Insulin-like growth factor type-I receptor (IGF-1R) dependent phosphorylation of ERK1/2 but not Akt (PKB) can be induced by picropodophyllin

Radu Vasilcanu, Daiana Vasilcanu, Bita Sehat, Shucheng Yin, Ada Girnita, Magnus Axelsson and Leonard Girnita

Department of Oncology and Pathology, Karolinska Institute, CCK, R8:04, SE-171 76 Stockholm, Sweden (R.V., D.V., B.S., S.Y., A.G., L.G.)

Department of Clinical Chemistry, Karolinska University Hospital, SE-17176 Stockholm, Sweden (M.A.)
Running title: PPP and ERK activation

Corresponding author: Leonard Girnita, MD, PhD
Department of Oncology-Pathology, Cellular and Molecular Tumor Pathology
Cancer Center Karolinska, CCK R8:04, Karolinska Institutet,
S-17176, Stockholm, Sweden
phone: 46-8-517 75242
fax: 46-8-321047
E-mail: Leonard.Girnita@ki.se

Number of text pages: 28
Number of tables: 0
Figures: 5
References: 45
Abstract: 206 words
Introduction: 773 words
Discussion: 992
List of non-standard abbreviations:

IGF-1R  insulin-like growth factor type-I receptor
PPP  picropodophyllin
PI3K  phosphatidylinositol-3 kinase
siRNA  Small interfering RNA
NEM  N-ethylmaleimide
SBS  Substrate Binding Site
Abstract

The initial event upon binding of insulin-like growth factor 1 (IGF-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. Recently, we found that the cyclolignan picropodophyllin (PPP) inhibits phosphorylation of IGF-1R and phosphatidyl-3 kinase (PI3K)/Akt (PKB) 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. Intriguingly, we show here that short treatments with PPP activate the intracellular 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 since PPP is not able to induce ERK phosphorylation in IGF-1R negative cells or in cells in which the receptor is knocked down by siRNA. Moreover, in the absence of Mdm2, an E3 ligase that previously has been shown 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.
INTRODUCTION

The insulin-like growth factor type-I receptor (IGF-1R) is important for transformation and proliferation of malignant cells (Baserga, 1995; Baserga, 1999; Baserga, 2000; Girnita et al., 2000a; Girnita et al., 2000b; Larsson et al., 2005; Yu and Rohan, 2000) and is crucial for preventing apoptosis (Baserga, 1995; Baserga, 1999; Baserga, 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; Baserga, 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; Girnita et al., 2005; Girnita et al., 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). Further, 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; Girnita et al., 2007). The interaction of Mdm2 and β-arrestin1 seems to have two functions in regulation of IGF-1R, one being downregulation (desensitization) of IGF-1R (Girnita et al., 2005) and the other one to control ERK1/2 activation and G1-S progression of the cell cycle (Girnita et al., 2007). Cells with a dominant-negative MDM2 construct or siRNA to β-arrestin1 do not exhibit IGF-1R dependent ERK phosphorylation and are arrested in the G1 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 (Colon et al., 2007; Conti et al., 2007; Girnita et al., 2006; Girnita et al., 2004; Guha et al., 2007; Razuvaev et al., 2007; Shields et al., 2007; Stromberg et al., 2006; Ulfarsson et al., 2005; Vasilcanu et al., 2004; Vasilcanu et al., 2006) but also downregulates the receptor (Vasilcanu et al., 2007). Even though PPP may have other effects, we can 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 to the control group (Menu et al., 2007). In spite of having a high anti-tumor 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 (Colon et al., 2007; Conti et al., 2007; Girnita et al., 2004; 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 recently demonstrated that PPP induces ubiquitination and downregulation of the IGF-1R (Vasilcanu et al., 2007). However, PPP does not affect signalling or degradation of the highly homologous insulin receptor (Fulzele et al., 2007; Girnita et al., 2004; Vasilcanu et al., 2007).
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 ERK pathway were lower or absent (Conti et al., 2007; Girnita et al., 2004). From a therapeutic point of view this imbalance in effects on the two major pathways could be favourable. Phosphorylation of ERK1/2 is namely important for the G1-S transition and inhibition of this reaction leads to G1 arrest (Hoshino et al., 2001; Johnson and Lapadat, 2002; Weber et al., 1997). Therefore, a comparably weak inhibition of ERKs may maintain cells in the cell cycle. Since cycling cells are more prone to apoptotic cell death compared to G1 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 if it even induces an activation of ERK phosphorylation.
METHODS

