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Interaction with Grb14 results in site-specific regulation of tyrosine phosphorylation of the insulin receptor

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The dynamics of interaction of the insulin receptor (IR) with Grb14 was monitored, in real time, in living human embryonic kidney cells, using bioluminescence resonance energy transfer (BRET). We observed that insulin rapidly and dose-dependently stimulated this interaction. We also observed that insulin-induced BRET between the IR and protein tyrosine phosphatase 1B (PTP1B) was markedly reduced by Grb14, suggesting that Grb14 regulated this interaction in living cells. Using site-specific antibodies against phosphorylated tyrosines of the IR, we showed that Grb14 protected the three tyrosines of the kinase loop from dephosphorylation by PTP1B, while favouring dephosphorylation of tyrosine 972. This resulted in decreased IRS-1 binding to the IR and decreased activation of the extracellular signal-regulated kinase pathway. Increased Grb14 expression in human liver-derived HuH7 cells also seemed to specifically decrease the phosphorylation of Y972. Our work therefore suggests that Grb14 may regulate signalling through the IR by controlling its tyrosine dephosphorylation in a site-specific manner.

Keywords: BRET; insulin receptor; Grb14; PTP1B; IRS-1; MAP kinases

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INTRODUCTION

The insulin receptor (IR) is a heterotetramer constituted of two extracellular α-subunits that bind to the hormone and two β-subunits that possess an intracellular tyrosine kinase (TK) activity. Binding of insulin to the IR results in autophosphorylation of each β-subunit on at least six different tyrosines. This autophosphorylation occurs first on three tyrosines located in the activation loop of the kinase domain (Y1158, 1162 and 1163), resulting in the stabilization of the kinase in an active conformation. Phosphorylations then occur on a tyrosine located in the juxtamembrane region (Y972) and on two tyrosines located in the carboxy-terminal region (Y1328, 1334). Whereas the precise role of the tyrosines located in the C-terminal domain remains debatable, Y972 seems to be a docking site for substrates such as IRS-1 and Shc (Combettes-Souverain & Issad, 1998). Tyrosine phosphorylation of intracellular substrates has an important role in the activation of signalling pathways (mitogen-activated protein (MAP) kinases, phosphatidylinositol 3-kinase (PI3-K)). After activation, the IR is internalized as a tyrosine-phosphorylated fully active kinase, which may still transmit signals at intracellular sites. Termination of the signal involves inactivation of the IR by dephosphorylation of the three tyrosines of the kinase domain (Tonks, 2003). PTP1B is a protein tyrosine phosphatase located in the endoplasmic reticulum that has an important role in the dephosphorylation of these tyrosines after internalization of the IR (Boute *et al*, 2003). Although dephosphorylation is clearly a crucial mode of regulation, other mechanisms also seem to be important in the control of the activity of TK receptors. Grb14 belongs to the Grb7-like family of adaptor proteins, thus far made up of Grb7, Grb10 and Grb14 (Daly, 1998). These adaptors seem to regulate positively or negatively signalling pathways elicited by different growth factor receptors. Several lines of evidence indicate that Grb14 has a negative role in the regulation of insulin signalling. However, the mechanism by which Grb14 inhibits signalling through the IR remains elusive. *In vitro* experiments have shown that Grb14 impairs the TK activity of the IR towards exogenous substrates but protects the tyrosine-phosphorylated IR from dephosphorylation by PTP1B (Bereziat *et al*, 2002). Paradoxical effects of Grb14 on the tyrosine phosphorylation and TK activity of the IR were observed in Chinese hamster ovary (CHO) cells, in which Grb14 either seems to have no effect (Kasus-Jacobi *et al*, 1998; Hemming *et al*, 2001) or increases the tyrosine phosphorylation of the IR (Bereziat *et al*, 2002), while inhibiting the effect of insulin on IRS-1 phosphorylation (Kasus-Jacobi *et al*, 1998; Hemming *et al*, 2001), activation of the

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extracellular signal-regulated kinase (ERK) pathway (Bereziat *et al*, 2002) and glycogen and DNA synthesis (Kasus-Jacobi *et al*, 1998). Definitive evidence for a negative role of Grb14 in insulin signalling was obtained with Grb14 knockout mice, which showed improved glucose tolerance and insulin sensitivity (Cooney *et al*, 2004). However, paradoxical effects were also observed, including a decrease in IR tyrosine phosphorylation associated with an increased tyrosine phosphorylation of IRS-1 in the liver of knockout mice. The decreased IR phosphorylation was attributed to removal of a potentially protective effect of Grb14 on dephosphorylation of the IR by PTP1B. However, the concomitant increase in the tyrosine phosphorylation of IRS-1 observed in the same tissue remained difficult to explain.

