Grb10 mediates insulin-stimulated degradation of the insulin receptor: a mechanism of negative regulation

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Ramos, Fresnida J., Paul R. Langlais, Derong Hu, Lily Q. Dong, and Feng Liu. Grb10 mediates insulin-stimulated degradation of the insulin receptor: a mechanism of negative regulation. Am J Physiol Endocrinol Metab 290: E1262–E1266, 2006. First published January 24, 2006; doi:10.1152/ajpendo.00609.2005.—Growth factor receptor-bound protein 10 (Grb10) is an adapter protein that interacts with a number of tyrosine-phosphorylated growth factor receptors, including the insulin receptor (IR). To investigate the role of Grb10 in insulin signaling, we generated cell lines in which the expression levels of Grb10 are either overexpressed by stable transfection or suppressed by RNA interference. We found that suppressing endogenous Grb10 expression led to increased IR protein levels, whereas overexpression of Grb10 led to reduced IR protein levels. Altering Grb10 expression levels had no effect on the mRNA levels of IR, suggesting that the modulation occurs at the protein level. Reduced IR levels were also observed in cells with prolonged insulin treatment, and this reduction was inhibited in Grb10-deficient cells. The insulin-induced IR reduction was greatly reversed by MG-132, a proteasomal inhibitor, but not by chloroquine, a lysosomal inhibitor. IR underwent insulin-stimulated ubiquitination in cells, and this ubiquitination was inhibited in the Grb10-suppressed cell line. Together, our results suggest that, in addition to inhibiting IR kinase activity by directly binding to the IR, Grb10 also negatively regulates insulin signaling by mediating insulin-stimulated degradation of the receptor.

RNA interference; ubiquitin; proteasome

INSULIN BINDING TO THE insulin receptor (IR) is the start of a multifunctional cascade regulating cellular activities, such as glucose metabolism, growth and proliferation, and gene translation. The interaction between insulin and the IR activates the intrinsic tyrosine kinase of the receptor and results in receptor autophosphorylation. These phosphorylated tyrosine residues act as binding sites for substrates such as insulin receptor substrate (IRS) proteins and Shc, which, when phosphorylated, recruit additional signaling molecules such as phosphatidylinositol 3-kinase (PI3-kinase) and growth factor receptor-bound protein 3 (Grb2), resulting in the activation of the PI3-kinase and MAPK pathways (reviewed in Ref. 19).

The activated IR also recruits binding proteins that serve to modulate signaling rather than mediate its transduction. One such protein is the adapter Grb10. Grb10 belongs to the Grb7/Grb10/Grb14 family of adapter molecules, which are characterized by a shared homology of several functional domains such as a proline-rich sequence, a pleckstrin homology domain, an Src-homology 2 (SH2) domain, and a unique region between the pleckstrin homology and SH2 domains, termed the BPS domain. The SH2 domain and the BPS region have been found to mediate the interaction between Grb10 and the IR (reviewed in Refs. 6, 10). Although a substantial body of work in a variety of cell culture and overexpression systems has been dedicated to understanding the role of Grb10 in regulating insulin and insulin-like growth factor I (IGF-I) signaling, there is continued debate on the true nature of the modulation and the mechanism by which it occurs. For example, it has been shown that Grb10 stimulates mitogenic signaling in PDGF-BB, IGF-I, and insulin action (18). On the other hand, suppression of Grb10 expression levels by RNA interference (RNAi) has been shown to increase both insulin (9) and IGF-I-stimulated Akt and MAPK phosphorylation (5), suggesting that endogenous Grb10 negatively regulates these signaling pathways. In addition, knockout of the Grb10 gene in mice results in overgrowth of both the embryo and the placenta, whereas transgenic overexpression of Grb10 results in postnatal growth retardation, again suggesting a negative role for Grb10 in insulin and/or IGF-I signaling in vivo (3, 15). Interestingly, the related adapter protein Grb14 has also been shown to inhibit insulin signaling, the structural basis of which may involve the BPS domain acting as a pseudo-substrate inhibitor for the IR (2, 4).