Reagents

PPP was synthesized as described (Buchardt et al., 1986) and following recrystallization its purity was 99.7%. For experimental purposes, PPP was dissolved in DMSO 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). 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 DMEM supplemented with 10%. P6, R-, R+, 46, 56 and 96 cell lines were cultured in the presence of G-418 (Promega).

Small interfering RNA (siRNA).

Chemically synthesized, double-strand siRNAs, with 19-nt duplex RNA and 2-nt 3’ dTdT overhangs, were purchased from Dharmaco (Lafayette, Colorado). The siRNA targeting the human IGF-1R sequence 5’-GCAGACACCUACCAAC AUCAUU-3’ was used to
deplete endogenous IGF-1R levels in 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 non-silencing RNA duplex (SmartPool, Dharmacon) was used as a control as previously described (Girnita et al., 2005).

**Transfections**

The BE melanoma cell line plated at subconfluent density in 6-cm dishes, were 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, Carlsbad, CA, USA). 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 10 mM N-ethylmaleimide (NEM) was added to the lysis buffer. Fifteen µl Protein G Plus-A/G agarose and 1µg 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 2,500 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 50-100 µg cell protein were analyzed by SDS-PAGE with a 7.5% or 10% separation gel. Molecular weight markers (BioRad, Stockholm, Sweden) were run simultaneously.

Following SDS-PAGE the proteins were transferred overnight to nitrocellulose membranes (Amersham, 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 HRP-labeled or a biotinylated secondary antibody (Amersham) for 1 h.

Following 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). The films were scanned by Fluor-S (BioRad).

**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-wells plates in 100 µl medium. After the incubation periods, 50 µl XTT labeling mixture was added to each well and incubated for additional 1 h. Spectrophotometric absorbance was measured at 492nm using an ELISA 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 while ERK phosphorylation is increased (data not shown). After serum starvation the cells were stimulated with different doses of PPP and/or IGF-1. Fig. 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. Surprisingly, if the cells are treated with PPP for 1h, phospho-ERK is drastically increased. After longer treatments with PPP (2-6h) 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. Fig. 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 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. Fig. 2A also shows that PPP does not cause activation of Akt (cf. 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% +/- 3.5; \( p = 0.56 \)) and PPP (16% +/- 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.

In order 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 stimulated with IGF-1. Fig. 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 (cf. Fig. 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 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 to the mock control. Fig. 3C also confirms that siRNA completely downregulated 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 activates 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 panel) demonstrating that the ERK signaling pathway is not a 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*
Recently it was demonstrated that IGF-1 induces ubiquitination of the IGF-1R and enhances its degradation (Girnita et al., 2003; Girnita et al., 2005; Vecchione et al., 2003). We identified Mdm2 to be an E3 ligase in this respect (Girnita et al., 2003). Just recently, we reported that that the action of Mdm2, as well as of its adapter protein β-arrestin1, is necessary for IGF-1 induced ERK activation (Girnita et al., 2007). Accordingly, it would be interesting to investigate whether the action of Mdm2 is involved in PPP-induced ERK signaling. We first analyzed if PPP (500 nM), administered for 1-10 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). Also, 5-10 min treatments with PPP resulted in IGF-1R ubiquitination (Fig. 4A).