The bioluminescence resonance energy transfer (BRET) technique has recently emerged as an important tool for the study of protein–protein interactions in living cells. We previously used this technique to study the interaction of the IR with PTP1B (Boute *et al*, 2003). In the present work, we use BRET to study, in living cells, the relationships between IR, Grb14 and PTP1B. We also show that, in cells transfected with PTP1B, Grb14 selectively protects the three tyrosines of the activation loop from dephosphorylation, while favouring the dephosphorylation of the tyrosine of the juxtamembrane domain of the IR.

RESULTS AND DISCUSSION

Monitoring IR–Grb14 interaction in living cells by BRET

To determine whether the interaction of the IR and Grb14 can be monitored by BRET, HEK293 cells were co-transfected with IR–Rluc and Grb14–yellow fluorescent protein (YFP) fusion proteins. In the absence of insulin, a low basal BRET signal could be detected. Insulin rapidly and dose-dependently increased this signal (Fig 1A). A plateau was reached about 10 min after addition of 100 nM insulin and 20 min after addition of 5 nM insulin. The kinetics of insulin-induced BRET closely parallel the kinetics of insulin-induced phosphorylation of the IR (supplementary information 1 online).

Fig 1B,C shows BRET signal between Grb14–YFP and either IR–Rluc or a kinase-dead mutant of the IR fused to luciferase (IR-K1030A–RLuc). Whereas insulin markedly increased BRET between IR–Rluc and Grb14–YFP, it had no effect on BRET between IR-K1030A–Rluc and Grb14–YFP. This result demonstrates that binding of insulin has no effect by itself, and that autophosphorylation of the IR is necessary to induce the Grb14–IR interaction. The specificity of insulin-induced BRET signal measured in these experiments was demonstrated by the low BRET signal obtained in cells expressing IR–Rluc and YFP alone (supplementary information 2 online).

Grb14 modulates the interaction of IR with PTP1B

In vitro experiments, using glutathione *S*-transferase-tagged Grb14, showed that Grb14 binding to the IR inhibited its dephosphorylation by purified recombinant PTP1B (Bereziat *et al*, 2002). We previously demonstrated that the interaction of IR and PTP1B can be monitored by BRET using IR–Rluc and a YFP-tagged substrate-trapping mutant of PTP1B (YFP–PTP1B-D181A). To analyse the effect of Grb14 on the interaction between IR and PTP1B in living cells, HEK cells were co-transfected with IR–Rluc, YFP–PTP1B-D181A and either untagged Grb14 or empty vector. As shown previously (Boute *et al*, 2003), a substantial basal BRET

