Grb14 belongs to the Grb7 family of adapters and was recently identified as a partner of the insulin receptor (IR). Here we show that Grb14 inhibits in vitro IR substrate phosphorylation. Grb14 does not alter the $K_m$ for ATP and behaves as an uncompetitive inhibitor for the IR substrate. Similar experiments performed with other members of the Grb7 family, Grb7 and Grb10, and with IGF-1 receptor argue in favor of a specific inhibition of the IR catalytic activity by Grb14. The IR-interacting domain of Grb14, the PIR, is sufficient for the inhibitory effect of Grb14, whereas the SH2 domain has no effect on IR catalytic activity. In Chinese hamster ovary (CHO) cells overexpressing both IR and Grb14, Grb14 binds to the IR as early as 1 min after insulin stimulation, and the two proteins remain associated. When interacting with Grb14, the IR is protected against tyrosine phosphatases action and therefore maintained under a phosphorylated state. However, the binding of Grb14 to the IR induces an early delay in the activation of Akt and ERK1/2 in CHO-IR cells, and ERK1/2 are less efficiently phosphorylated. These findings show that Grb14 is a direct inhibitor of the IR catalytic activity and could be considered as a modulator of insulin signaling.

Activation of receptor tyrosine kinases by ligand binding results in the autophosphorylation of multiple tyrosine residues of the cytoplasmic domain of the receptors. Phosphorylated tyrosyl residues act then as binding motifs for multiple intracellular effectors, which interact through their Src homology 2 (SH2) domains, and initiate various signaling pathways (1). Among the receptor tyrosine kinases, the insulin receptor (IR) behaves specifically since the main effectors, the IRSs and Shc families of proteins, bind through their phosphotyrosine binding domains (2). IRSs and Shc act then as scaffolding proteins, recruiting SH2-containing effectors. Searching for new proteins able to bind to the intracellular domain of the IR led to the identification of a new family of proteins, the Grb7 family of molecular adapters. This family comprises Grb7, Grb10, and Grb14 (3). These proteins possess a C terminus SH2 domain, which is implicated in the interaction with a number of receptor tyrosine kinases and also with different signaling proteins (for a review, see Ref. 3). However, in addition to the SH2 domain, another region of the protein is required for the binding to the IR. This region, located upstream from the SH2 domain, was called BPS (for between pleckstrin homology (PH) and SH2 (4)) or PIR (for phosphorylated insulin receptor-interacting region (5)). The BPS/PIR domain is only conserved among the Grb7 family of proteins (4–6). Although the SH2 domain is sufficient for the association between Grb7 and the IR, the binding of Grb10 is mediated by both domains SH2 and BPS/PIR (4, 6–10). In the case of Grb14, the SH2 domain is dispensable for the interaction with the IR, the PIR being the main interacting region (5). Interestingly, Grb14 interacts specifically with the IR regulatory tyrosine kinase loop (5). By contrast, Grb7 and Grb10 interact with the IR through distinct domains since, in addition to the activation loop, they also bind to the juxtamembrane site and to the C terminus (6, 8–10).

The biological role of the members of the Grb7 family of proteins is not yet elucidated. Various studies have suggested a role for Grb7 in the regulation of cell migration (11–15). Grb10 is expressed as six different isoforms (16). It is found associated with mitochondria where it interacts with Raf1 and is likely to be involved in the regulation of apoptosis (17, 18). The role of the Grb7 family of proteins in the insulin signal transduction is still not fully understood. At the present time, available data are discordant suggesting either a positive or a negative role of Grb10 on insulin-induced mitogenesis (16, 19). We recently identified Grb14 as a novel effector of insulin signaling (5). Grb14 is specifically expressed in insulin-sensitive tissues, and insulin induces Grb14 binding to the IR in vivo in rat liver. In CHO-IR cells, the overexpression of Grb14 leads to an inhibition of insulin actions such as DNA and glycogen synthesis or tyrosine phosphorylation of proteins (5, 20). However, as already reported for IRS-1, this inhibitory effect could be due to the sequestration of downstream insulin signaling effectors (21, 22). Thus, the positive or negative role of Grb14 on insulin signaling has not been clearly established.

