BIOCHEMICAL PHARMACOLOGY 72 (2006) 1355–1366

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Interaction between the insulin receptor and Grb14: A dynamic study in living cells using BRET

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ARTICLE INFO

Article history:
Received 3 May 2006
Accepted 19 July 2006

Keywords:
BRET
Insulin receptor
Grb14
PTP1B
IRS-1
MAP kinases

Abbreviations:
IR, insulin receptor
Grb, growth factor receptor binding
TK, tyrosine kinase
PTP1B, protein tyrosine phosphatase 1B
IRS-1, insulin receptor subtrate 1
Shc, Src homology collagen
MAP kinases, mitogen activated protein kinases
ERK, extracellular signal-regulated kinases
PI3-kinase, phosphoatidyl inositol 3-kinase
PH, pleckstrin homology

ABSTRACT

Grb14 is a molecular adaptor that binds to the activated insulin receptor (IR) and negatively regulates insulin signaling. We have studied the dynamics of interaction of the IR with Grb14, in real time, in living HEK cells, using bioluminescence resonance energy transfer (BRET). Insulin rapidly and dose-dependently stimulated this interaction. Removing insulin from the incubation medium only resulted in a modest decrease in BRET signal, indicating that the interaction between the IR and Grb14 can remain long after insulin stimulus has disappeared. BRET saturation experiments indicated that insulin markedly increases the affinity between IR and Grb14, resulting in recruitment of the adaptor to the activated IR. In addition, using both BRET and co-immunoprecipitation experiments, we demonstrated that insulin induced the dimerization of Grb14, most likely as a result of simultaneous binding of two Grb14 molecules on the activated IR. We also investigated the relationships between IR, Grb14 and the protein tyrosine phosphatase PTP1B. We observed that insulin-induced BRET between the IR and PTP1B was markedly reduced by Grb14, suggesting that Grb14 regulated this interaction in living cells. Using site-specific antibodies against phosphorylated tyrosines of the insulin receptor, 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 ERK pathway. Our work suggests that Grb14 may regulate signalling through the insulin receptor by controlling its tyrosine-dephosphorylation in a site-specific manner.

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.07.018

SH2, Src homology 2
BPS/PIR, (between PH and SH2/phosphorylated insulin receptor interacting region)
Rluc, *Renilla* luciferase
YFP, yellow fluorescent protein
BRET, bioluminescence resonance energy transfer
mBU, milliBRET unit
PTPα/ε, protein tyrosine phosphatase α/ε
IGF-1, insulin-like growth factor-1
WGL, wheat germ lectin

1. Introduction

Insulin is a peptidic hormone produced by the β cell of the pancreas that plays a crucial role in the regulation of energy metabolism. It stimulates the uptake of nutrients (glucose, amino acids and fatty acids) by cells, accelerates their conversion into macromolecules (glycogen, proteins and triglycerides) and inhibits the degradation of these macromolecules. At the cellular level, insulin acts on its target tissues (liver, muscle and adipose tissue) through a plasma membrane receptor. The insulin receptor (IR) is a glycoprotein composed of two α-subunits and two β-subunits linked by disulfide bonds (Fig. 1). Binding of insulin to the α-subunits results in autophosphorylation of each β-subunit on several tyrosines. This autophosphorylation occurs first on three tyrosines located in the activation loop of the kinase domain (Y1158, 1162 and 1163), resulting in the stabilisation 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 C-terminal region (Y1328, 1334). Among these tyrosines, Y972 clearly appears as a docking site for substrates such as IRS-1 (insulin receptor substrate-1) and Shc (Src homology collagen protein) [1], which play crucial roles in the activation of signalling pathways, such as ERK (extracellular signal-regulated kinases) and PI3-kinase (phosphatidyl inositol 3-kinase) pathways (Fig. 1). Termination of the signal involves inactivation of the IR by dephosphorylation of the 3 tyrosines of the kinase domain [2]. PTP1B (protein tyrosine phosphatase 1B) is a protein tyrosine phosphatase located on the surface of the endoplasmic reticulum that plays a major role in the dephosphorylation of these tyrosines after internalisation of the IR [2,3]. Besides dephosphorylation, other mechanisms, involving molecular adaptors, also appear to play important roles in the control of the activity of tyrosine kinase (TK) receptors. Grb14 (growth factor receptor binding protein 14) belongs to the Grb7-like family of adaptor proteins made up thus far of Grb7, Grb10 and Grb14 [4]. These proteins share a lack of intrinsic enzymatic activity and display highly conserved structural features. They contain a small proline-riche SH3 (Src-homology 3) domain-binding motif, a RA (Ras-associating) domain, a central consensus pleckstrin homology (PH) domain, a BPS/PIR (between PH and SH2/phosphorylated receptor interacting region) domain and a conserved SH2 (Src-homology 2) domain [5]. These adaptors can regulate either positively or negatively signaling pathways elicited by different growth factors receptors [6,7].

Grb14 appears to be preferentially expressed in classical insulin-target tissues, such as liver, skeletal muscle, white adipose tissue, and in more recently described insulin-sensitive organs such as pancreas and brain [8]. Several lines of evidence indicate that Grb14 plays a negative role in the regulation of insulin signaling [8–11]. In pathological states, such as diabetes and obesity, insulin effect on its target tissues is markedly reduced. This insulin resistance is often associated with a decrease in the TK activity of the receptor, resulting in alterations in insulin signaling. Grb14 expression has been shown to be increased in white adipose tissue of rodent models of insulin-resistance and in human type 2 diabetic patients [12]. However, the mechanism by which Grb14 inhibits signalling through the IR remains elusive. *In vitro* experiments have shown that Grb14 may impair the TK activity of the IR towards exogenous substrates but protects the tyrosine-phosphorylated IR from dephosphorylation by PTP1B [10]. Paradoxical effects of Grb14 on the tyrosine-phosphorylation state and TK activity of the IR were observed in CHO cells, in which Grb14 appears either to have no effect [8,9] or to increase the tyrosine-phosphorylation state of the IR [10], while inhibiting insulin effect on IRS-1 phosphorylation [8,9], activation of the ERK pathway [10], glycogen and DNA synthesis [8]. Further evidence for a negative role of Grb14 in insulin signalling was obtained with Grb14 knock-out mice, which displayed improved glucose tolerance and insulin sensitivity [11]. However, paradoxical effects were also observed, including a decrease in the tyrosine phosphorylation of the IR associated with an increased tyrosine phosphorylation of IRS-1 in the liver of knock out mice. The decreased phosphorylation of the IR 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.

