PPP (16% $\pm$ 4.5; p = 0.44) treatment; however, this increase was not statistically significant.

Next, the BE cells were exposed to 500 nM PPP for longer time periods. As shown in Fig. 2B, the maximal ERK activation was reached after 20 min, after which the phospho-ERK levels declined.

To elucidate whether the PPP-induced ERK activation is mediated via the IGF-1R, we compared the effects on P6 and R− cells. P6 is a mouse fibroblast cell line overexpressing human IGF-1R, and R− is an IGF-1R-deficient cell line. After serum starvation, the cells were treated with different doses of PPP or were stimulated with IGF-1. Figure 3A shows that ERK is activated significantly by short exposures to PPP even though the effects are lesser than those observed in glioblastoma and melanoma cell lines (compare Figs. 1 and 2). The strongest ERK phosphorylation was induced by a 20-min exposure to 2500 nM PPP, which reached a level of 60% of that induced by IGF-1 (Fig. 3A). Like the case of glioblastoma and melanoma cells, PPP did not activate Akt (data not shown). As expected, in R− cells, only serum and not IGF-1 could induce ERK activation (Fig. 3B). PPP did not affect the phospho-ERK levels in these cells (Fig. 3B). These data suggest that PPP-induced ERK activation requires IGF-1R expression. To further investigate this, we treated BE cells with siRNA targeting IGF-1R (Fig. 3C). After serum starvation, the cells were treated with different doses of PPP or were stimulated with IGF-1. The mock-transfected cells responded to both IGF-1 and PPP with ERK activation. In the IGF-1R siRNA-transfected cells, there was no clear increase in phospho-ERK compared with the mock control. Figure 3C also confirms that siRNA completely down-regulated the IGF-1R.

To verify that ERK is not an off-target of IGF-1R siRNA, we stimulated both the siRNA- and mock-treated cells with serum or IGF-1. IGF-1 could activate ERKs only in mock-transfected cells whereas upon stimulation with serum, phosphorylation of ERKs increased strongly in both mock- and IGF-1R siRNA-transfected BE cells (Fig. 3C, right), demonstrating that the ERK signaling pathway is not an siRNA off-target. These data provide further support for the notion that PPP-induced ERK activation is dependent on the IGF-1R.

PPP Induces IGF-1R Ubiquitination and ERK Activation in an Mdm2-Dependent Manner. It has been demonstrated that IGF-1 induces ubiquitination of the IGF-1R and enhances its degradation (Girnita et al., 2003, 2005; Vecchione et al., 2003). We identified Mdm2 to be an E3 ligase in this respect (Girnita et al., 2003). We reported that the action of Mdm2 and of its adapter protein β-arrestin1 is necessary for IGF-1-induced ERK activation (Girnita et al., 2007). Therefore, it would be interesting to investigate whether the action of Mdm2 is involved in PPP-induced ERK signaling. We first analyzed whether PPP (500 nM), administered for 1 to 10 min, could induce IGF-1R ubiquitination. BE cells were serum-starved and then exposed to PPP or IGF-1. Cell lysates were immunoprecipitated for ubiquitin and blotted for IGF-1R. As demonstrated previously (Girnita et al., 2005), a 10-min stimulation with IGF-1 induced ubiquitination of the receptor (Fig. 4A). In addition, 5- to 10-min treatments with PPP resulted in IGF-1R ubiquitination (Fig. 4A).

Next, we investigated the effects of constructs expressing deleted *MDM2* ($MDM2_{1–400}$, lacking the ligase domain) or wild-type (WT) *MDM2* ($MDM2_{1–491}$) on PPP-induced ERK activation. The $MDM2_{1–400}$ construct has been demonstrated

to exert dominant-negative (DN) effects on IGF-1R ubiquitination and IGF-1-induced ERK activation, whereas WT-*MDM2* increases these activities (Girnita et al., 2005, 2007). The expression of truncated DN Mdm2 and overexpression of WT Mdm2 was confirmed (data not shown). As shown in Fig. 4B, transfection of an empty vector into BE serum-starved cells does not change the ERK responses to IGF-1 or PPP. In the cells transfected with the DN-*MDM2* construct, ERK is no longer activated in response to PPP or IGF-1 (Fig. 4B). On the other hand, overexpression of MDM2 in the cells transfected with WT-*MDM2* ($MDM2_{1-491}$) induces an increased ERK activation compared with mock-transfected cells.