The present study focuses on the elucidation of the molecular mechanisms implicated in the effects of Grb14 on insulin signaling. To get insight on the role of the association between Grb14 and the IR regulatory kinase loop, we determined whether Grb14 binding could alter the IR catalytic activity in an in vitro system. We demonstrate here that Grb14 inhibits
the IR substrate phosphorylation activity and that this inhibitory effect is mediated through the PIR domain. In vivo in CHO-IR cells, Grb14 overexpression induces alterations in the activation by insulin of the Akt and ERK pathways. In addition, experiments performed with Grb7 and Grb10 and with the insulin-like growth factor-1 (IGF-1) receptor argue in favor of a specific inhibitory effect of Grb14 on the IR catalytic activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides were purchased from Invitrogen. Monoclonal anti-phosphotyrosine horseradish peroxidase-conjugated antibody and polyclonal antibodies against the IR β-subunit were purchased respectively from Amersham Biosciences, Inc. and Santa Cruz Biotechnology. Polyclonal antibodies directed against the phosphorylated forms of ERK1/2 and Akt (Ser-473) were from Promega and New England Biolabs, respectively. Polyclonal antibodies against ERK1/2 and Akt were from Transduction Laboratories and Santa Cruz Biotechnology. Monoclonal anti-Myc and anti-PTP1B antibodies were from Invitrogen and Oncogene Science, respectively. Polyclonal anti-Grb14 antibodies were described previously (5). All chemicals were from Sigma. Culture media and geneticin (G418) were from Invitrogen, and hygroycin was from Roche Molecular Biochemicals.

**Cell Lines and Culture Conditions**—CHO-IR and CHO-IR/Grb14 cell lines were cultured as previously (5). HEK293 cells were cultured exponentially (5). HEK293-122 cells were cultured in medium (4.5 g/liter glucose) supplemented with 1 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum in the presence of 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and geneticin (G418) from Invitrogen. Cells were plated at 5 × 10⁵ cells/ml.

**Partial Purification of Insulin and IGF-1 Receptors**—Culture medium from CHO-IR or CHO-IR/Grb14 cells were harvested (10 g of cells/ml) and processed as described previously (23). Briefly, induced bacteria were lysed by sonication in PLC lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 10% leupeptin, 100 µg/ml pepstatin A, 20 µg/ml leupeptin, 100 µg/ml protease inhibitors (Complete, Roche Molecular Biochemicals), 10% glycerol, and 1% Triton X-100 for 60 min at 4 °C. The lysates were clarified by a centrifugation at 15,000 × g for 15 min at 4 °C. The pellets were washed in ice-cold H₂O and boiled in 20 µl of Laemmli sample buffer (24). The samples were separated by reducing SDS-PAGE (10% resolving gels) and visualized by autoradiography. Blots were analyzed by densitometry and quantified (Quantity One from Bio-Rad).

**Tyrosine Kinase Activity in Vitro Assays**—The IR and IGF-1 receptor (IGF-1R) tyrosine kinase activity was measured essentially as described previously (25). Aliquots of the WGA-purified receptors (0.5 nM final concentration) were incubated for 1 h at room temperature in the presence or absence of 100 nM insulin or IGF-1 in 30 mM HEPES buffer (pH 7.6), 30 mM NaCl, 0.1% Triton X-100, and 0.05% bovine serum albumin. The reaction was stopped by addition of 10 mM HEPES (pH 7.6), 30 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol. The lysates were cleared by a centrifugation at 15,000 × g for 15 min at 4 °C. For direct Western blots equal amounts of proteins were subjected to SDS-PAGE analysis and immunodetected with the indicated antibodies. The immunoreactive bands were revealed using the ECL detection kit (Amersham Biosciences, Inc.). The autoradiograms were analyzed by densitometry and quantified (Quantity One from Bio-Rad). For immunoprecipitations, after a preclearing step the lysates were incubated overnight at 4 °C with anti-Grb14 or anti-PTP1B antibodies in the presence of protein A-agarose (Upstate Biotechnology) or protein G-Sepharose (Sigma). After extensive washing in lysis buffer, the resulting immunoprecipitates were analyzed by SDS-PAGE as described above.