Grb14 is not a substrate of the insulin receptor TK activity. Interaction between Grb14 and the IR mainly involves the

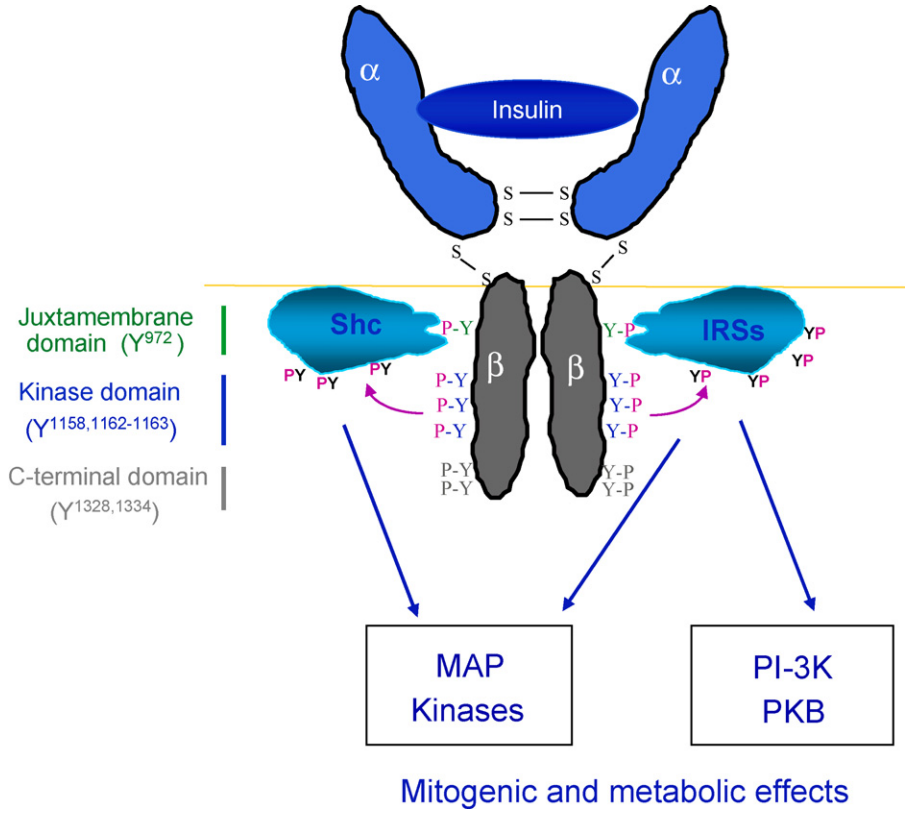

Fig. 1 – The insulin receptor and its dowstream signaling pathways. Binding of insulin to the α-subunit of the IR induces a conformational change that brings its two β-subunits in close proximity, allowing *trans*-phosphorylation of one β-subunit by the other on the three tyrosines of the activation loop (Y1158, 1162 and 1163), resulting in the stabilisation of the kinase in an active conformation. Phosphorylations then occur on a tyrosine located in the juxtamembrane domain (Y972) and on two tyrosines located in the C-terminal domain (Y1328, 1334). pY972 serves as a docking site for substrates such as IRS-1 and Shc, allowing activation of dowstream signalling pathways.

PIR/BPS domain of Grb14 and the regulatory tyrosine kinase loop of the IR [8]. The PIR/BPS domain was shown to be a largely unfolded region of Grb14 which could undergo some degree of folding in the presence of its physiological partner [13,14]. Bérézіat et al. showed that the PIR /BPS domain of Grb14 was sufficient for its inhibitory effect on IR substrate phophorylation *in vitro*, whereas the SH2 domain had no effect on IR catalytic activity [10]. More recently, determination of the crystal structure of the Grb14 PIR/BPS region in complex with the TK domain of the IR revealed that the N-terminal portion of the PIR/BPS region binds as a pseudosubstrate inhibitor in the substrate peptide binding groove of the kinase [15]. Although these studies have brought important insights into the molecular mechanisms of interaction between IR and Grb14, information concerning the dynamics of interaction between IR and Grb14 in living cells is very scarce. In addition, the relationships between Grb14 and PTP1B with regards to the negative regulation of the IR remain poorly defined.

The BRET technique is an important tool for the study of protein–protein interactions in living cells. In order to study the interaction between two partners, one of the partners is fused to Renilla luciferase (Rluc) and the other partner is fused to a yellow fluorescent protein (YFP). The luciferase is excited with its cell permeable substrate, coelenterazine. If the two parnters interact (if the distance between the two partners is less than 100 Å), an energy transfer can occur between the Rluc and the YFP, resulting in the emission of a fluorescent signal by the YFP. We previously used this technique to study ligand induced conformational changes within insulin and IGF-1 (insulin-like growth factor-1) receptors [16,17], as well as to monitor the dynamics of interaction of the IR and the IGF-1R with PTP1B in living cells [3,17]. We also studied the interaction of the IR with the receptor-like plasma membrane protein tyrosine phosphatases α (PTPα) and ε (PTPε) [18]. More recently, we showed that this technique can also be used to monitor the interaction between IR and Grb14 in real time, in intact living cells, and we demonstrated that Grb14 modulates the interaction of IR with PTP1B, resulting in site-specific dephosphorylation of the IR [19].

2. Materials and methods

All materials have been described previously [3,18,19], except anti-Rluc antibody (Chemicon International, Temecula, CA).

2.1. *Expression vectors*

IR-Rluc, IR-YFP, YFP-PTP1B-wt, YFP-PTP1B-D181A and Grb14-YFP cDNAs have been described previously [3,19]. Grb14-Rluc

was obtained by insertion of the coding sequence of rat Grb14 into the pcDNA3 *Rluc* vector (Promega, Madison, WI).

2.2. BRET experiments

HEK-293 cells were transfected exactly as described previously [3] 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, where all BRET measurements were carried out on the following day. Results were expressed in mBRET Units (mBU) as described previously [3].