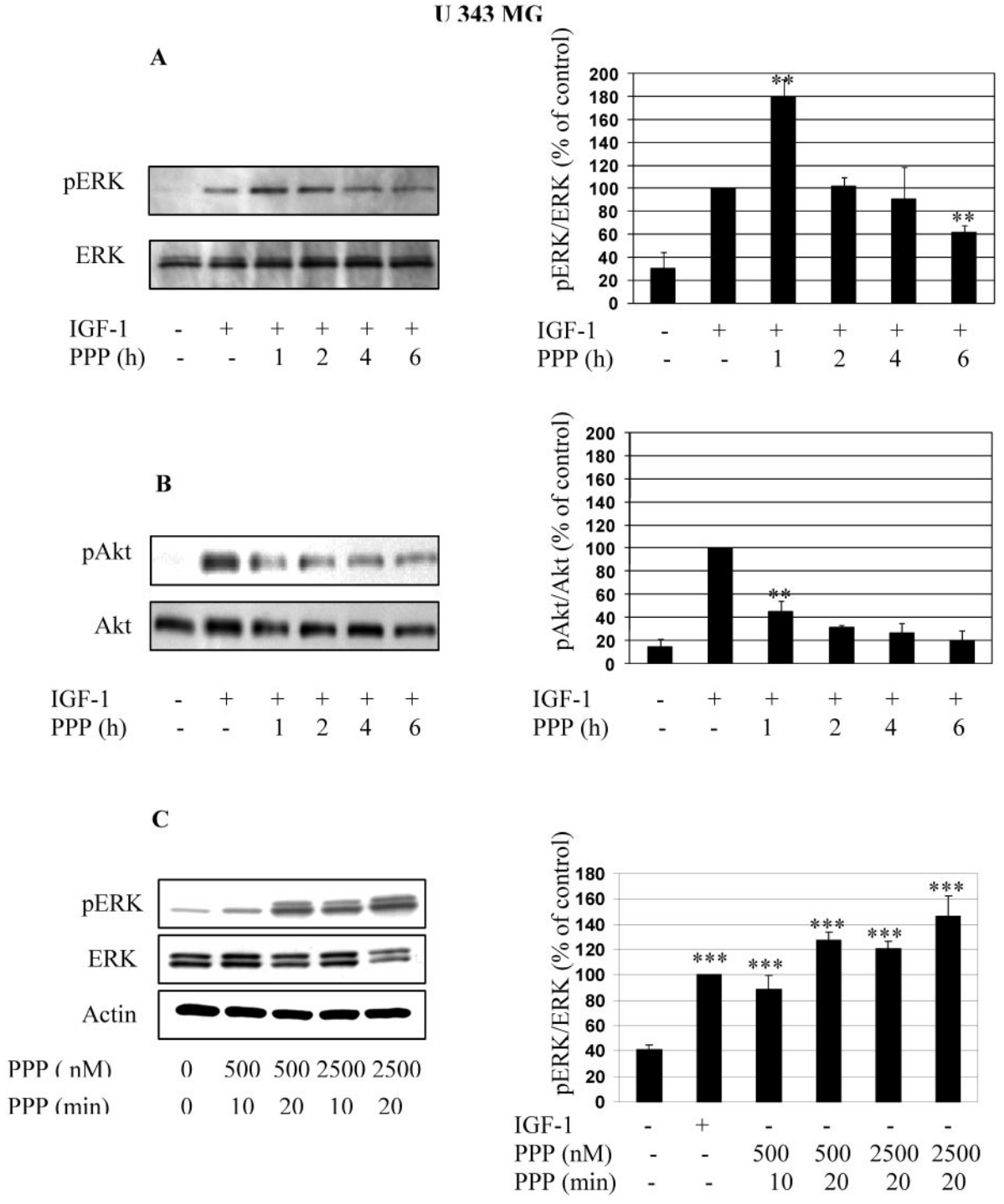

Fig. 1. PPP activates ERK independent of IGF-1 in glioblastoma cells. A. U-343MG cells were serum-starved for 24 h and then treated with PPP (500 nM) for 0 to 6 h and finally stimulated with IGF-1 (50 ng/ml) for 10 min. The cell lysates were analyzed for phosphorylated and total ERK1/2. B, cell lysates from the experiment described in A were analyzed for phosphorylated and total Akt by Western blot. The signals were quantified by densitometry, and the mean and S.D. values of three experiments are shown. **, $p < 0.02$ less than control + IGF-1; ***, $p < 0.002$ less than control + IGF-1 as determined by paired t test. C, U-343MG cells were serum-starved and then treated with IGF-1 for 10 min or PPP (500 or 2500 nM) for 0, 10, or 20 min. The cell lysates were analyzed for phosphorylated and total ERK1/2. Quantifications of signals were made and also include the measurement of ERK1/2 in cells treated with IGF-1 for 10 min. Diagram shows means and S.D. values of three separate experiments.

We also down-regulated Mdm2 by siRNA, and the results from this experiment show that the decrease in Mdm2 expression (Fig. 4D) impaired both IGF-1 and PPP-induced ERK phosphorylation (Fig. 4C, left). In this system, we also tested whether the ERK signaling pathway is functional by stimulating the cells with serum (Fig. 4C, right).

To investigate whether PPP-induced ERK activation contributes to cell death, we compared the viability response to PPP treatment of the *MDM2* siRNA-transfected cells with the mock-transfected cells. Transfected BE cells were treated with different concentrations of PPP (0–2.5 μM) for 24 h and then analyzed for survival using XTT assay. Figure 4D shows that mock-transfected cells died in a dose-dependent manner with an IC_{50} value of approximately 0.5 μM. In contrast, the *MDM2* siRNA-transfected cells exhibited a significantly reduced cell death in the dose interval 0.5 to 2.5 μM. The IC_{50} value was increased from 0.5 to 2.4 μM as a consequence of knockdown of MDM2. It was also verified that the siRNA transfection decreased the levels of MDM2. Taken together, the results presented in Fig. 4 strongly suggest the involvement of Mdm2-dependent IGF-1R ubiquitination in PPP-induced ERK activation.