**RESULTS**

**Grb14 Inhibits the IR Substrate Phosphorylation**—A role for Grb14 on IR catalytic activity was investigated in vitro using partially purified IR and recombinant GST-Grb14. The effect of Grb14 was first tested in vitro on the kinetics of IR autophosphorylation (Fig. 1A). In the absence of Grb14, IR was markedly phosphorylated after 2 min of exposure to insulin. A maximal increase was observed after 5 min, and the phosphorylation was maintained for 30 min. The addition of 120 nM GST-Grb14 induced a slight decrease in IR autophosphorylation, which was significant only after 30 min (Fig. 1A). The IR kinase activity was then measured as the phosphorylation of

**Polyprotein Phosphatase Phosphatase in Vitro Assays**—Aliquots of the WGA-purified IR were activated by insulin as described above and phosphorylated for 30 min at room temperature in a kinase buffer (30 mM HEPES (pH 7.6), 30 mM NaCl, 8 mM MgCl₂, 4 mM MnCl₂, 100 mM ATP) in the presence or absence of 1 µg of GST-Grb14 (120 nM final concentration). The dephosphorylation reaction was performed with 1.5 µg of the protein tyrosine phosphatase PTP1B (Upstate Biotechnology) for 40 min at room temperature in an assay buffer containing 30 mM HEPES (pH 7.6), 30 mM NaCl, 5 mM dithiothreitol, 2.5 mM EDTA, and 0.7% bovine serum albumin in the presence or absence of 2 mM orthovanadate. The reaction was stopped by addition of 4 × stop buffer, and the samples were separated by reducing SDS-PAGE. IR phosphorylation was visualized by Western blotting using anti-phosphotyrosine antibodies, analyzed by densitometry, and quantified (Quantity One from Bio-Rad).

**Immunoprecipitation and Western Blotting**—Confluent CHO-IR or CHO-IR/Grb14 cells were serum-deprived for 24 h and stimulated or not with insulin (100 nM) for various periods of time. Cells were solubilized at 4 °C in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM NaF, 1% Triton X-100, 0.1% bovine serum albumin, 1 µg/ml peptide A, 2 µg/ml bovine serum albumin, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM phenylthiourea (transducant). Lysates were cleared by a centrifugation at 15,000 × g for 15 min at 4 °C. For direct Western blots equal amounts of proteins were subjected to SDS-PAGE analysis and immunodetected with the indicated antibodies. The immunoreactive bands were revealed using the ECL detection kit (Amersham Biosciences, Inc.). The autoradiograms were analyzed by densitometry and quantified (Quantity One from Bio-Rad).
the synthetic substrate poly(Glu:Tyr) (4:1) in the presence of increasing concentrations of GST-Grb14. As shown in Fig. 1B, GST-Grb14 strikingly inhibited IR catalytic activity. A 40% inhibition was observed for the addition of 2 nM GST-Grb14, and the activity was abolished for a concentration of 20 nM. The addition of up to 200 nM GST did not modify IR catalytic activity. Thus, the inhibitory effect of GST-Grb14 was specific to the Grb14 protein. The molecular adaptors Shc and SH2-B are also known to bind to the phosphorylated IR (5, 26–29). As shown on Fig. 1B, these adaptors did not alter IR catalytic activity, suggesting that Grb14 acts as a direct and potent inhibitor of IR substrate phosphorylation.