2.3. Analysis of IR phosphorylation sites

HEK cells were transfected in 35 mm dishes 48 h before the experiments. After insulin stimulation, insulin receptors were

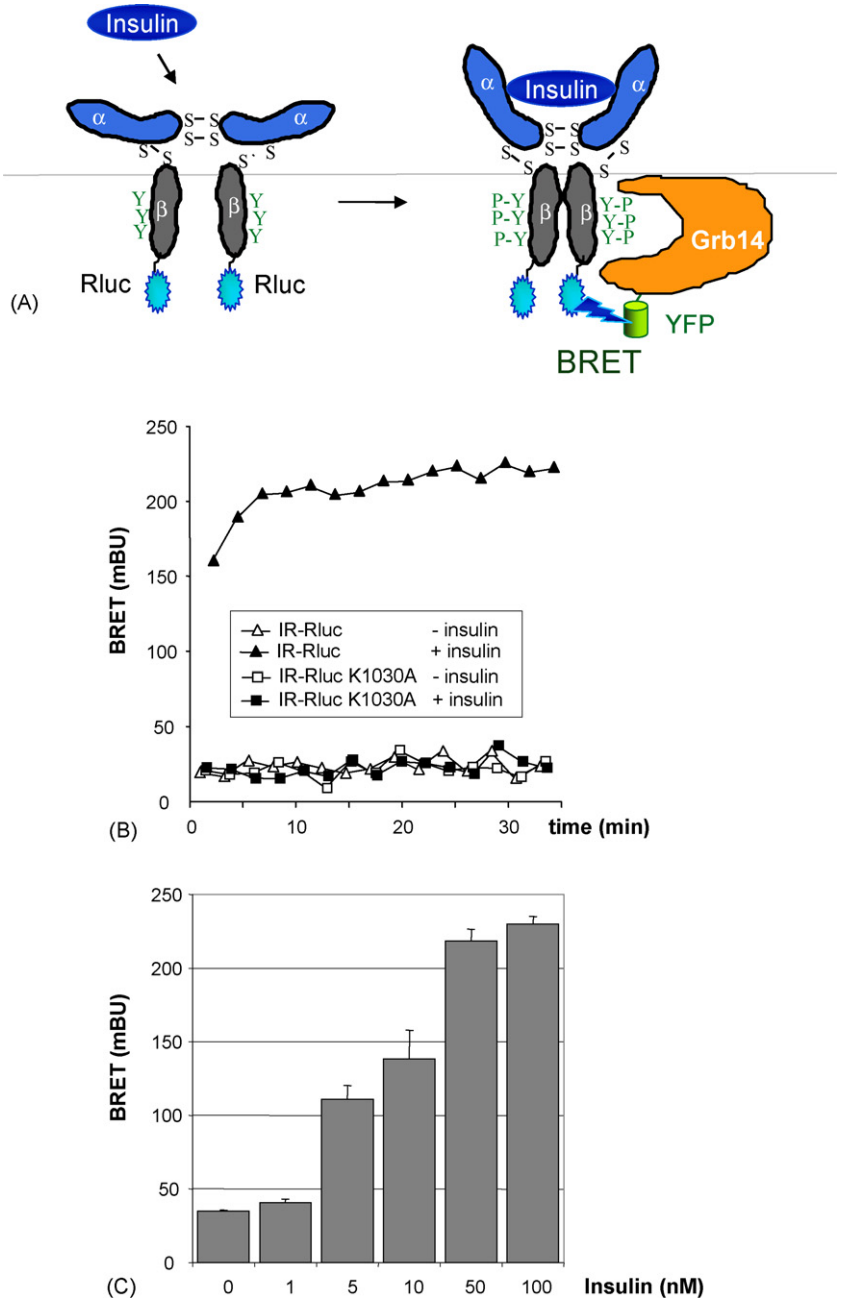

Fig. 2 – Dynamics of interaction between the IR and Grb14 in intact living cells (adapted from [19]). (A) The interaction between IR and Grb14 can be monitored in living cells by BRET using IR-Rluc and Grb14-YFP fusion proteins. (B) HEK cells were co-transfected with IR-Rluc (either wild-type or kinase-dead (K1030A) mutant) and Grb14-YFP. Cells were pre-incubated for 15 min in presence of coelenterazine (5 μM) and then stimulated with 100 nM of insulin. The dynamics of interaction between IR and Grb14 can be monitored in real time for more than 30 min. (C) Dose-dependent effect of insulin on BRET signal between IR and Grb14. Basal and insulin-stimulated BRET were measured 20 min after insulin addition. Results are means ± S.E.M. of two independent experiments.

partially purified on wheat germ lectin agarose beads or immunoprecipitated with anti-GFP antibody [18] in buffer containing 1 mM of freshly dissolved orthovanadate.

2.4. Statistical analysis

Statistical analyses were performed using a Student's *t*-test for paired values.

3. Results

3.1. Study of the dynamics of interaction between IR and Grb14 in living cells using BRET

In order to monitor the interaction between IR and Grb14 in living cells by BRET, HEK-293 cells were co-transfected with IR-Rluc and Grb14-YFP fusion proteins (Fig. 2A). In absence of insulin, a low basal BRET signal could be detected. Insulin rapidly increased this signal (Fig. 2B). In contrast, insulin had no effect on BRET signal when a kinase-dead version of IR-Rluc (IR-K1030A-Rluc) was used (Fig. 2B). This result demonstrates that binding of insulin to the IR has no effect by itself, and that autophosphorylation of the IR is necessary to induce interaction of Grb14 with the IR. Fig. 2C shows the BRET signal between IR-Rluc and Grb14-YFP, measured 20 min after stimulation with increasing doses of insulin. We observed that insulin dose-dependently stimulates the interaction between IR and Grb14. A half-maximal effect was observed at about 5 nM of insulin, which also corresponds to the EC50 of insulin for IR autophosphorylation [16].

Fig. 3A shows the evolution of IR-Grb14 interaction after removing of insulin from the incubation medium. We observed that removing insulin from the incubation medium only resulted in a modest decrease in BRET signal. Quantification of three independent experiments (Fig. 3B) showed that removing of insulin results in a statistically significant decrease in BRET at all time point considered, although this decrease is only of 6% at time 5 min and 18% at time 20 min. Comparison of Figs. 2B and 3A suggests that the rate of association after adding insulin is much higher than the rate of dissociation after removing insulin. Indeed, whereas it only took 2 min to observe an increase in BRET of