IGF-1R Domains Responsible for PPP-Induced ERK Activation: Relationship with IGF-1R Ubiquitination. We next sought to identify IGF-1R domains required for the PPP-induced ERK signaling. For this purpose we used R− and R+ (R− cells stably transfected with WT IGF-1R) cells as negative and positive controls, respectively. In addition, we used 46 cells that are R− cells stably transfected with an IGF-1R construct possessing a mutation in the substrate binding site (SBS) (Y950F), which does not recruit and activate Shc and IRS-1, the two major transducers of IGF-1R signaling. We also tested 56 cells, which are R− cells stably transfected with IGF-1R lacking the C-terminal domain and 96 cells being R− cells expressing IGF-1R with both SBS mutation (Y950F) and truncated C terminus. After serum starvation, the cells were treated with PPP or stimulated with IGF-1. All of these cell variants, with the exception of R−, exhibit IGF-1 stimulated phosphorylation of IGF-1R (Girnita et al., 2007). Five-minute stimulation with IGF-1 induces IGF-1R ubiquitination in cells expressing full-length IGF-1R (R+ and 46); however, the absence of the C terminus and of the whole receptor abrogates this modification (56, 96, and R− cells, respectively) (Fig. 5A). As shown, PPP is able to induce IGF-1R ubiquitination in the same cell lines (Fig. 5A). Next, we compared the effect of IGF-1 and PPP on phospho-ERK and phospho-AKT. Cells were treated with these agents for different time intervals between 2 and 60 min. R+ cells were responsive to IGF-1, with an activity peak at 5 to 10 min (Fig. 5B, left). Consistent with previously reported data (Girnita et al., 2007) and in agreement with the pattern of IGF-1R ubiquitination, ERK1/2 is slightly but clearly phos-