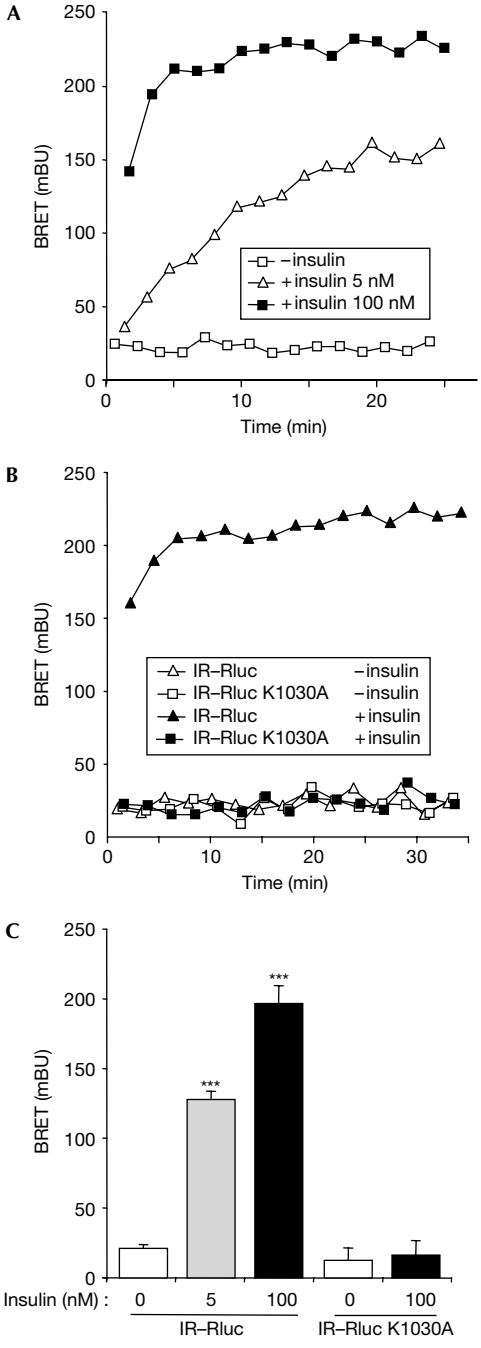

Fig 1 | Dynamics of interaction between the insulin receptor and Grb14 in intact living cells. (**A**) Bioluminescence resonance energy transfer (BRET) was monitored in human embryonic kidney cells coexpressing IR–Rluc and Grb14–YFP. Cells were preincubated for 15 min in the presence of coelenterazine (5 μM) and then stimulated with 5 or 100 nM of insulin. IR, insulin receptor; YFP, yellow fluorescent protein. (**B**) Interaction of Grb14 with the wild-type and kinase-dead mutant. (**C**) Basal and insulin-stimulated BRET at time 20 min. Results are means ± s.e.m. of seven (IR–Rluc) or two (IR–Rluc-KD) independent experiments (***$P < 0.001$ when compared with basal condition).