Next we investigated the effects of constructs expressing deleted MDM2 (MDM21-400, lacking the ligase domain) or wild type (WT) MDM2 (MDM21-491) on PPP-induced ERK activation. The MDM21-400 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; Girnita et al., 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 not longer activated in response to PPP or IGF-1 (Fig. 4B). On the other hand, overexpression of MDM2 in the cells
transfected with WT-\textit{MDM2} (MDM2_{1-491}) induces an increased ERK activation as compared to mock transfected cells.

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

To investigate whether PPP-induced ERK activation contributes to cell death, we compared the viability response to PPP treatment of the \textit{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 IC50 of around 0.5 µM. In contrast, the \textit{MDM2} siRNA-transfected cells exhibited a significantly reduced cell death in the dose interval 0.5–2.5 µM. The IC50 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.

\textit{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 utilized 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 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 as well as 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 2-60 min. R+ cells were responsive to IGF-1 with an activity peak at 5-10 min (Fig. 5B, left panels). Consistent with previous reported data (Girnita et al., 2007), and in agreement with the pattern of IGF-1R ubiquitination, ERK1/2 is slightly but clearly phosphorylated by IGF-1 in 46 cells, whereas in 56, 96 and R- cells IGF-1 induces essentially no ERK1/2 phosphorylation (left panels of Fig 5B). 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, 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.

Left panels of Fig. 5B 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), as well as the IGF-1R deficient cells (R-) were not responsive (Fig. 5B, left panels). Right panels of Fig. 5B clearly demonstrate that PPP has no stimulatory effect on Akt phosphorylation in any of the cell lines.
DISCUSSION

The IGF-1R plays an essential role in malignant processes in three different ways: (1) acting as a promoting factor; (2) as an anti-apoptotic factor; and (3) being quasi-obligatory for establishment and maintenance of the malignant phenotype (Ahlen et al., 2005; All-Ericsson et al., 2002; Baserga, 1995; Baserga, 1999; Le Roith et al., 1999). Several signaling pathways, including MAPK pathways and 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., 2006; Girnita et al., 2004; Menu et al., 2007; Menu et al., 2006).

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., 2007).

Signaling by ligand-activated receptor tyrosine kinases (RTKs) can trigger a wide range of intra-cellular signaling pathways leading to specific responses such proliferation or differentiation. A subset of signal transducers requires the receptors to be internalized for full activation. Thus, endocytic trafficking of activated EGFR plays a critical role in 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 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 downregulation 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 downregulation 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 appears also dependent on Mdm2. Three findings are supporting this mechanism: (i) inhibition of Mdm2 by siRNA impaired ERK activation; (ii) aberrant expression of a dominant-negative MDM2 construct inhibited ERK phosphorylation and; (iii) an IGF-1R construct defective in Mdm2 binding (Girnita et al., 2007) is unable to activate ERK. Recently, we showed that IGF-1 induced ubiquitination of IGF-1R needs the C-terminus 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 ubiquitination 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. While PPP inhibits IGF-1 induced IGF-1R phosphorylation (Colon et al., 2007; Conti et al., 2007; Girnita et al., 2004; Vasilcanu et al., 2004) the present results suggest that PPP induces IGF-1R ubiquitination without receptor phosphorylation. Since 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. Tyr 1136), 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 Y1136 has accelerated ubiquitination and degradation (Sehat et al., 2007). Furthermore the cells with mutated Y1136 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 mimicks phosphorylation.

Accordingly, the present study adds a new aspect on 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 appears that the effect is IGF-1R specific, since 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., 2006). 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 Rb that in turn induces cyclin E expression required for S phase entry (Chambard et al., 2006).