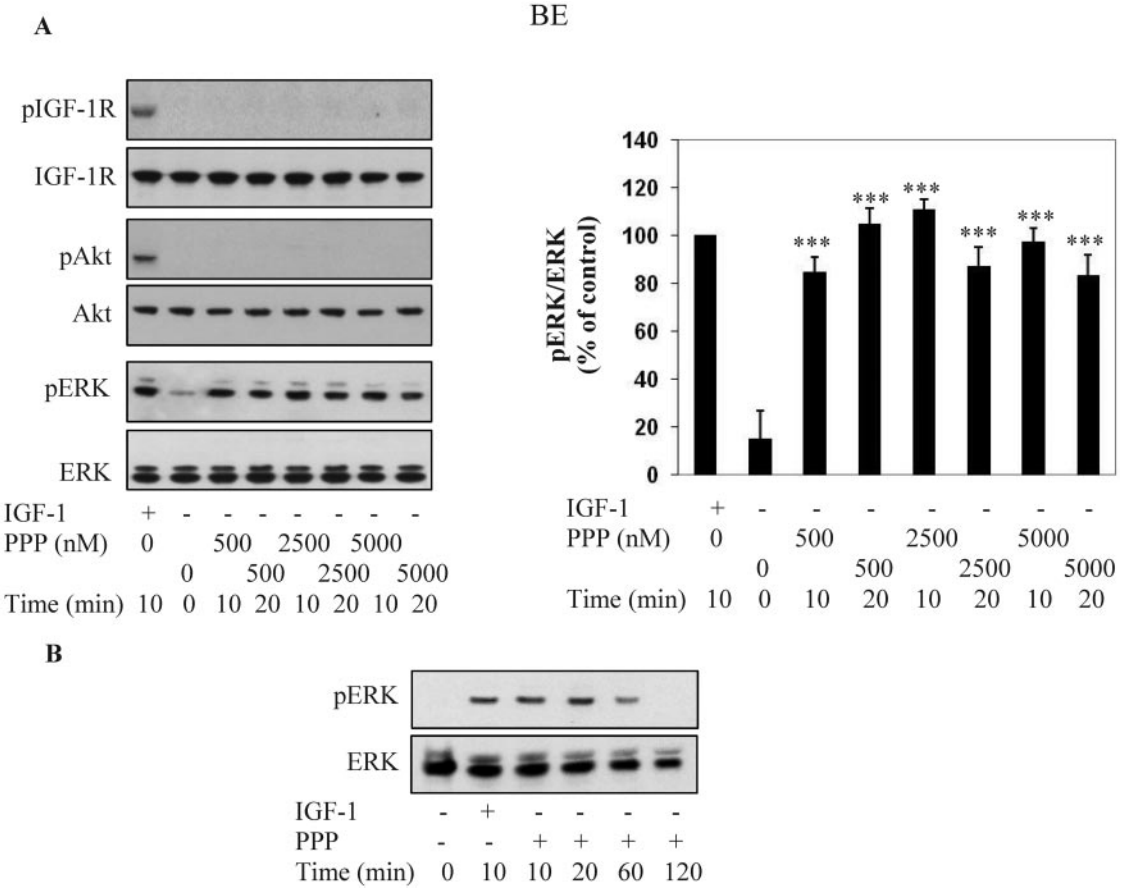

Fig. 2. PPP-induced activation of ERK in human melanoma cells. A, BE cells were serum-starved for 24 h and treated with IGF-1 for 10 min or PPP (500, 2500, or 5000 nM) for 0, 10, or 20 min and then assayed for IGF-1R, ERK1/2, and Akt phosphorylation as described in Fig. 1. Quantifications were made for phospho-ERK1/2. Diagram shows means and S.D. values of three separate experiments. ***, $p < 0.002$ versus unstimulated control as determined by paired t test. B, a separate experiment shows the effects on ERK phosphorylation induced by longer treatments with PPP (up to 120 min).

phorylated by IGF-1 in 46 cells, whereas in 56, 96, and R− cells. IGF-1 induces essentially no ERK1/2 phosphorylation (Fig. 5B, left). In line with our previous study (Girnita et al., 2007; Sehat et al., 2007), these data suggest that the C-terminal domain of IGF-1R is important for ERK activation. Regarding the effect on Akt phosphorylation, PPP was not able to induce Akt activation. Furthermore, IGF-1 could induce Akt phosphorylation only in R+ and 56 cells. However, compared with R+ cells, Akt phosphorylation is greatly impaired in 46 cells and absent in R− (Fig. 5B). In 96 cells,