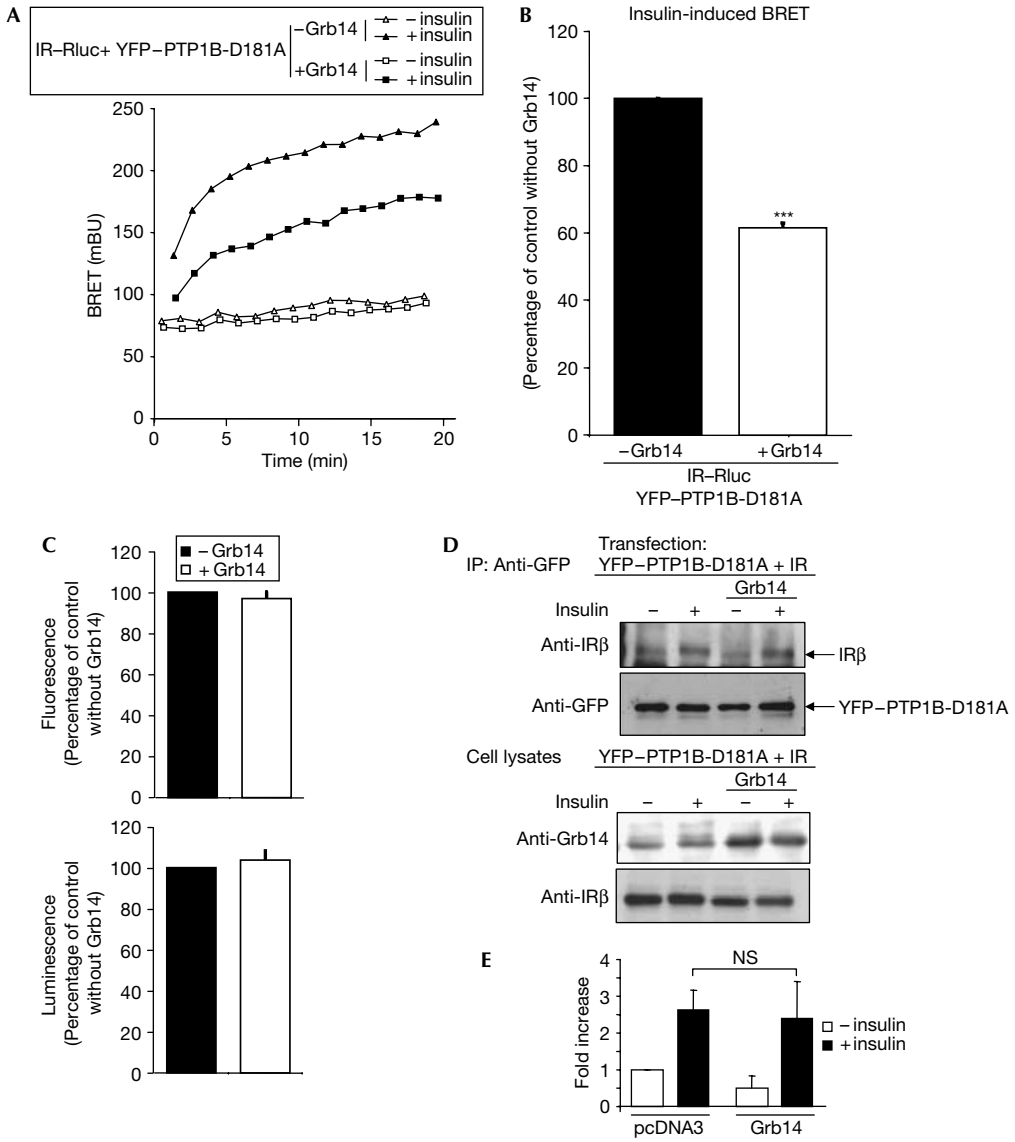

Fig 2 | Grb14 inhibited bioluminescence resonance energy transfer between the insulin receptor and PTP1B. (**A**) Human embryonic kidney cells were co-transfected with IR–Rluc and YFP–PTP1B-D181A, and either Grb14 or empty vector (pcDNA3). Bioluminescence resonance energy transfer (BRET) was monitored during 20 min in the absence or presence of 100 nM insulin. IR, insulin receptor; YFP, yellow fluorescent protein. (**B**) Insulin-induced BRET (increase BRET above basal) at time 20 min. Results are means ± s.e.m. of three independent experiments and are expressed as a percentage of control cells (cells not transfected with Grb14; $***P<0.001$). (**C**) YFP–PTP1B-D181A fluorescence and IR–Rluc luminescence as a percentage of control cells. (**D**) HEK cells were co-transfected with IR and YFP–PTP1B-D181A, and either Grb14 or empty vector (pcDNA3). Cells were stimulated or not stimulated with 100 nM insulin for 10 min and lysed. YFP–PTP1B-D181A was immunoprecipitated with an anti-green fluorescent protein (GFP) antibody. The amount of IR co-precipitated with YFP–PTP1B-D181A was evaluated with an anti-IRβ antibody. (**E**) Densitometric analysis of IRβ signals. The data presented are means ± s.e.m. of three independent experiments. NS, not significant.

signal was detected, and insulin markedly increased this signal (Fig 2A). In cells transfected with Grb14, basal BRET signal was unaltered, but insulin-induced BRET signal was markedly reduced (about 40%; Fig 2A,B), despite similar levels of IR–Rluc and YFP–PTP1B-D181A expression (Fig 2C). This suggests that Grb14 can affect the interaction between IR and PTP1B. However, BRET depends not only on the distance between two partners, but also on their relative orientation (Lacasa *et al*, 2005). Therefore, the Grb14-induced decrease in BRET may either reflect a disruption of the interaction between IR–Rluc and YFP–PTP1B-D181A or a conformational change that resulted in a lower efficiency of energy transfer. In an attempt to discriminate between these two possibilities, YFP–PTP1B-D181A was precipitated with an anti-green fluorescent protein (GFP) antibody, and the amount of IR co-immunoprecipitated with PTP1B was evaluated using an anti-IRβ antibody. We observed that the amount of IR

co-immunoprecipitated with PTP1B was similar whether the cells were co-transfected with Grb14 or not (Fig 2D). Although we cannot exclude that these results reflect the lower sensitivity of the co-immunoprecipitation procedure compared with the non-invasive BRET technique, the absence of any significant effect of Grb14 in these co-immunoprecipitation experiments may also indicate that Grb14-induced decrease in BRET reflects subtle conformational changes in the interaction between IR and PTP1B, rather than disruption of this interaction.