ERK activity plays also important roles in induction of apoptosis and cell cycle arrest, usually occurring in G2. Many cytotoxic agents induce a prolonged activation of ERK required for induction of apoptosis or cell cycle arrest (Chambard et al., 2006; Hsu et al., 2005; Tang et al., 2002; Xiao and Singh, 2002). The role of ERK activity in these cellular responses has been confirmed using MEK1 inhibitors. As cycling cells are more prone to apoptotic cell death compared to G1 arrested ones, an agent which 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 as well as their downstream signaling pathways being active or inactive, dependent on 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, JBC 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, while it did not activate Akt phosphorylation. This ERK activation appears to be specific for IGF-1R since it was not obtained in IGF-1R deficient cells.
REFERENCES


Footnotes

This study was supported by grants from the Swedish Cancer Society, the Cancer Society in Stockholm, the Swedish Research Council, the Swedish Children Cancer Society, Ingabritt and Arne Lundberg’s research foundation, UICC International Cancer Technology Transfer Fellowship", Alex and Eva Wallström’s foundation and the Karolinska Institute.

Shucheng Yin current address: Department of Otolaryngology, Zhongnan Hospital of Wuhan University, Wuhan, 430071, China
FIGURE LEGENDS

Fig. 1  PPP activates ERK independent of IGF-1 in glioblastoma cells.

A. U-343MG cells were serum starved for 24h and then treated with PPP (500 nM) for 0-6 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 blott.

The signals were quantified by densitometry and the mean and SDs 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 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 of ERK1/2. Quantifications of signals were made and also include measurement of ERK1/2 in cells treated with IGF-1 for 10 minutes. Diagram shows means and SDs of three separate experiments.

Fig.2  PPP induced activation of ERK in human melanoma cells.

A. BE cells were serum starved for 24h 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 SDs 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.).
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 SDs 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 SDs 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 24h 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 24h and then stimulated with IGF-1 or serum for 10 min (right panel). Cell lysates were analyzed for IGF-1R and phosphorylated and total ERK1/2.

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 panels), DN-MDM2 (left middle panels) or WT-MDM2 (right middle panels). 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 24h 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 SDs 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 -2.5 µM (** p< 0.02 and *** p< 0.002) compared to mock-transfected cells as determined by paired t test.

**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-terminus truncated cells (56, R- stably transfected with IGF-1R and lacking the C-terminal), Y950F C-terminus truncated cells (96, R- stably transfected with IGF-1R with a mutation in SBS and lacking the C-terminal) and R- (IGF-1R KO) 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.
Fig. 1
Fig. 2
### Data Table

**IP: Ubiq**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**IB: IGF-1R**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**IGF-1R**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Western Blot

**Mock**

<table>
<thead>
<tr>
<th>Test Condition</th>
<th>pERK</th>
<th>ERK</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1 (min)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PPP (min)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Mdm2 DN**

<table>
<thead>
<tr>
<th>Test Condition</th>
<th>pERK</th>
<th>ERK</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1 (min)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>PPP (min)</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

**Mock**

<table>
<thead>
<tr>
<th>Test Condition</th>
<th>pERK</th>
<th>ERK</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1 (min)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PPP (min)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Mdm2 wt**

<table>
<thead>
<tr>
<th>Test Condition</th>
<th>pERK</th>
<th>ERK</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1 (min)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PPP (min)</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

### Western Blot

**Mock**

<table>
<thead>
<tr>
<th>Test Condition</th>
<th>pERK</th>
<th>ERK</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1 (min)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>PPP (min)</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

**siRNA MDM2**

<table>
<thead>
<tr>
<th>Test Condition</th>
<th>pERK</th>
<th>ERK</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1 (min)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PPP (min)</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

**Mock**

<table>
<thead>
<tr>
<th>Test Condition</th>
<th>pERK</th>
<th>ERK</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1 (min)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>PPP (min)</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

**siRNA MDM2**

<table>
<thead>
<tr>
<th>Test Condition</th>
<th>pERK</th>
<th>ERK</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1 (min)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PPP (min)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Graph

**Cell Survival (%)**

<table>
<thead>
<tr>
<th>PPP (μM)</th>
<th>Mock</th>
<th>siRNA MDM2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**GAPDH**

**MDM2**

**Fig. 4**
Figure 5