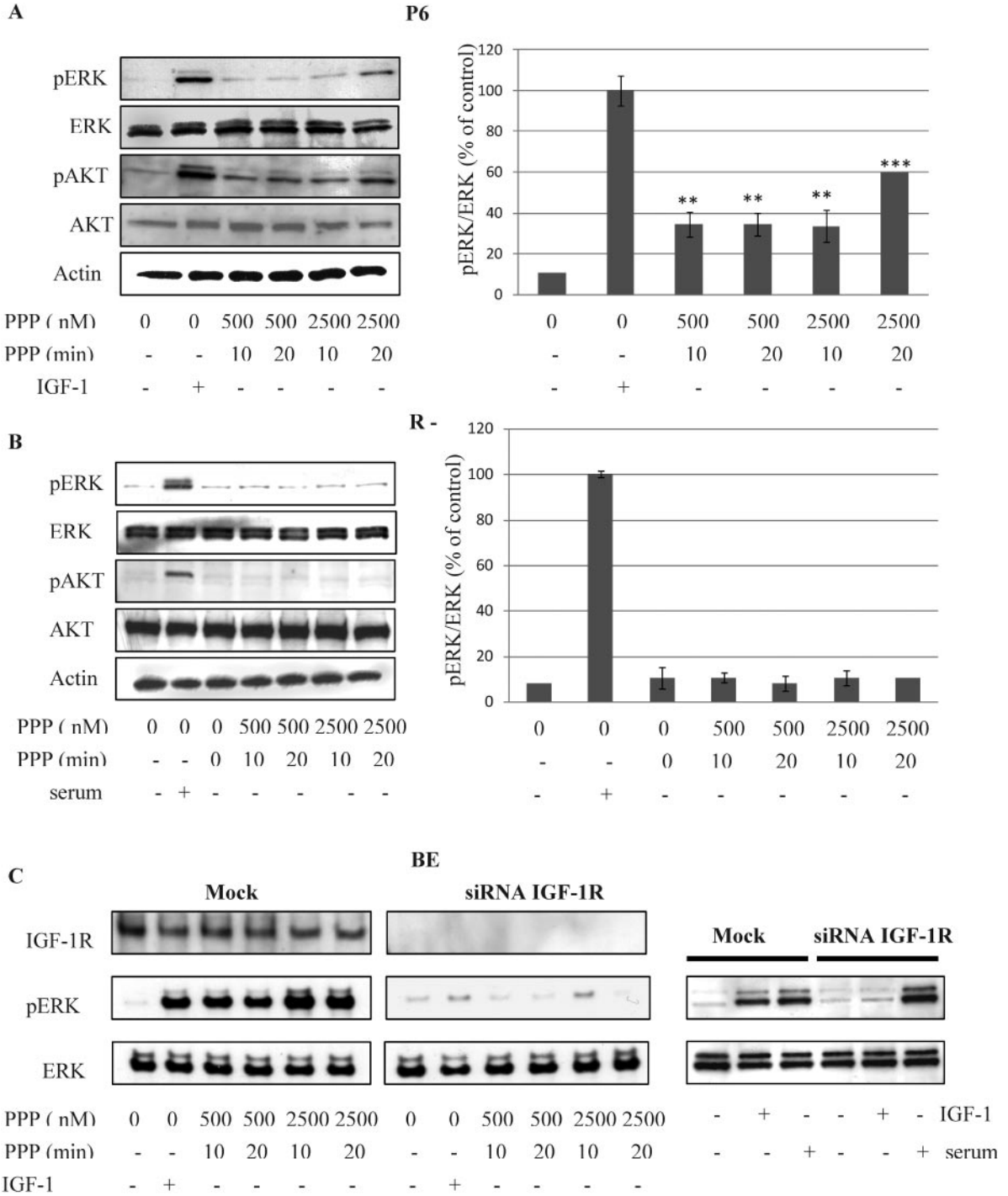

Fig. 3. PPP-induced ERK activation requires IGF-1R. A, Mouse fibroblasts overexpressing human IGF-1R (R+ cells) were serum-starved for 24 h and treated with IGF-1 for 10 min or PPP (500 or 2500 nM) for 10 or 20 min and then assayed for ERK1/2 phosphorylation. Quantifications were made, and graph shows means and S.D. values of three separate experiments. **, $p < 0.02$ and ***, $p < 0.002$ versus unstimulated control, as determined by paired t test. B, IGF-1R null (−/−) mouse fibroblasts (R−) were serum-starved and treated with serum for 10 min or IGF-1 for 10 min or PPP (500 or 2500 nM)) for 10 or 20 min and then assayed for ERK1/2 phosphorylation. Quantifications were made, and graph shows means and S.D. values of three separate experiments. **, $p < 0.02$ and ***, $p < 0.002$ versus unstimulated control as determined by paired t test. C, BE cells were transfected with IGF-1R siRNA or control siRNA, serum-starved for 24 h, and then treated with IGF-1 for 10 min or PPP (500 or 2500 nM) for 0, 10, or 20 min. In a separate experiment, transfected BE cells were serum-starved for 24 h and then stimulated with IGF-1 or serum for 10 min (right). Cell lysates were analyzed for IGF-1R and phosphorylated and total ERK1/2.

with combined mutations, Akt phosphorylation cannot be induced by IGF-1. These data suggest that the recruitment of Shc and IRS proteins is critical only for the Akt pathway but not for ERK activation upon IGF-1R stimulation.

Figure 5B, left, shows that PPP induces ERK phosphorylation in R+ cells. The 46 cells (with mutation of SBS) also responded but with a maximal level at 30 min. Cells expressing C-terminal-truncated IGF-1R (56 and 96) and the IGF-1R-deficient cells (R−) were not responsive (Fig. 5B, left). Figure 5B, right, clearly demonstrates that PPP has no stimulatory effect on Akt phosphorylation in any of the cell lines.