We have previously shown that Grb10 can physically disrupt the interaction of IRS-1/2 with the IR resulting in the inhibition of PI3-kinase signaling (20). In this study, we show that suppression of endogenous Grb10 expression by RNAi led to increased IR protein levels without alteration of IR mRNA levels. We also show that in Grb10-deficient cells, the insulin-induced decrease in IR protein levels, as well as ubiquitination of the IR, is inhibited and/or delayed. Together, our results suggest that modulation of IR protein levels by the ubiquitin-proteasomal degradation pathway may provide an additional mechanism by which Grb10 negatively regulates insulin signaling.

MATERIALS AND METHODS

Buffers. Buffer A consisted of 50 mM HEPES, pH 7.6, 150 mM NaCl, and 0.1% Triton X-100. Buffer B consisted of 50 mM HEPES, pH 7.6, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM NaF, 1 mM sodium pyrophosphate, 1 mM Na3VO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, serine/threonine phosphatase inhibitor cocktail (Sigma), tyrosine phosphatase inhibitor cocktail (Sigma), and microcystin LR (CalBiochem). Buffer C consisted of 50 mM Tris, pH 7.5, 1% NP-40, 150 mM NaCl, 2 mM EGTA, 100 mM NaF, 10 mM Na3P2O7, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, serine/threonine phosphatase cocktail (Sigma), tyrosine phosphatase cocktail (Sigma), and microcystin LR.

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Cells were seeded to HA-tagged hGrb10 (CHO/IR/HA-hGrb10) (described in Ref. 11). Human embryonic kidney (HeLa) cells stably expressing IR (HeLa/IR), and CHO/IR stably expressing hGrb10 (CHO/IR/Grb10) Cells were treated with or without 50 μM MG-132 and chloroquine treatment. Cells were treated with or without 10 μM MG-132 for 4 h in serum-free medium (DMEM + 10% FBS + 1% penicillin-streptomycin (PS) for 4 h and then treated with 100 nM insulin for 0, 4, 8, 16, or 20 h. Cells were lysed in ice-cold buffer B. Cell lysates were centrifuged at 12,000 g for 10 min at 4°C, and the protein concentration of the clarified lysates was determined by Bradford assay. The lysates were diluted with lysis buffer and 2× SDS loading buffer, heated to 95°C for 5 min, and briefly centrifuged. An equal amount of protein per sample was separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted using antibodies to the IR, Grb10, and tubulin. Fluorescence-conjugated secondary antibodies were used to visualize the protein bands.

**Time course of insulin treatment.** Cells were incubated with serum-free medium [DMEM + HEPES, pH 7.6, + 1% penicillin-streptomycin (PS)] for 4 h and then treated with 100 nM insulin for 0, 4, 8, 16, or 20 h. Cells were lysed in ice-cold buffer B. Cell lysates were centrifuged at 12,000 g for 10 min at 4°C, and the protein concentration of the clarified lysates was determined by Bradford assay. The lysates were diluted with lysis buffer and 2× SDS loading buffer, heated to 95°C for 5 min, and briefly centrifuged. An equal amount of protein per sample was separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted using antibodies to the IR, Grb10, and tubulin. Fluorescence-conjugated secondary antibodies were used to visualize the protein bands.

**MG-132 and chloroquine treatment.** Cells were treated with or without 10 μM MG-132 for 4 h in growth medium (DMEM + 10% FBS + 1% PS) for 4 h and then treated with 100 nM insulin for 0, 4, 8, 16, or 20 h. Cells were lysed in ice-cold buffer B. Cell lysates were centrifuged at 12,000 g for 10 min at 4°C, and the protein concentration of the clarified lysates was determined by Bradford assay. The lysates were diluted with lysis buffer and 2× SDS loading buffer, heated to 95°C for 5 min, and briefly centrifuged. An equal amount of protein per sample was separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted using antibodies to the IR, Grb10, and tubulin. Fluorescence-conjugated secondary antibodies were used to visualize the protein bands.