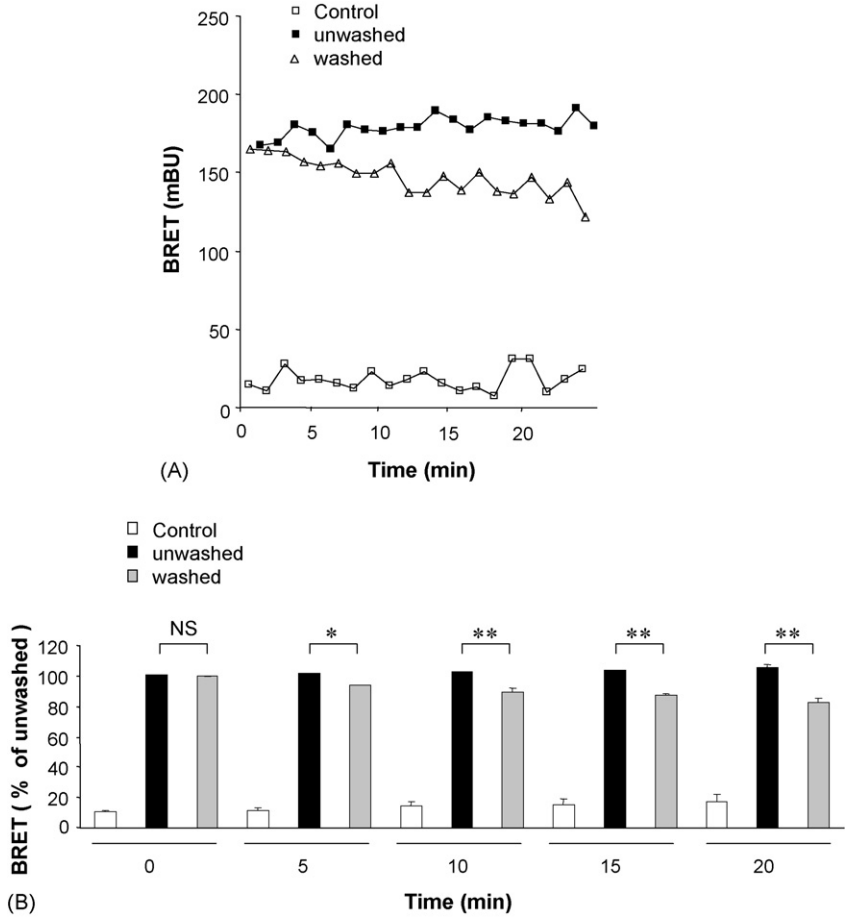

Fig. 3 – HEK cells were co-transfected with IR-Rluc and Grb14-YFP. (A) Cells were pre-incubated 20 min in absence (control condition) or presence of 100 nM insulin. 20 min after insulin addition, cells were washed three times in PBS (phosphate buffer saline) containing either 0 (washed condition) or 100 nM insulin (unwashed condition) and then incubated for a further 20 min in absence (washed condition) or presence of 100 nM insulin (unwashed condition). BRET measurements were started immediately after the third wash. (B) BRET signal was expressed as % of BRET in unwashed condition at different time-points. Results are means ± S.E.M. of three independent experiments ($^{*}p < 0.05$, $^{**}p < 0.01$, respectively).

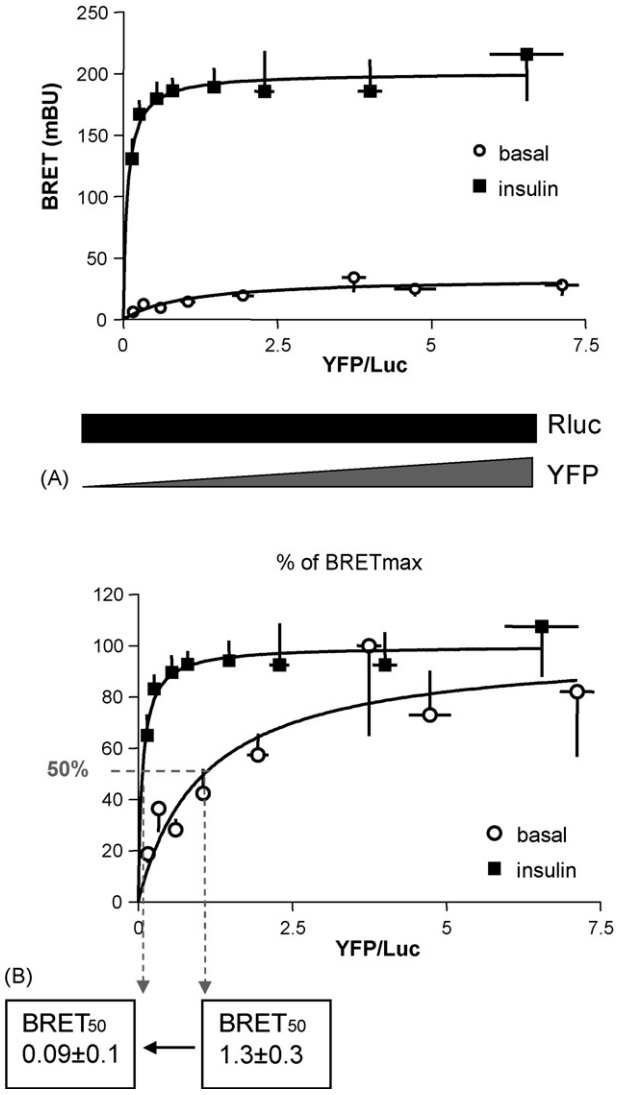

Fig. 4 – Saturation curves of BRET between insulin receptor and Grb14. HEK cells were co-transfected with a constant amount of cDNA coding for IR-Rluc (300 ng/well) and increasing amount of cDNA coding for Grb14-YFP (25–1500 ng/well). (A) 48 h after transfection, BRET signal, luciferase and fluorescence levels were measured. BRET signals were plotted as a function of the ratio of Grb14-YFP fluorescence to IR-Rluc luminescence. The results presented are from nine independent experiments. The curves were fitted using non-linear regression equation assuming a single binding site (GraphPad Prism). (B) Results are presented as percent of the BRETmax in basal and insulin-stimulated conditions.

about 140 mBU (i.e. an increase in BRET at a rate of approximately 70 mBU/min, Fig. 2B), it took 20 min to observe a decrease in BRET of about 45 mBU (i.e. a decrease in BRET at a rate of 2.25 mBU/min, Fig. 3A). These results suggest that the interaction between the IR and Grb14 is quite stable and remains long after the insulin stimulus has disappeared.

3.2. *Quantitative BRET analysis*

We have shown that insulin induces a marked increase in BRET signal between IR-Rluc and Grb14-YFP. BRET signal not only depends on the distance between the luminescent and flurorescent proteins, but also on their relative orientation [20]. Therefore, an insulin induced BRET signal could theoretically reflect either a recruitment of Grb14 to the activated receptor or a conformational change within pre-associated complexes containing insulin receptors and Grb14. We used quantitative BRET analysis to further characterize the effect of insulin on the interaction of the IR with Grb14. Quantitative BRET analysis has been used to assess receptor oligomerisation [21,22] as well as characterization of the association between the IR and receptor-like protein tyrosine phosphatases [18]. This analysis is based on the principle that the level of energy transfer detected for a constant donor concentration should rise with increasing concentrations of acceptor. When all donor molecules are engaged by an acceptor, the energy transfer reaches a plateau. BRET saturation curves are constructed by increasing the acceptor concentration for a constant donor concentration. The maximal level reached (BRETmax) reflects the total number of donor–acceptor complexes. The relative amount of acceptor giving 50% of the maximal energy transfer ($BRET_{50}$) reflects the relative affinity of the donor- and acceptor-fusion proteins.