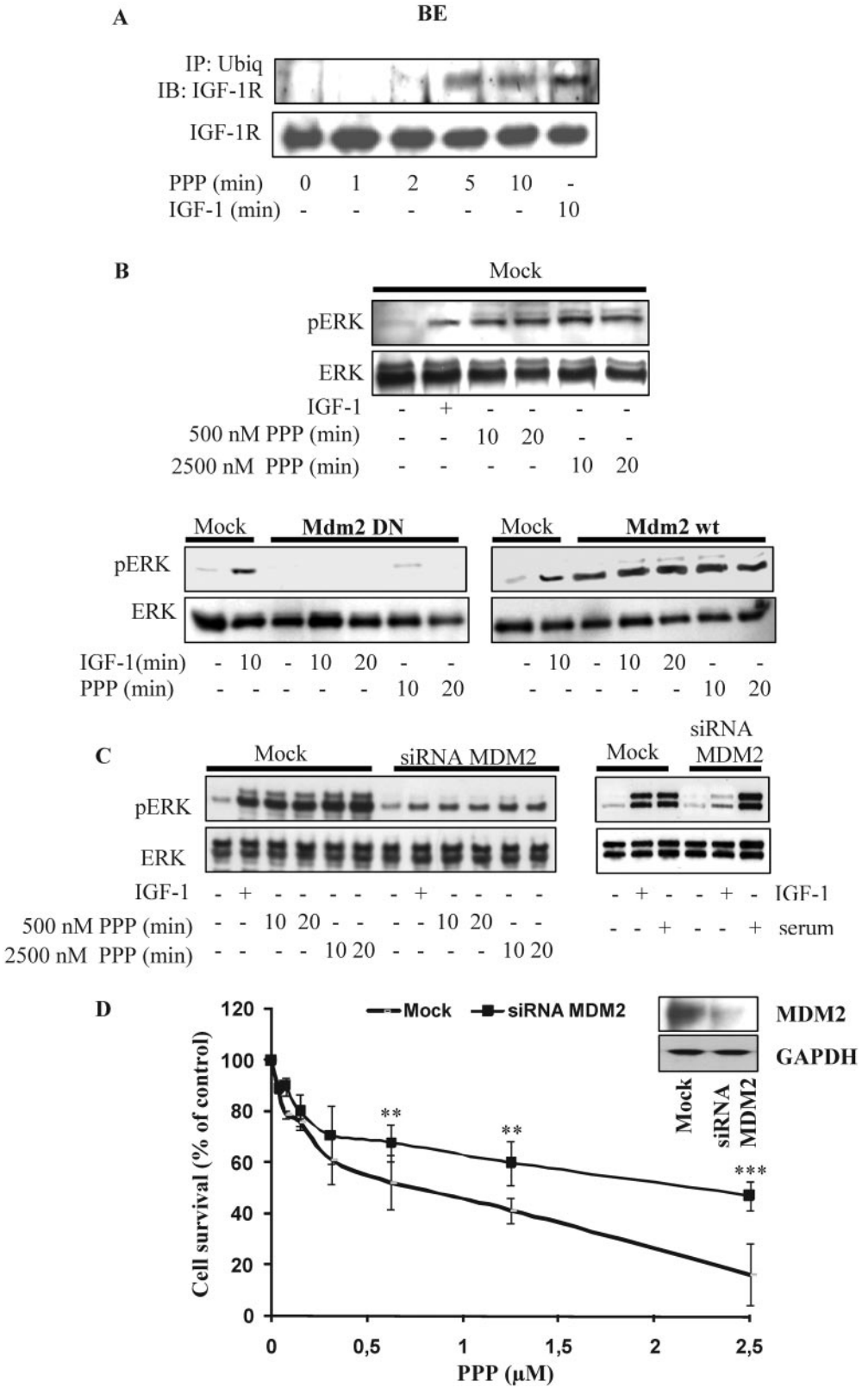

Fig. 4. PPP-induced ERK activation requires Mdm2-dependent ubiquitination of IGF-1R. A, BE cells were serum-starved for 24 h and then were treated with PPP (500 nM) for indicated times or IGF-1 for 10 min. Cell lysates were immunoprecipitated with ubiquitin antibody and analyzed for IGF-1R β-subunit by Western blotting. B, BE cells were transfected with an empty vector (top), DN-*MDM2* (left, middle), or WT-*MDM2* (right, middle). Cells were then serum-starved and treated with PPP (500 nM) for indicated times or IGF-1 for 10 min, and ERK1/2 phosphorylation was determined. C, knockdown of MDM2 decreases PPP-induced ERK1/2 phosphorylation. BE cells were transfected with siRNA-targeting *MDM2* mRNA or control siRNA. The cells were serum-depleted for 24 h and then treated with IGF-1, serum, or PPP as indicated. ERK1/2 phosphorylation and total ERK were determined. D, knockdown of MDM2 decreases PPP-induced cell death. BE cells were transfected as described in C. The cells were then treated with PPP (0–2.5 μM) for 24 h and analyzed for cell survival. Means and S.D. values from five wells of each experimental situation are shown. MDM2 siRNA-transfected cells were significantly more resistant to PPP given at concentrations of 0.6 to 2.5 μM (**, $p < 0.02$, and ***, $p < 0.002$) compared with mock-transfected cells as determined by paired t test.

Discussion

The IGF-1R plays an essential role in malignant processes in three ways: 1) acting as a promoting factor; 2) acting as an antiapoptotic factor; and 3) being quasiobligatory for establishment and maintenance of the malignant phenotype (Baserga, 1995, 1999; Le Roith et al., 1999; All-Ericsson et al., 2002; Ahlén et al., 2005). Several signaling pathways, including MAPK pathways and the PI3K pathway, are activated by IGF-1 and IGF-2.

Phosphorylation is the major event inducing IGF-1R signaling. PPP impairs IGF-1-induced IGF-1R phosphorylation in tumor cells in vitro and in vivo, decreases Akt activity, and causes malignant cell death, leading to tumor regression (Girnita et al., 2004, 2006; Menu et al., 2006, 2007).

The accurate intramolecular mechanism of PPP is still unknown, but previous data indicated abrogation of phosphorylation of tyrosine residue 1136 in the activation loop of the kinase (Vasilcanu et al., 2004). Furthermore, a recent study demonstrated that PPP also increases degradation of the receptor (Vasilcanu et al., 2008).

Signaling by ligand-activated receptor tyrosine kinases can trigger a wide range of intracellular signaling pathways, leading to specific responses such as proliferation or differentiation. A subset of signal transducers requires the receptors to be internalized for full activation. Thus, endocytic trafficking of activated epidermal growth factor receptor plays a critical role in the activation of ERK1/2 signaling (Vieira et al., 1996). Likewise, a close relationship between tyrosine kinase receptor trafficking and signaling was demonstrated for Trk receptor family (Jullien et al., 2002). In the case of IGF-1R, Chow et al. (1998) demonstrated that IGF-1R internalization is crucial for signaling via the Shc/MAPK pathway but not for the IRS-1/PI-3K pathway (Chow et al., 1998). A core mechanism responsible for IGF-1R down-regulation is represented by interaction between IGF-1R and the Mdm2 E3 ligase (Girnita et al., 2003). Mdm2 associates with and ubiquitinates the IGF-1R (Girnita et al., 2003). Mdm2-dependent ubiquitination of IGF-1R seems to have two major biological effects: one being down-regulation or desensitization of IGF-1R (Girnita et al., 2005), and the other one to mediate IGF-1R-dependent ERK activation (Girnita et al., 2007).