Site-specific regulation of IR dephosphorylation by Grb14

As our results suggested that Grb14 may modulate the way IR and PTP1B interact, we analysed the consequences of the presence of Grb14 expression on specific tyrosine-phosphorylation sites of the IR. HEK cells were co-transfected with IR–Rluc and the wild-type version of YFP–PTP1B (YFP–PTP1B-wt), with or without Grb14. After cell lysis, receptors were partially purified on wheat germ lectin (WGL) agarose beads and submitted to western blotting using an anti-phosphotyrosine antibody. As shown in Fig 3A, insulin-induced tyrosine phosphorylation of IR was similar in cells co-transfected with PTP1B alone and with both PTP1B and Grb14. To analyse whether Grb14 modified the phosphorylation state of specific tyrosine residues, immunodetection was carried out using antibodies that recognize either the tris-phosphorylated form of the activation loop of the IR (anti-$pY^{1158,1162,1163}$) or the phosphorylated Y972 located in the juxtamembrane domain of the IR (anti-pY^{972}). In cells co-transfected with PTP1B and Grb14, phosphorylation of the three tyrosines of the activation loop was higher than in cells transfected with only PTP1B. In contrast, phosphorylation of Y972 was markedly reduced by co-transfection with Grb14 (Fig 3A,B). Altogether, these results indicate that Grb14 specifically protects the three tyrosines located in the activation loop of the IR from PTP1B activity, while favouring the dephosphorylation of Y972. Grb14 and PTP1B are both known to bind to the kinase domain of the activated IR (Kasus-Jacobi *et al*, 1998; Tonks, 2003). Therefore, the presence of Grb14 may impair the docking of the active site of PTP1B on the phosphotyrosines of the catalytic loop and cause displacement of PTP1B activity towards pY^{972}. This displacement of PTP1B from the catalytic loop towards the juxtamembrane domain of the IR may explain why we observed a reduction in BRET signal (Fig 2A,B) but no significant changes in the amount of IR co-precipitated with the substrate-trapping mutant PTP1B-D181A (Fig 2D,E). A similar result was also observed in cells transfected with IR and Grb14 only, indicating that this effect can be mediated by endogenous PTP1B and/or other PTPases (supplementary information 3 online).

Modification of IR downstream signalling by Grb14

Y972 is the main docking site for IRS-1 on the activated IR. We therefore analysed the effect of Grb14 on IRS-1 recruitment to the IR. HEK cells were co-transfected with YFP-tagged IR (IR–YFP), IRS-1, PTP1B-wt and Grb14. The IR was immunoprecipitated using an anti-GFP antibody (Fig 4A). The amount of IRS-1 co-precipitated with the activated IR was markedly reduced in cells co-transfected with Grb14. This suggests that, by favouring the dephosphorylation of Y972, Grb14 reduced the amount of IRS-1 recruited to the insulin-stimulated IR. A similar effect of Grb14 was also observed even in the absence of co-transfected PTP1B