As the phosphorylation of IR induces a conformational change that allows access to the ATP and to the substrate binding sites (30), we determined whether the binding of Grb14 altered the kinetic parameters of the IR catalytic activity. First, increasing concentrations of ATP were tested in the presence or absence of 4 nM GST-Grb14 fusion protein. In the absence of Grb14, kinetics data obtained were in the range of values reported in the literature (31, 32). In the presence of Grb14 the $K_m$ for ATP was not altered, but the $V_{max}$ was decreased by 30%, suggesting that Grb14 acts as a noncompetitive inhibitor for ATP (Fig. 2A). Similar experiments were then performed using increasing amounts of the synthetic substrate poly(Glu:Tyr) (4:1) in the presence of 0, 4, and 16 nM GST-Grb14. Three parallel straight lines were obtained on the Lineweaver-Burk plot, indicating that Grb14 modified both $K_m$ and $V_{max}$ values (Fig. 2B). The $K_m$ for the synthetic substrate was only slightly reduced by Grb14, but the $V_{max}$ was decreased by 70–80%. These kinetics data are representative of an uncompetitive inhibitor. Taken together these results show that Grb14 reduces IR-catalyzed reactions without blocking the access to the ATP and to the substrate binding sites.

Grb14 Is a Specific Inhibitor of the IR Catalytic Activity—The IGF-1R is a member of the tyrosine kinase family of growth factor receptors and is closely related to the IR (33). Grb14 interacts with the activated IGF-1R in vitro and in the two-hybrid system (not shown). The effect of GST-Grb14 was then tested on in vitro IGF-1R catalytic activity. As reported in Fig. 3, the IGF-1R catalytic activity was less sensitive than IR to the inhibitory effect of Grb14: the addition of 6 nM GST-Grb14 decreased by 35% the IGF-1R catalytic activity compared with 80% for the IR. Thus, Grb14 is a weaker inhibitor of the IGF-1R than of the IR kinase activity.

Grb7 and Grb10 are closely related to Grb14. To determine whether these two proteins could also act as IR tyrosine kinase inhibitors, the effect of the three Grb proteins was measured in the same assays and illustrated in Fig. 4. Whatever the amount of GST-Grb7 tested, the inhibitory effect was less than 50%,
showing that Grb7 is a poor inhibitor of IR catalytic activity. In the case of Grb10, increasing the amount of Grb10 to 60 nM of fusion protein allowed a maximal inhibition of 80%. However, the entire dose-response curve was shifted to the right when compared with the Grb14 curve, indicating that the IR was less sensitive to the inhibitory effect of Grb10. The half-maximal inhibition of IR kinase activity was obtained for 11 nM Grb10 compared with 3–4 nM for Grb14. These results are in favor of a specific inhibitory effect of Grb14 on IR catalytic activity.

The in Vitro Inhibitory Effect of Grb7/10/14 Is Mediated by the PIR—The Grb7/10/14 proteins are characterized by a succession of interacting domains: an N-terminal proline-rich motif (PP), a central PH domain, a PIR (or BPS) (4, 5), and a C-terminal SH2 domain (3) (Fig. 5A). To determine whether a particular domain of Grb14 was responsible for its inhibitory effect, the IR catalytic activity was measured in the presence of increasing amounts of the various Grb14 domains expressed as GST fusion proteins. The Grb14 N-terminal region, deleted of the PIR and SH2 domains, did not modify the in vitro IR catalytic activity (data not shown). However, as shown in Fig. 5B, the GST-PIR of Grb14 strongly inhibited the IR kinase activity: a 55% inhibition was observed for 12 nM GST-PIR, and the kinase activity was fully inhibited for a concentration of 120 nM. In contrast, the GST-SH2 Grb14 fusion protein did not alter the IR catalytic activity (Fig. 5C). Similar experiments were performed using the different domains of Grb7 and Grb10. As reported for Grb14, the inhibitory effect of Grb7 and Grb10 on the IR kinase activity was due to the PIR, the SH2 domain alone having no effect (Fig. 5). Moreover, as reported for the full-length proteins, the PIR domains of Grb7 and Grb10 were less efficient than the PIR of Grb14.

All together these results show that the members of the Grb7 family of proteins inhibit in vitro the IR catalytic activity. Since Grb14 is more efficient in inhibiting IR catalytic activity than on IGF-1Rs, we suggest that Grb14 is a specific IR inhibitor. Interestingly, PIR, the new interacting domain, is implicated in the inhibitory effect of these proteins.