HEK-293 cells were co-transfected with a constant amount of IR-Rluc and increasing concentrations of Grb14-YFP. As shown in Fig. 4A, the curves behave as hyperbolic functions reaching a saturation level. The BRET max values were 34.2 ± 4.9 and 200.8 ± 5.4 mBU in absence and presence of 100 nM insulin, respectively. These saturation curves were also presented as percentage of BRETmax. As shown in Fig. 4B,

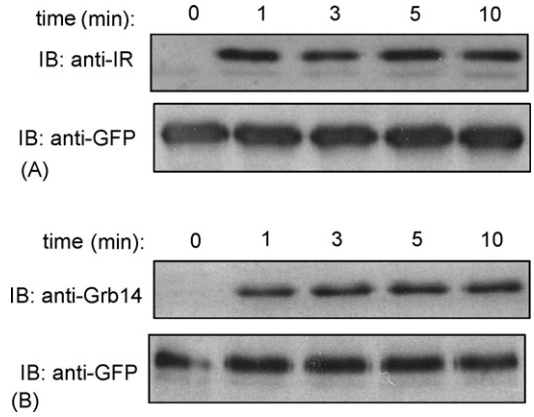

Fig. 5 – Co-immunoprecipitation of the activated insulin receptor and Grb14. Cells were co-transfected with either untagged IR and YFP-tagged Grb14 (A), or YFP-tagged IR and untagged Grb14 (B). Cells were incubated in absence or presence of 100 nM of insulin and lysed after different time of stimulation. Immunoprecipitation was performed using an anti-GFP antibody. The amount of untagged IR or Grb14 co-precipitated with YFP-tagged Grb14 or IR was evaluated by immunobotting using anti-IRβ or anti-Grb14 antibody, respectively. Insulin rapidly (1 min) induces the recruitment of Grb14 to the activated IR.

insulin induced a marked shift of the curve to the left, suggesting an increased affinity of Grb14 for the activated IR. The $BRET_{50}$ values were significantly reduced by insulin (1.299 ± 0.309 mBU in absence versus 0.0923 ± 0.015 mBU in presence of insulin, $p < 0.01$). This indicates that upon insulin stimulation, the relative affinity of Grb14 for the IR is increased by at least 14-fold. Such an increase in affinity of Grb14 for the IR results in the recruitment of Grb14 to the activated IR, as demonstrated by co-immunoprecipitation, with an anti-GFP antibody, of either untagged-IR with Grb14-YFP (Fig. 5A), or untagged-Grb14 with IR-YFP (Fig. 5B). In the absence of insulin, co-immunoprecipitation of the untagged partner with the YFP-tagged partner could not be detected, in agreement with a very low affinity between IR and Grb14 in the basal state. Insulin-induced increase in affinity resulted in rapid association between the two partners, as demonstrated by co-immunoprecipitation of the insulin-stimulated untagged-IR with Grb14-YFP (Fig. 5A), and, reciprocally, of untagged-Grb14 with the insulin-stimulated IR-YFP (Fig. 5B).

3.3. *Dimerization of Grb14*

Grb10 has been shown previously to be capable of dimerization or tetramerization in cells, in solution, and in crystals [23,24]. To date, there is no available information concerning the dimerization status of Grb14. To determine whether dimerization of Grb14 molecules can be detected by BRET, HEK-293 cells where co-transfected with the untagged version of IR, a Rluc-tagged version of Grb14 (Grb14-Rluc) and the Grb14-YFP construct (Fig. 6A). Cells were stimulated with 100 nM of insulin and BRET measurements were performed in real time (Fig. 6B). We observed that, in the absence of insulin, a small basal BRET signal could be detected. Insulin increased this signal by two- to three-fold (Fig. 6C). The effect of insulin is likely to result from