Based on our present study, PPP-induced ERK activation seems also dependent on Mdm2. Three findings are supporting this mechanism: 1) inhibition of Mdm2 by siRNA impaired ERK activation; 2) aberrant expression of a dominant-negative *MDM2* construct inhibited ERK phosphorylation; and 3) an IGF-1R construct defective in Mdm2 binding (Girnita et al., 2007) is unable to activate ERK. We have shown that IGF-1-induced ubiquitination of IGF-1R needs the C-terminal domain of the receptor. Furthermore, C-terminal-truncated IGF-1R is not degraded by the proteasomes but exclusively by the lysosomes (Sehat et al., 2007). In addition, this mutant receptor is not able to activate ERK signaling either (Girnita et al., 2007). Our present results demonstrate that C-terminal-truncated IGF-1R also abrogates PPP-induced ERK signaling. Thus, in case of IGF-1R ubiquitination and ERK activation PPP exhibits similar effects to ligand stimulation. On the other hand, PPP inhibits phosphorylation of Akt, which is consistent with the fact that ubiquiti-

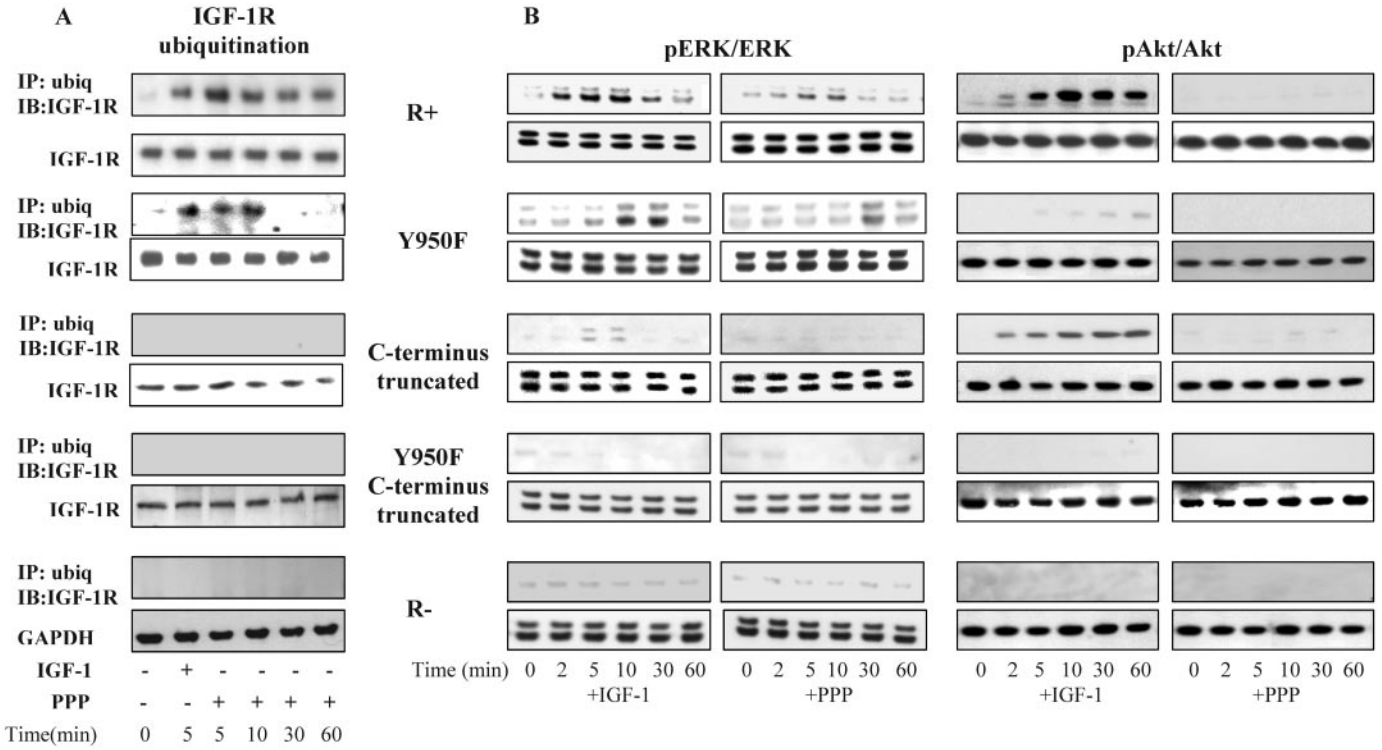

Fig. 5. PPP-induced IGF-1R ubiquitination and ERK activation is dependent of IGF-1R C-terminal domain. R+ (R− overexpressing human IGF-1R), Y950F (46, R− stably transfected with IGF-1R with a mutation in SBS), C-terminal-truncated cells (56, R− stably transfected with IGF-1R and lacking the C terminus), Y950F C-terminal-truncated cells (96, R− stably transfected with IGF-1R with a mutation in SBS and lacking the C terminus), and R− (IGF-1R knockout) cells were serum-depleted for 24 h and then treated with IGF-1 or 500 nM PPP for 0, 2, 5, 10, 30, and 60 min. IGF-1R ubiquitination, ERK, and Akt phosphorylation were analyzed.

nation of IGF-1R is not required for Akt activation (Girnita et al., 2007; Sehat et al., 2007).