Grb14 Rapidly and Stably Associates with the Activated IR—To further delineate the mechanism of Grb14 inhibition in a cellular model, we studied the kinetics of Grb14 effects in the CHO-IR/Grb14 cell line. We first determined the kinetics of binding of Grb14 to the IR. CHO-IR/Grb14 cells were exposed...
cells were stimulated with insulin (100 nM) for various periods of time and immunoprecipitated using anti-Grb14 antibodies and immunodetected with anti-IR antibodies (upper blot). After stripping, the blot was reprobed with anti-Myc antibodies to verify the efficiency of the immunoprecipitation (Grb14 contained a Myc tag). These blots are representative of four different experiments. B and C, kinetics of IR tyrosine phosphorylation. CHO-IR and CHO-IR/Grb14 cells were stimulated with insulin (100 nM) for various periods of time and solubilized. Cell lysates were immunodetected with anti-phosphotyrosine antibodies (B) and then with anti-IRβ antibodies (C). These blots are representative of four different experiments. 
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Fig. 6. A, kinetics of Grb14 binding to IR receptors. CHO-IR/Grb14 cells were stimulated with insulin (100 nM) for various periods of time as indicated. Lysates were immunoprecipitated with anti-Grb14 antibodies and immunodetected with anti-IR antibodies (upper blot). After stripping, the blot was reprobed with anti-Myc antibodies to verify the efficiency of the immunoprecipitation (Grb14 contained a Myc tag). These blots are representative of four different experiments. B and C, kinetics of IR tyrosine phosphorylation. CHO-IR and CHO-IR/Grb14 cells were stimulated with insulin (100 nM) for various periods of time and solubilized. Cell lysates were immunodetected with anti-phosphotyrosine antibodies (B) and then with anti-IRβ antibodies (C). These blots are representative of four different experiments. 

**Experimental Procedures**

IR phosphorylation was maximal only after 10 min and then remained maximal throughout the 90 min of the experiment.

Since the rapid dephosphorylation of the IR after insulin stimulation in CHO-IR cells could be due to the action of tyrosine phosphatases, we then tested whether the interaction of Grb14 with the phosphorylated IR could block the tyrosine phosphatase action. For this experiment, we used the tyrosine phosphatase PTP1B, which was reported to bind to the activated IR and to inhibit insulin action (35-37). WGA-purified IRs were activated by insulin, and the autophosphorylation reaction was performed in the presence of either 200 nM GST or 100 nM GST-Grb14. The phosphorylation state of the IR was measured 40 min after addition of the PTP1B recombinant using anti-phosphotyrosine antibodies (Fig. 7A). In agreement with recent studies (38), PTP1B strikingly decreased IR tyrosine phosphorylation (Fig. 7A, lanes 3 and 4). After SDS-PAGE analysis IR autophosphorylation was visualized by Western blotting using anti-phosphotyrosine antibodies. This blot is representative of five different experiments. IP, immunoprecipitation.

**Fig. 7. Effect of Grb14 on insulin-induced PTP1B action.** A, in vitro PTP1B dephosphorylation of IR. WGA-purified IR were stimulated (lanes 2-7) or not (lane 1) with insulin (100 nM) and phosphorylated as described under "Experimental Procedures" in the presence of 1 μg of GST (lanes 1-4) or 1 μg of GST-Grb14 (lanes 5-7). Dephosphorylation was performed by the addition of 1.5 μg of the recombinant PTP1B (lanes 3 and 4 and lanes 6 and 7) in the presence or absence of orthovanadate (lanes 4 and 7). After SDS-PAGE analysis IR autophosphorylation was visualized by Western blotting using anti-phosphotyrosine antibodies. This blot is representative of two different experiments. IP, immunoprecipitation.
interaction, but the expression of Grb14 suppressed this effect. All together these experiments suggest that, in vitro and in cell lines, the binding of Grb14 on the activated IR hinders the action of tyrosine phosphatases, therefore maintaining the IR under a phosphorylated state.