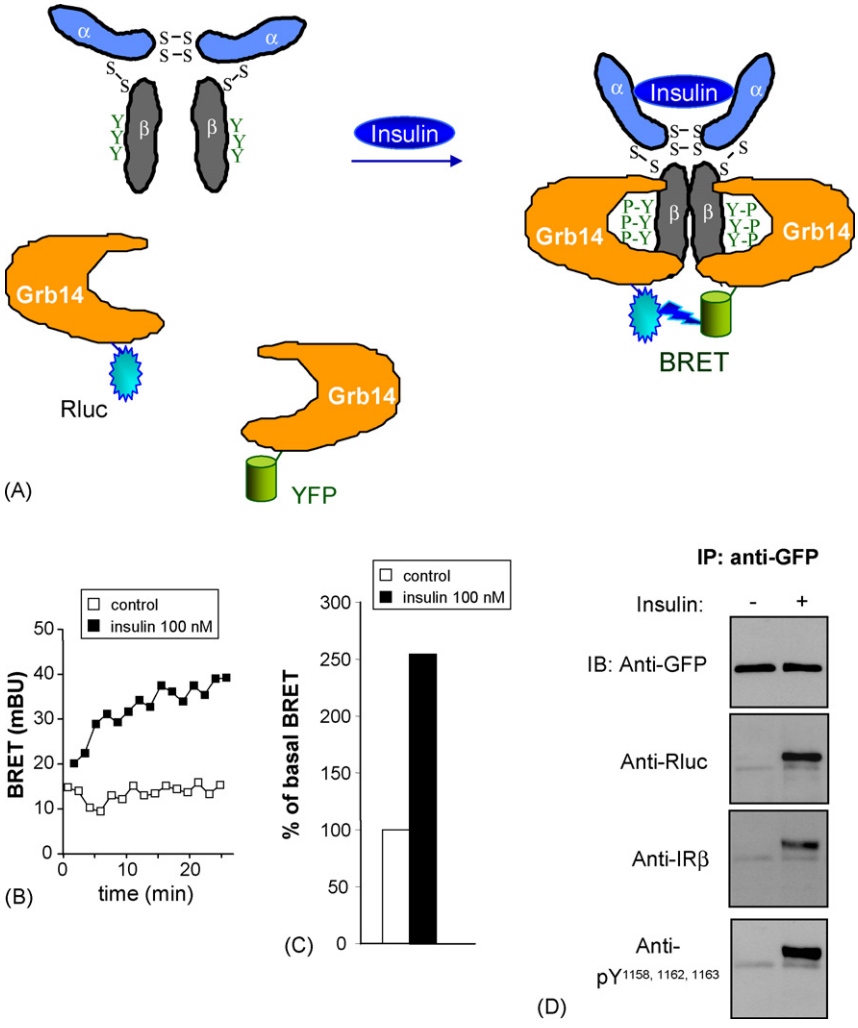

Fig. 6 – Dimerization of Grb14 is living cells. (A) HEK cells were co-transfected with cDNAs coding for untagged IR (300 ng/well), Grb14-Rluc (300 ng/well) and Grb14-YFP (50 ng/well). (B) BRET signal was monitored in the absence or presence of 100 nM insulin. (C) Basal and insulin-induced BRET signal at time 20 min expressed as percentage of the basal value. (D) Cells transfected as described in A were incubated in absence or presence of 100 nM insulin. Grb14-YFP was immunoprecipitated with an anti-GFP antibody. In the presence of insulin, Grb14-Rluc and the activated (tris-phosphorylated) IR were co-immunoprecipitated with Grb14-YFP, demonstrating that two Grb14 molecules can bind simultaneously to the activated IR. The data shown are representative of three independent experiments.

dimerization induced by the recruitment of Grb14 to each β-subunit of the IR. Indeed, although insulin effect on BRET between Grb14 molecules (two- to three-fold) appeared to be lower than insulin effect on BRET between IR and Grb14 (about 10-fold), it must be kept in mind that BRET competent dimers (Grb14-Rluc/Grb14-YFP) only represent a fraction of the possible combinations of tagged-Grb14 molecules recruited to the activated IR. Insulin-induced dimerization of Grb14 on the IR was further demonstrated by co-immunoprecipitation, with an anti-GFP antibody, of a complex containing Grb14-Rluc, the tyrosine-phosphorylated IR and Grb14-YFP (Fig. 6D). Therefore, our results are in line with biochemical and structural studies indicating that the adapter proteins Grb10 and APS (Adapter protein containing a PH and a SH2 domain), which were both crystallised in dimeric forms, engaged two phosphorylated IR kinase molecules [24,25], and with the model proposed by Depetris et al. [15], based on crystalysation studies of the PIR/BPS and SH2 domains of Grb14.

3.4. *Grb14 modulates the interaction between IR and PTP1B*

Grb14 and PTP1B are both believed to interact with the activation loop of the IR [2,8,15], raising the possibility that they may compete for binding to the IR. *In vitro* experiments, using GST-tagged Grb14, showed that Grb14 binding to the IR inhibited its dephosphorylation by purified recombinant PTP1B [10]. However, the relationships between IR, Grb14 and PTP1B had never been studied in living cells. We previously demonstrated [3] that the interaction between IR and PTP1B can be monitored by BRET using IR-Rluc and a YFP-tagged substrate-trapping mutant of PTP1B (YFP-PTP1B-D181A). We therefore analysed the effect of Grb14 on the interaction between IR and PTP1B in living HEK cells co-transfected with IR-Rluc and YFP-PTP1B-D181A, in the absence or presence of untagged-Grb14 (Fig. 7A). We observed (Fig. 7B; [19]) that in cells transfected with Grb14,

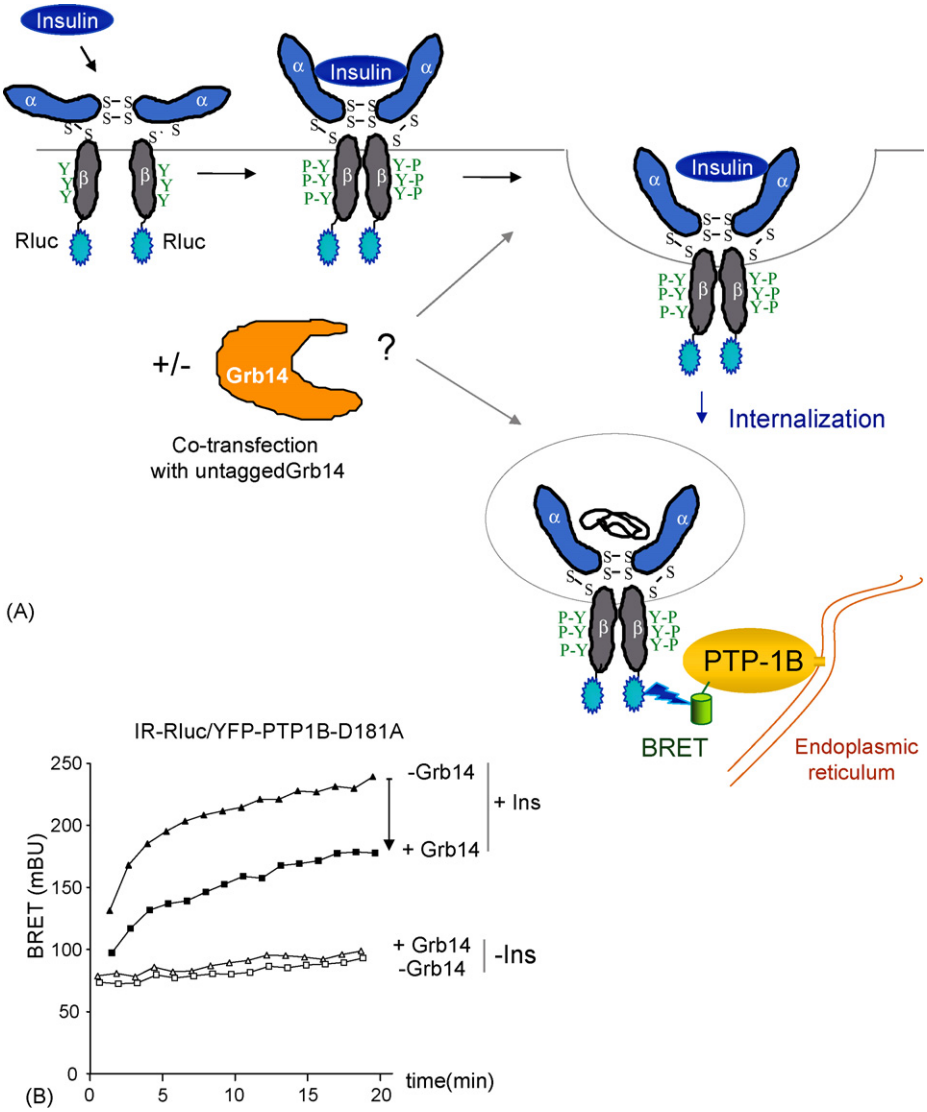

Fig. 7 – Grb14 inhibited BRET between IR and PTP1B (adapted from [19]). (A) HEK cells were co-transfected with IR-Rluc and YFP-PTP1B-D181A, and either Grb14 or empty vector (pcDNA3). (B) BRET signal was monitored during at least 20 min in absence or presence of 100 nM insulin. In the presence of Grb14, insulin-induced BRET between IR and PTP1B was markedly decreased.