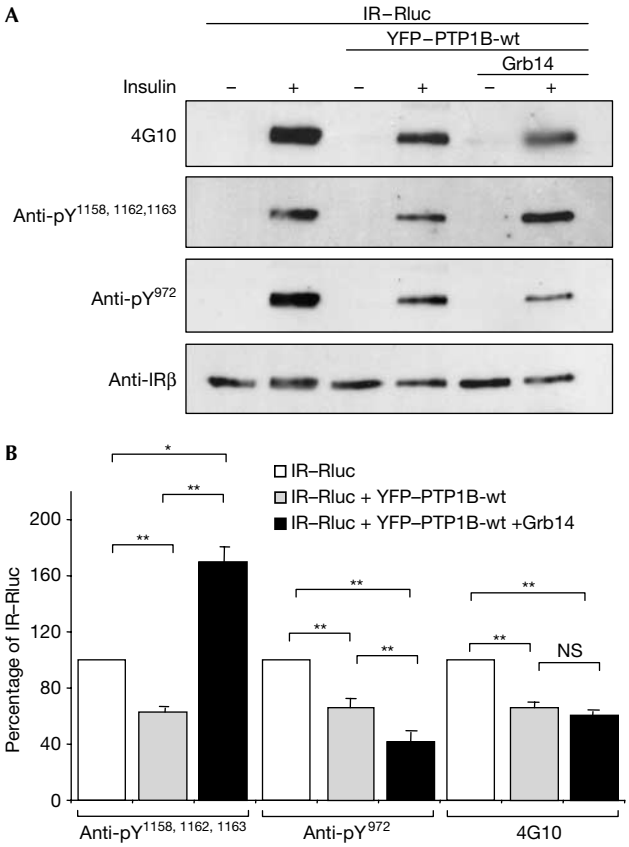

Fig 3 | Grb14 regulates the dephosphorylation of the IR in a site-specific manner. Human embryonic kidney cells co-transfected with IR–Rluc alone or in combination with YFP–PTP1B-wt and Grb14 were stimulated or not stimulated with 100 nM insulin for 10 min and lysed. (**A**) The phosphorylation of IR partially purified on wheat germ lectin was evaluated by western blotting using a general anti-phosphotyrosine antibody (4G10), or site-specific phospho-antibodies (anti-$pY^{1158,1162,1163}$ and anti-pY^{972}). The amount of receptors loaded in each lane was evaluated using an anti-IRβ antibody. Results are representative of five independent experiments. (**B**) Densitometric analysis of the anti-phosphotyrosine signals corrected for the anti-IR signal. The data presented are means ± s.e.m. of three to four independent experiments (*$P<0.05$; **$P<0.01$). IR, insulin receptor; NS, not significant; YFP, yellow fluorescent protein.

(supplementary information 4 online). As an interaction of an NPXY motif in Grb14 and the phosphotyrosine-binding domain of IRS-1 has been reported (Rajala & Chan, 2005), the inhibitory effect of Grb14 on IR/IRS-1 association could also be due to binding of Grb14 to IRS-1. We observed that Grb14 mutated in the NPXY motif (Grb14-Y200F) also induced dephosphorylation of Y972 and inhibition of the interaction of IR and IRS-1, indicating that these effects cannot be attributed to the direct binding of Grb14 to IRS-1 (supplementary information 5 online).

Recruitment of IRS-1 to the activated IR transduces signals that result in the activation of intracellular signalling pathways, including the ERK1/2 (Myers *et al*, 1994) and the protein kinase B (PKB) pathways (Sun *et al*, 1993). We (unpublished observations) and others (Shin *et al*, 2002) found that the PKB pathway is

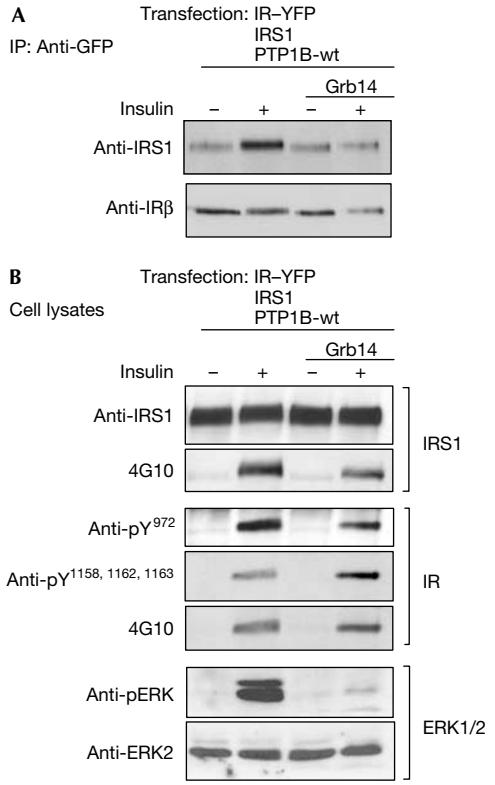

Fig 4 | Grb14 reduces insulin-stimulated association between IR and IRS-1. Human embryonic kidney cells were co-transfected with IR–YFP, IRS-1 and PTP1B, with or without Grb14. Cells were stimulated with 100 nM insulin for 10 min and lysed. (**A**) The IR was immunoprecipitated with an anti-green fluorescent protein (GFP) antibody. The amount of IRS-1 co-precipitated with the IR was evaluated with an anti-IRS-1 antibody. (**B**) Crude cell extracts from the same cells were submitted to western blotting and immunodetected with anti-IRS-1, 4G10 (general anti-phosphotyrosine), anti-$pY^{1158,1162,1163}$, anti-pY^{972}, anti-pERK1/2 and anti-ERK2 antibodies. ERK, extracellular signal-regulated kinase; IR, insulin receptor; YFP, yellow fluorescent protein.

constitutively activated in HEK293 cells. We therefore studied the consequences of Grb14 expression on ERK1/2 activation in cells co-transfected with IR–YFP, IRS-1, PTP1B-wt and Grb14. As shown in Fig 4B, phosphorylation of ERK1/2 was reduced in the presence of Grb14, confirming that the reduction of Y972 phosphorylation had consequences not only on the recruitment of IRS-1 but also on downstream signalling pathways. Although the activation of PKB cannot be evaluated in HEK cells, we observed that Grb14 markedly inhibits the phosphorylation of IRS1 on Y612, which is involved in the binding of PI3-K and activation of PKB (supplementary information 5 online).