**Grb14 Impairs Insulin Signaling Pathways**—The effect of Grb14 overexpression on the activation of two main insulin signaling effectors, Akt and ERKs, was studied in the CHO-IR and CHO-IR/Grb14 cell lines. Whole cell lysates were analyzed by Western blotting using antibodies directed against the activated phosphorylated forms of the proteins. In the CHO-IR cell line, the kinetics of Akt and ERK1/2 activation observed are in agreement with previous reports (Fig. 8) (39–42). Maximal insulin-induced Akt phosphorylation was not different in the two cell lines. However, the overexpression of Grb14 delayed by 5–10 min the maximal activation of Akt (Fig. 8, A and B). The activation of ERK1 and ERK2 was also delayed in the presence of Grb14: maximal phosphorylation was observed after 5 min of insulin stimulation in CHO-IR cells, whereas it was only observed after 10 min in CHO-IR/Grb14 cells (Fig. 8, C–E). In addition, after insulin induced a 3- and 5-fold increase, respectively, in ERK1 and ERK2 phosphorylation in CHO-IR cells, a 2- and 2.5-fold increase was only observed in CHO-IR/Grb14 cells. Thus, the activation of ERKs was significantly decreased in the presence of Grb14. These effects could not be attributed to variations in the total amounts of Akt and ERK1/2 since they were similarly expressed in both CHO-IR and CHO-IR/Grb14 cells (data not shown). All the experiments in CHO-IR/Grb14 cells were reproduced in different clones expressing various levels of Grb14 and led to similar conclusions (data not shown).

**DISCUSSION**

The molecular adapter Grb14 was recently identified as a binding partner of the IR. The interaction between Grb14 and IR involves a new interaction domain, the PIR, which specifically binds to the regulatory kinase loop of the IR (5). To investigate whether this novel interaction had functional consequences, we studied the IR kinase activity in the presence of purified recombinant Grb14 in vitro and showed that Grb14 is a direct inhibitor of the IR catalytic activity.

Crystallographic studies have shown that the IR regulatory kinase loop adopts a different conformation when it is phosphorylated. In the basal state, one of the tyrosine residues of the dephosphorylated regulatory kinase loop is bound to the active site and exerts an autoinhibition on the receptor (43). After autophosphorylation, this autoinhibition is released, and the phosphorylated regulatory kinase loop is stabilized in an open conformation, allowing access to the ATP and to the substrate binding sites (30). This open conformation facilitates the interaction between the regulatory kinase loop and downstream signaling proteins like Grb14. Indeed, Grb14, which binds to the phosphorylated IR, only slightly decreases IR autophosphorylation. In the presence of Grb14, the $K_m$ for ATP is not modified, whereas the $V_{max}$ is decreased. In addition, increasing concentrations of Grb14 decrease both the $K_m$ and $V_{max}$ for a synthetic substrate, suggesting that Grb14 behaves as an uncompetitive inhibitor (44). Thus, the binding of Grb14 does
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not block the access of ATP and of the substrate to their respective sites but rather maintains the kinase under an inactive form. A possible explanation for the inhibitory effect of Grb14 could be that conformational modifications occur during the phosphorylation reaction and that Grb14 binding alters these modifications and blocks the phosphoryl transfer from ATP to the substrate.