insulin-induced BRET signal between IR-Rluc and YFP-PTP1B-D181A was markedly reduced (by about 40%). This suggested that Grb14 could indeed affect the interaction between IR and PTP1B. However, BRET not only depends on the distance between two partners, but also on their relative orientation [18]. 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. Immunoprecipitation experiments using an anti-GFP antibody indicated that the amount of IR that can be co-immunoprecipitated with YFP-PTP1B-D181A was similar whether the cells have been co-transfected or not with Grb14 [19]. These results suggested that the Grb14-induced decrease in BRET (Fig. 7B) reflect subtle conformational changes in the interaction between IR and PTP1B, rather than disruption of this interaction.

3.5. *Grb14 differentially regulates the dephosphorylation of specific phosphotyrosine residues on the IR*

Since Grb14 appeared to modulate the way IR and PTP1B interact, we analysed, in cells co-transfected with IR-Rluc and the wild-type version of YFP-PTP1B, the consequences of the presence of Grb14 expression on specific tyrosine-phosphorylation sites of the IR, 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}). We observed that in cells co-transfected with both Grb14 and PTP1B, 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. 8; [19]). These results suggest that Grb14 specifically protects the three tyrosines located in the activation loop of the IR from PTP1B activity, while favouring the dephosphorylation of Y972. Therefore, the presence of Grb14 may impair the docking of the active site of PTP1B on the phospho-tyrosines of the catalytic loop and cause displacement of PTP1B activity towards pY^{972} (Fig. 9C).

3.6. *Modulation of IR downstream signalling by Grb14*

Since Grb14 seemed to specifically increase the dephosphorylation of Y972 by PTP1B, the major docking site for IRS-1 on the activated IR [1], we 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. After insulin stimulation, the IR was immunoprecipitated using an anti-GFP antibody. We observed that the amount of IRS-1 co-precipitated with the activated IR was markedly reduced in cells co-transfected with Grb14 (Fig. 9A). This suggests that by favouring the dephosphorylation of Y972, Grb14 reduced the amount of IRS-1 recruited to the insulin-stimulated IR. Recruitment of IRS-1 to the activated IR transduces signals that result in the activation of intracellular signalling pathways, including the ERK1/2 [26] and the PKB (protein kinase B) pathways [27]. We (unpublished observations) and others [28] found that the PKB pathway is constitutively activated in

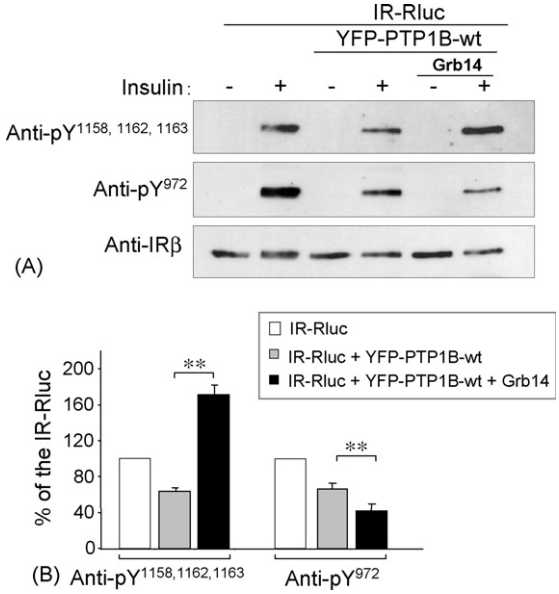

Fig. 8 – Grb14 regulates the dephosphorylation of the IR in a site-specific manner (adapted from [19]). HEK cells were co-transfected with IR-Rluc alone or in combination with YFP-PTP1B-wt and Grb14. Cells were stimulated or not with 100 nM insulin for 10 min and lysed. (A) After partial purification of IR on WGL, 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 IRβ-antibody. Results are representative of five independent experiments. (B) Densitometric analysis of the anti-phosphotyrosine signals corrected by the anti-IR signal. The data presented are means ± S.E.M. of three to four independent experiments ($^{**}p < 0.01$).

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. 9B (upper blot), phosphorylation of ERK1/2 was reduced in the presence of Grb14, confirming that the reduction of phosphorylation of Y972 had consequences not only on the recruitment of IRS-1 but also on downstream signalling pathways. Although the activation of PKB could not be directly evaluated in HEK cells, we observed that Grb14 markedly inhibits the phosphorylation of IRS-1 on Y612, which is involved in the binding of PI-3 kinase and activation of PKB (Fig. 9B, lower blot). In addition to IRS-1, pY^{972} also serves as a docking site for binding of Shc to the activated IR. Therefore, although the recruitment of Shc to the activated IR has not been evaluated in this study, it is likely that Grb14 also reduces Shc recruitment and phosphorylation by the IR, thereby contributing to inhibition of downstream signalling (Fig. 9C).

Grb10 has also been reported to inhibit the interaction between IRS-1/2 and the IR, resulting in impaired downstream insulin signalling [29]. However, this inhibitory effect was attributed to physical disruption of the IR/IRS interaction rather than to dephosphorylation of Y972. Differences in the