Grb10 has also been reported to inhibit the interaction of IRS-1/2 with the IR, resulting in impaired downstream insulin signalling (Wick *et al*, 2003). However, this inhibitory effect was attributed to physical disruption of the IR/IRS interaction rather than to dephosphorylation of Y972. Differences in the molecular mechanism of binding of Grb10 and Grb14 to the IR have been described, particularly with regard to the respective roles of their SH2 (Src homology 2) and BPS (between Pleckstrin homology and SH2) domains. Indeed, whereas Grb14 is believed to bind to the IR by engaging both its SH2 and BPS domains on the three phosphotyrosines of the activation loop (Depetris *et al*, 2005), Grb10 is likely to bind to pY972 through its SH2 domain (Frantz *et al*, 1997) and to the activation loop through its BPS domain (Depetris *et al*, 2005). These differences may have a role in the different mechanisms by which Grb10 and Grb14 regulate IR/IRS interaction. In agreement with this notion, overexpression of Grb10 does not decrease the phosphorylation of Y972 of the IR in CHO cells, and even seems to increase it (Wick *et al*, 2003).

To determine whether the effects of Grb14 on site-specific dephosphorylation of the IR could also be detected in a more relevant cellular system, we studied the effect of increasing amounts of transfected Grb14 on the endogenous IR in the human liver cell line HuH7. We observed that increasing the amount of Grb14 specifically decreases the amount of pY972. The lowest amount of transfected Grb14 had no apparent effect on the phosphorylation of the activation loop, and at higher amounts, a slight but not significant decrease was observed. Densitometric analysis of three independent experiments indicates that increasing Grb14 amount by 2.0 ± 0.2-fold (an increase found in tissues of rodent models of insulin resistance and human type II diabetic patients; Cariou *et al*, 2004) significantly decreased the $pY^{972}/pY^{1158,1162,1163}$ ratio. Increasing the amount of Grb14 by 5.0 ± 0.9-fold further decreased the $pY^{972}/pY^{1158,1162,1163}$ ratio (Fig 5A,B). At these higher levels of Grb14 expression, a decrease in insulin-induced PKB phosphorylation was also observed, indicating that Grb14 also affects the PI3-K/PKB pathway in insulin target cells (Fig 5C).

Therefore, a site-specific dephosphorylation of the endogenous IR, associated with inhibition of downstream insulin signalling, is also observed when Grb14 expression increases in human liver cells. Although care should be taken when extrapolating results from cultured transfected cells, this result strongly suggests that our observations may be relevant to pathophysiological situations of insulin resistance.

Altogether, our work suggests that Grb14 binding to the activated IR modifies the interaction of IR with PTP1B. This impairs the dephosphorylation of the three tyrosines of the activation loop by PTP1B, while favouring the dephosphorylation of tyrosine 972. As a recent structural study suggested that Grb14 may act as a pseudosubstrate inhibitor for the IR kinase (Depetris *et al*, 2005), it is also possible that an inhibition of the IR catalytic activity leads to the decreased phosphorylation of tyrosine 972. This site-specific regulation of the tyrosine phosphorylation of the IR may explain some of the paradoxical effects of Grb14 observed previously. In addition, our results clearly demonstrate that considering the global phosphotyrosine content of the IR as an index of its signalling capability may be misleading, as the phosphorylation of different domains can be regulated in opposite ways.

Speculation

Site-specific regulation of IR dephosphorylation may permit fine-tuning of insulin signalling. Subcellular fractionation studies have shown that after insulin stimulation, most of the tyrosine-phosphorylated IRS-1 is recovered associated with intracellular membranes, probably associated with the internalized IR (Kelly & Ruderman, 1993). A desensitization mechanism, involving the release of tyrosine-phosphorylated IRS-1 from the endomembrane