The inhibitory effect of Grb14 is mediated through its PIR, the IR-interacting domain. The Grb14 SH2 domain, which binds very poorly to the IR, did not alter its catalytic activity. The adaptor SH2-B was also described to interact with the activated IR, and this association is mediated by the SH2 domain, which binds to the activation loop of the receptors (5, 28, 29). Interestingly, the binding of SH2-B does not modify the in vitro IR catalytic activity. This implies that a protein bound to the activated IR tyrosine kinase loop does not necessarily inhibit the kinase activity. The interaction of SH2-B and Grb14 with the activated IR is then likely to involve different mechanisms. It has been shown that mutations of the same tyrosine residues of the kinase loop alter the interaction of both Grb14 and SH2-B (Refs. 28 and 29 and data not shown). The SH2 domain of SH2-B binds to phosphorylated tyrosine residues. This suggests that either the PIR of Grb14 does not bind to phosphorylated tyrosine residues or that it uses another mechanism in addition to a phosphotyrosine association. In favor of the first hypothesis, a recent paper reported that a tri-phosphorylated peptide of the IR, containing the three tyrosine residues of the regulatory loop, was unable to compete for the interaction between the PIR of Grb10 and the phosphorylated IR kinase domain (45). However, a definitive answer should be given by crystallographic studies of the PIR domain bound to the phosphorylated IR regulatory loop.

It was recently shown that PTP1B selectively recognized the Tyr(P)-1162 phosphotyrosyl residue of the tyrosine kinase loop (46). In addition, the IR binding of PTP1B is necessary for its dephosphorylating effect (47). As Grb14 is known to bind to the same motif, a competition for binding, and thus for IR dephosphorylation, is then likely to occur. In the CHO-IR/Grb14 cell line, the overexpression of Grb14 should favor the IR-Grb14 interaction and thus decrease IR dephosphorylation by PTP1B. Accordingly, we observed in vitro and in CHO-IR cells that IR interacting with Grb14 was maintained under its phosphorylated form because the presence of Grb14 prevented the tyrosine phosphatases action. Protein tyrosine phosphatases and Grb14 are then two kinds of inhibitors of the IR that act through different mechanisms. Phosphatases inactivate the receptor by dephosphorylation, whereas the binding of Grb14 on the phosphorylated tyrosine kinase loop inhibits its catalytic activity. The role of these two kinds of inhibitors is likely to be determined by their respective level of expression and affinity for the receptor.

The in vitro inhibitory effect of Grb14 on IR catalytic activity is reflected in vivo in insulin signaling in a CHO-IR/Grb14 cell line. In these cells, Grb14 rapidly binds to the IR. This leads to a significant delay in the insulin-induced activation of ERKs and a decrease in their maximal phosphorylation. This alteration can account for the decrease in DNA synthesis previously reported in this cell line (5). Grb14 also induces a delay in Akt phosphorylation but does not affect its maximal phosphorylation. This defect is weak when compared with the huge inhibition of glycogen synthesis measured in CHO-IR/Grb14 cells (5). Two hypotheses can explain this apparent discrepancy. First, insulin-mediated activation of glycogen synthesis implicates other signaling pathways in addition to the phosphatidylinositol 3-kinase/Akt pathway (48). On the other hand, the activation state of Akt was estimated using anti-phospho-Ser-473. However, as recently shown, Ser-473 phosphorylation of Akt does not always correlate with its activity (49). Thus, the direct inhibitory effect of Grb14 on IR catalytic activity documented in vivo is likely to be responsible for the alterations in the early steps of insulin signaling and to account for the inhibition of the distal actions of insulin.

The Grb14 inhibitory effect is less pronounced in CHO-IR/Grb14 cells than in vitro using purified proteins. In addition to the presence of the cellular machinery that could interfere with the IR-Grb14 association in the CHO model, this difference could also be attributed to variations in the ratio of Grb14 and IR expression in the two systems. In vitro the inhibitory effect of Grb14 on the IR tyrosine kinase activity was observed for a stoichiometry of IR-Grb14 ranging from 1:6 to 1:20, which could be compatible with a physiological phenomenon. However, the ratio of Grb14 and IR expression in CHO-IR/Grb14, as well as in animal tissues, cannot be easily determined. It should be noted that the expression of Grb14 is restricted to insulin-sensitive tissues and that insulin induces in vivo Grb14 binding to liver IR, suggesting that this interaction is physiologically relevant (5).