IGF-1R signaling is controlled by two major events: receptor phosphorylation, and receptor ubiquitination. Although PPP inhibits IGF-1-induced IGF-1R phosphorylation (Girnita et al., 2004; Vasilcanu et al., 2004; Colón et al., 2007; Conti et al., 2007), the present results suggest that PPP induces IGF-1R ubiquitination without receptor phosphorylation. Because IGF-1R phosphorylation is required for its ubiquitination (Sehat et al., 2007), the existence of a causal relationship between the two actions of PPP on IGF-1R seems to be uncertain. One possibility is that PPP is acting as an inhibitor of IGF-1-induced IGF-1R phosphorylation; this inhibition, which targets specific residues (e.g., Tyr1136), in addition to decreasing the IGF-1R tyrosine kinase activity, is also able to elicit IGF-1R ubiquitination, internalization, and degradation. In line with this hypothesis, we recently demonstrated that IGF-1R with mutated Tyr1136 has accelerated ubiquitination and degradation (Sehat et al., 2007). Furthermore the cells with mutated Tyr1136 did not exhibit any Akt phosphorylation but intact phosphorylation of ERK1/2 (Sehat et al., 2007). This suggests that an entirely active IGF-1R kinase is required for Akt phosphorylation, whereas ERK1/2 activation, via IGF-1R ubiquitination, can occur despite impaired kinase activity. In the absence of the ligand, we were not able to detect any PPP-induced IGF-1R phosphorylation (by Western blot), yet the receptor was ubiquitinated. Therefore, we cannot completely exclude a PPP-induced conformational change of the receptor, which mimics phosphorylation.

Therefore, the present study adds a new aspect to the mechanism of action of PPP. A drug discovered to inhibit IGF-1R activity surprisingly is found to also serve as a temporal inducer of ERK signaling. The PPP-induced ERK activation was observed in two different types of human malignant cell lines, glioblastoma and melanoma cells, and in murine cell lines. This suggests that the ERK induction is not cell type-specific. On the other hand, it seems that the effect is IGF-1R-specific, because neither IGF-1R knockout cells (R−) nor cells with knockdown receptor (siRNA-transfected) were responsive.

Many observations have suggested that a sustained ERK activation is an obligatory event for growth factor-induced cell cycle progression (Chambard et al., 2007). A key step for ERK-dependent cell cycle entry is the formation of an active cyclin D-CDK4/6 complex. The CDK4/6 kinase activity releases E2F from retinoblastoma protein that in turn induces cyclin E expression required for S-phase entry (Chambard et al., 2007).

ERK activity also plays important roles in the induction of apoptosis and cell cycle arrest, usually occurring in G_2. Many cytotoxic agents induce a prolonged activation of ERK required for induction of apoptosis or cell cycle arrest (Tang et al., 2002; Xiao and Singh, 2002; Hsu et al., 2005; Chambard et al., 2007). The role of ERK activity in these cellular responses has been confirmed using mitogen-activated protein kinase kinase-1 inhibitors. Because cycling cells are more prone to apoptotic cell death compared with G_1-arrested ones, an agent that simultaneously causes inhibition of Akt phosphorylation and preserves or enhances the ERK activity should be an efficient inducer of apoptotic cell death. This theory is supported by our results, which demonstrate that PPP-induced ERK activation is partially responsible for the PPP-induced cell death: when MDM2 is blocked by siRNA, the PPP effect on ERK activation is impaired, and the MDM2 siRNA-transfected cells were significantly more resistant to PPP.

The present study of PPP-mediated signaling is, to our knowledge, the first one demonstrating that an IGF-1R inhibitor could partially activate the IGF-1R-dependent ERK signaling pathways. This is an interesting concept, suggesting an inhibitor-modulating effect on signaling. The traditional model described the receptors and their downstream signaling pathways being active or inactive, depending on the presence or absence of the ligand stimulation. According to this model, a receptor inhibitor would inactivate all of its signaling. However, the receptor conformation activating one of the signaling cascades (i.e., PI3K/Akt) may be distinct from that activating the other one (i.e., MAPK/ERK). The latter one might instead, at least partially, be dependent on ubiquitination of the receptor (Girnita et al., 2007). Such dual effects would allow better fine-tuning possibilities of IGF-1R signaling. Our present results indicate that PPP interferes with the two signaling branches through different mechanisms, one being inhibition of IGF-1R kinase-dependent Akt pathway, and the other one the induction of receptor ubiquitination with consecutive ERK activation. Such differential effects may have therapeutic advantages.

In summary, the IGF-1R inhibitor PPP causes temporary activation of ERK, probably through Mdm2-induced ubiquitination of IGF-1R, although it did not activate Akt phosphorylation. This ERK activation seems to be specific for IGF-1R because it was not obtained in IGF-1R-deficient cells.

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