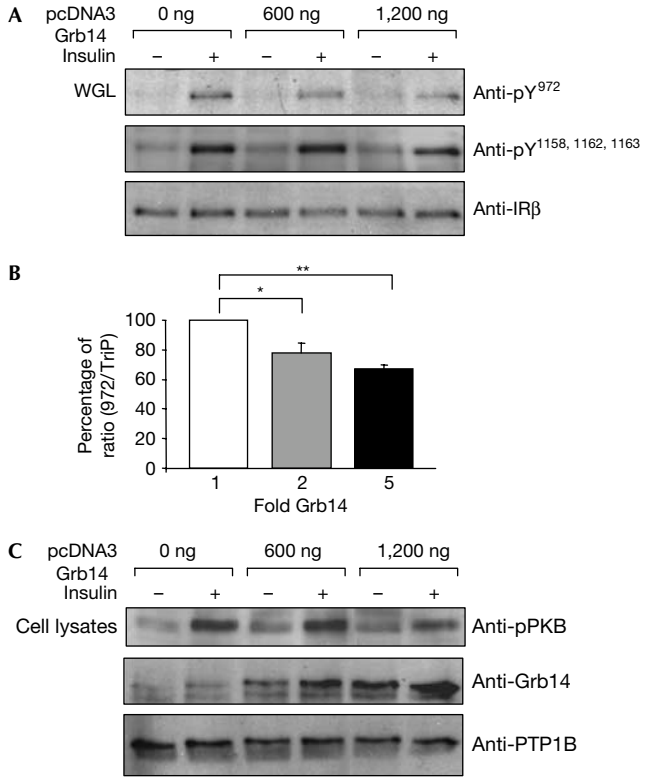

Fig 5 | Effect of Grb14 in liver-derived HuH7 cells. HuH7 cells transfected with different amounts of Grb14 complementary DNA were stimulated or not stimulated with 100 nM insulin for 10 min and lysed. (**A**) After partial purification of insulin receptor (IR) on wheat germ lectin, the phosphorylation of the IR was evaluated by western blotting using site-specific phospho-antibodies (anti-$pY^{1158,1162,1163}$ and anti-pY^{972}). The amount of receptors loaded in each lane was evaluated using an anti-IRβ antibody. (**B**) After densitometric analysis of the anti-$pY^{1158,1162,1163}$ and anti-pY^{972} phosphotyrosine signals, the $pY^{972}/pY^{1158,1162,1163}$ ratio was calculated. The data presented are means ± s.e.m. of three independent experiments (*$P<0.05$; **$P<0.01$). (**C**) Cell extracts were submitted to western blotting. The effect of insulin on the phosphorylation of protein kinase B (PKB) was evaluated using an anti-pPKB antibody. The expression levels of Grb14 and PTP1B proteins in these cells are also shown.

to the cytosol, has been proposed (Heller-Harrison *et al*, 1995). Signalling at endomembranes by internalized TK receptors has recently been shown to be of considerable importance. Indeed, internalized TK receptors can activate both Ras–MAP kinase (Bivona & Philips, 2003) and PI-3K (Sato *et al*, 2003) pathways on endoplasmic reticulum and Golgi membranes. Therefore, by increasing the dephosphorylation of tyrosine 972 by PTP1B, Grb14 may favour the release of IRS-1 from the endomembrane and thereby reduce endomembrane signalling by the IR.

METHODS

Materials. All materials have been described previously (Boute *et al*, 2003), except anti-IRS-1 (UBI, Charlottesville, CA, USA), anti-pERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Erk2 (UBI), anti-pY^{972} and anti-$pY^{1158,1162,1163}$ (Biosource International, Camarillo, CA, USA) and anti-pPKB (Cell Signaling Technology, Danvers, MA, USA) antibodies.

Expression vectors. IR–Rluc, IR–YFP, YFP–PTP1B-wt and YFP–PTP1B-D181A complementary DNAs have been described previously (Boute *et al*, 2003). Rat Grb14 coding sequence was inserted into pEYFP-N1 (Clontech, Mountain View, CA, USA).

BRET experiments. HEK293 cells were transfected exactly as described previously (Boute *et al*, 2003) using 300 ng of each cDNA construct, unless otherwise stated in the figure legend. One day after transfection, cells were transferred into 96-well microplates. All BRET measurements were carried out in these plates the following day, and results were expressed in mBRET units (mBU), as described previously (Boute *et al*, 2003).

Analysis of IR phosphorylation sites. HEK cells were transfected in 35 mm dishes 48 h before the experiments. After insulin stimulation, IRs were partly purified on WGL agarose or immunoprecipitated with anti-GFP antibody (Lacasa *et al*, 2005) in buffer containing 1 mM of freshly dissolved orthovanadate.

Statistical analysis. Statistical analysis was carried out using a Student's *t*-test for paired values.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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