IGF-IR and IR are two closely related receptors. Insulin and IGF-1 bind to their specific receptors with high affinity but can also bind to the reciprocal receptor with a lower affinity. The stimulation of IGF-1R and IR induces the activation of the same intracellular effectors (50). Furthermore, both receptors display the same heterotetrameric structure and an amino acid similarity in the range of 40–85% in different domains, the highest degree of homology being found in the tyrosine kinase domain (51). Interestingly, in the in vitro kinase assay Grb14 is 3–10 times less effective on IGF-1R than on IR catalytic activity. This suggests that Grb14 is a more specific inhibitor of the IR kinase activity. Grb14 was recently identified as a new binding partner of the FGF receptor (52). Overexpression of Grb14 induced only a slight decrease in FGF-stimulated DNA synthesis, but the expression of the Grb14 R466K mutant, containing an inactive SH2 domain, led to an enhanced DNA synthesis in response to FGF. Since this Grb14 mutant did not bind to the FGF receptor, the improvement of FGF-induced proliferation is likely to be due to interactions with downstream effectors (52). This suggests that Grb14 can inhibit receptor tyrosine kinase signaling through distinct mechanisms and further supports the idea of a specific inhibition of IR catalytic activity by Grb14.

We demonstrated that Grb14 is the most potent inhibitor of the Grb7 family of adapters toward the IR kinase activity. Grb7 induces a maximal inhibition of 40%, and the IR catalytic activity is less sensitive to the inhibitory effect of Grb10 than that of Grb14. However, remarkably for all three Grbs the inhibitory effect on IR catalytic activity is mediated by the PIR, in agreement with a recent paper reporting an in vitro inhibitory effect of the BPS domain of Grb10 on the IR catalytic activity (45). It has been shown that the PIR and SH2 domains of the various Grb proteins are differentially implicated in the interaction with the IR (6). The SH2 domain is responsible for the binding of Grb7 to the IR, whereas both PIR and SH2 are equally important in the Grb10-IR interaction, and the PIR is the main binding domain in the IR-Grb14 interaction. In addition, the analysis of the interaction between IR tyrosine mutants and the PIR revealed that for the three Grb proteins the PIR preferentially associates with the phosphorylated kinase domain (4–6). All together these observations support a direct link between the binding ability and inhibitory action of the PIRs, and also a greater specificity of Grb14 among the Grb proteins in the inhibition of the IR catalytic activity.

The mechanism of inhibition of Grb14 on IR catalytic activity
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... displays analogies with the SOCS inhibition of JAK2 signaling (53, 54). The SOCS family of proteins is a family of negative regulators of cytokine signal transduction (53, 54). It has been shown that SOCS-1 and SOCS-3 specifically bind to the phosphorylated activation loop of JAK2 and inhibit JAK2 signaling and tyrosine kinase activity. The association with JAK2 requires the SH2 domain and the immediate N-terminal region of SOCS-1 (extended SH2 subdomain), and the inhibitory effect requires an additional N-terminal domain (tyrosine kinase inhibitory region) (53, 54). In contrast, the SH2 domain is dispensable for the Grb14-IR interaction. In addition, another difference is that Grb14 does not seem to be inducible by insulin (not shown). Interestingly, it was recently reported that the SOCS proteins SOCS-1, SOCS-3, and SOCS-6 can also act as negative regulators of insulin signaling (57, 58). SOCS-3 expression is induced by insulin (59) and, by competition for binding to the same site on the IR, SOCS-3 inhibits insulin-induced STAT-5B activation (57). SOCS-1 and SOCS-6 inhibit insulin signaling through a different mechanism: they inhibit IR-directed phosphorylation of IRS-1 \textit{in vitro} and insulin-dependent activation of ERK1/2 and Akt in transfected hepatoma cells (58).

In conclusion, these data allow us to propose that Grb14 exerts a rapid negative feedback loop modulating insulin signal transduction. After insulin receptor activation, Grb14 rapidly binds to the phosphorylated kinase loop through its PIR, inhibits the IR catalytic activity, and decreases intracellular insulin signaling. This study describes the molecular mechanism of Grb14 action, and further experiments are currently under way to delineate the effect of Grb14 under physiological conditions.

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