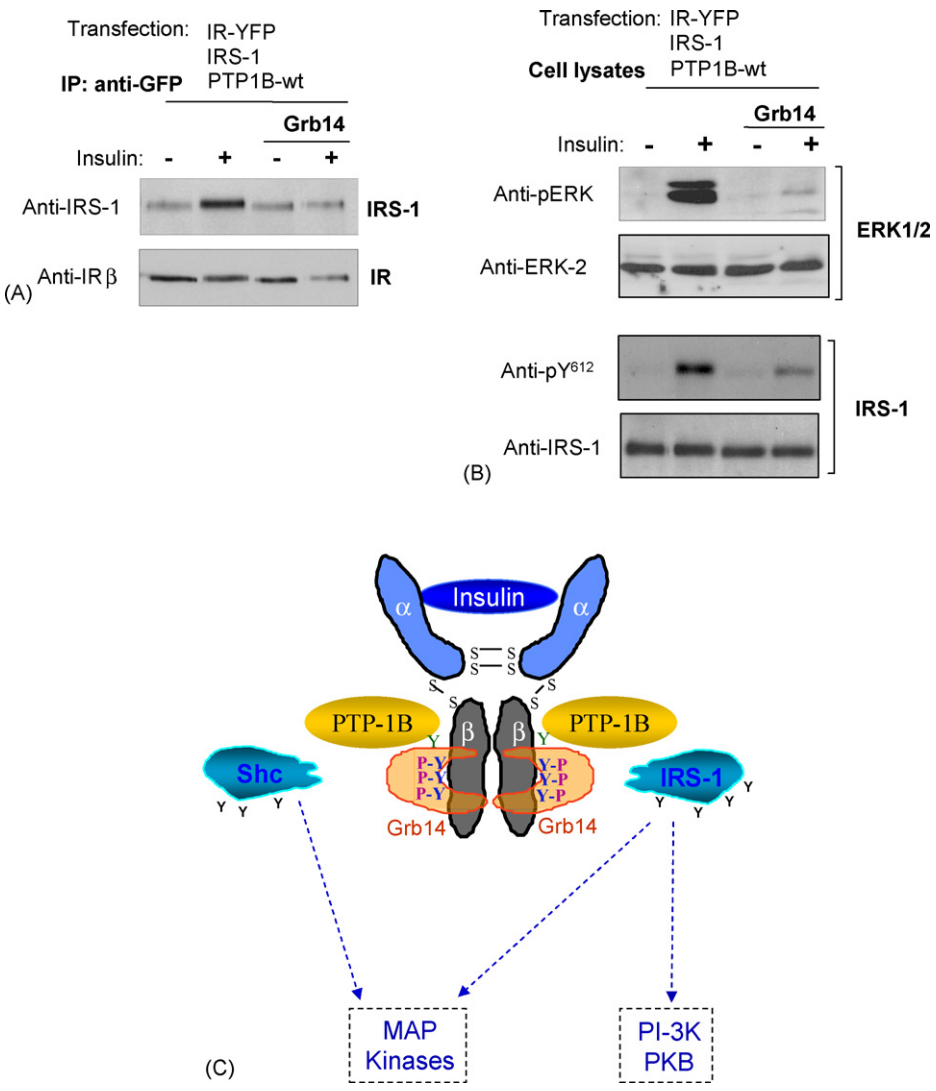

Fig. 9 – Grb14 reduces insulin-stimulated association between IR and IRS-1 (adapted from [19]). HEK cells were co-transfected with IR-YFP, IRS-1, PTP1B, with or without Grb14. Cells were stimulated with 100 nM insulin for 10 min and lysed. (A) The IR was immunoprecipitated with an anti-GFP antibody. The level 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-pERK1/2, anti-ERK2, anti-pY^{612}IRS-1 and anti-IRS-1 antibodies. (C) Model for the regulation of insulin signaling by Grb14. Binding of Grb14 to the activation loop of the IR results in site-specific dephosphorylation of tyrosine 972 by PTP1B. This reduces the binding of insulin receptor substrates to the activated IR, resulting in inhibition of downstream signaling pathways. In addition, the PIR/BPS region of Grb14 behaves as a pseudosubstrate inhibitor of the kinase of the IR [15], further contributing to inhibition of insulin signaling.

molecular mechanism of binding of Grb10 and Grb14 to the IR have been described, particularly with regard to the respective role of their SH2 and BPS 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 [15], Grb10 is likely to bind to pY972 through its SH2 domain [30] and to the activation loop through its BPS domain [15]. These differences may play 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 would even appear to increase it [29].

4. Discussion

Altogether, our work suggests that insulin-induced tyrosine-phosphorylation of the IR markedly increases the affinity of Grb14 for the activation loop of the IR. This would result in the rapid and stable binding of two Grb14 molecules on the two β-subunits of the activated IR. Binding of Grb14 appears to modulate the interaction of PTP1B with the IR. This impairs the dephosphorylation of the three tyrosines of the activation loop by PTP1B, while favouring the dephosphorylation of tyrosine 972. Although it cannot be excluded that Grb14 increases the specific activity of PTP1B towards phosphotyr-

osine 972, it is likely that Grb14-induced dephosphorylation of this tyrosine simply reflects mass action displacement of PTP1B activity toward this residue, due to shielding of the three tyrosines of the kinase domain by Grb14. This site-specific regulation of the tyrosine phosphorylation of the IR may explain some of the paradoxical effects of Grb14 observed previously [8–11]. In addition, our results clearly demonstrate that considering the global phospho-tyrosine content of the IR as an index of its signalling capability may be misleading, since the phosphorylation of different domains can be regulated in opposite ways.

Since the receptor can be deactivated by protein tyrosine phosphatases, the biological function of Grb14 as a negative regulator of insulin signaling remains intriguing. Interestingly, the insulin receptor is known to be internalized as a tyrosine-phosphorylated, potentially fully active kinase [31]. Grb14 has been proposed to function as a pseudosubtrate inhibitor that impairs the tyrosine-kinase activity of the phosphorylated receptor [15]. Therefore, it is possible that Grb14 serves to inhibit the kinase activity of the insulin receptor during its trafficking from the cell surface towards an intracellular dephosphorylation compartment, thereby precluding promiscuous tyrosine-phosphorylation of irrelevant intracellular proteins. Alternatively, Grb14 may simply serve as a chaperone or as an adapter that helps the activated receptor to move to the endoplasmic reticulum for its deactivation by PTP1B.

Our work suggests that by regulating IR dephosphorylation in a site-specific manner, Grb14 may permit fine-tuning of insulin signalling. Thus, the relative amounts of Grb14 and PTP1B expressed in a given cell could play an important role in the control of kinetics of deactivation of insulin signalling pathways. However, the level of expression of other protein tyrosine phosphatases may also be important. Plasma membrane tyrosine phosphatases, such as PTPα/ε, can also regulate the activity of the IR [18]. In addition, TC-PTP (T cells protein tyrosine phosphatase), which, like PTP1B, is targeted to the endoplamic reticulum, has been shown to differentially regulate the phosphorylation of Y972 of the IR [32], Therefore, further work aimed to investigate the effect of Grb14 on the interaction between IR and PTPα/ε or TC-PTP may be of considerable interest for a better understanding of the mechanism of deactivation of the insulin receptor.

Acknowledgments

This work was supported by the Institut de Recherche Servier, the Association pour la Recherche sur le Cancer, the Ligue contre le Cancer (Comité de Paris) and the Ministère de la Recherche (ACI no. 02 2 0537/8).

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