**Ubiquitination assay.** HeLa/IR or HeLa/IR/ΔGrb10 cells were seeded to ~90% confluent on 100-mm plates. The cells were washed with 5 ml of 1× PBS, and 8 ml of serum-free medium (DMEM + HEPES, pH 7.6, + 1% PS + 0.5% BSA) was added to each plate. The cells were incubated at 37°C, 5% CO2, overnight. The next day, cells were pretreated with 50 μM MG-132 for 30 min at 37°C, 5% CO2, before they were stimulated with 100 nM insulin for 0, 2, or 4 h. After stimulation, the cells were lysed with ice-cold buffer C. The lysates were centrifuged at 12,000 g for 10 min at 4°C. The clarified lysates were incubated with anti-IR antibody on ice for 2 h. The antibody complexes were incubated with protein A-Sepharose for 1 h, 4°C, with end-over-end shaking. The beads were washed three times with ice-cold buffer D and then resuspended in 2× SDS loading buffer and heated at 95°C for 5 min. The proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-ubiquitin or anti-IR antibodies. Fluorescence-conjugated secondary antibodies were used to visualize the proteins.

**Reverse transcriptase PCR.** RNA was isolated from HeLa/IR or HeLa/IR/ΔGrb10 cells by TRIzol reagent according to the manufacturer’s instructions. We performed first-strand synthesis using a kit from Invitrogen. We then analyzed the cDNA by PCR using the following primers specific for Grb10, IR, and GAPDH: 5′-GATCTGGGAACCCCTGGTG-3′ for Grb10 forward and 5′-CGTGAGCA-CAGGGGGC-3′ for Grb10 reverse, 5′-GCGAATTCTCTTGAGAGCTGGGG-3′ for IR forward and 5′-CAGCGTCGACATCTCCAGGAA-3′ for IR reverse, 5′-ACACAGTCCCATGCATCAC-3′ for GAPDH forward and 5′-TCCACACCCCTGTTGCTGTA-3′ for GAPDH reverse. The PCR products were normalized using GAPDH levels and visualized by agarose gel electrophoresis and ethidium bromide staining.

**RESULTS**

To uncover the functional role of endogenous Grb10, we generated HeLa/IR cells in which the expression of Grb10 is suppressed by RNAi (HeLa/IR/ΔGrb10) (9). We found that suppression of Grb10 expression in the HeLa/IR/ΔGrb10 cells resulted in a marked increase in IR protein levels (Fig. 1A, top). This finding suggests that, in addition to binding to IR and directly inhibiting IR downstream signaling (11), Grb10 may negatively regulate IR signaling by modulating the expression levels of IR. To further test this hypothesis, we examined IR...
levels in CHO/IR cells stably overexpressing human Grb10 (CHO/IR/Grb10). We found that overexpression of Grb10 led to a decrease in IR protein levels (Fig. 1B). To determine whether the Grb10-mediated reduction of IR protein levels is a result of regulation of mRNA, we analyzed the levels of IR mRNA in HeLa/IR cells and HeLa/IR/ΔGrb10. RT-PCR experiments revealed no difference in IR mRNA levels in these two cell lines (Fig. 1C), suggesting that the regulation of IR levels occurred at the protein level.

Recent studies showed that Grb10 plays a role in proteasomal degradation of the VEGF-2 receptor and IGF-I receptor (12, 17). To determine whether the Grb10-dependent degradation of IR is through the proteasomal pathway, we tested the effect of proteasomal inhibitor MG-132 on IR protein levels in HeLa/IR and HeLa/IR/ΔGrb10 cells. Treatment of HeLa/IR cells with MG-132 resulted in a significant increase in IR protein levels, suggesting that IR is regulated by the proteasomal degradation pathway (Fig. 2). On the other hand, MG-132 had no effect on IR protein levels in HeLa/IR/ΔGrb10 cells (Fig. 2). Together, these results suggest that Grb10 may play a role in mediating IR degradation through the proteasomal pathway.

Because Grb10 has been shown to bind to IR on insulin stimulation, we wanted to test whether insulin treatment affects IR protein levels. Time course studies revealed that prolonged insulin treatment resulted in a decrease in IR protein levels starting at 4 h, with significant effects at 16 and 20 h (Fig. 3). However, insulin treatment of HeLa/IR/ΔGrb10 cells did not result in a significant decrease in IR levels (Fig. 3). Together, these results suggest a role for Grb10 in negatively regulating insulin signaling by reducing the levels of IR.

To further confirm that insulin stimulates IR degradation through the proteasomal pathway, we pretreated HeLa/IR cells with the proteasomal inhibitor MG-132 or the lysosomal inhibitor chloroquine. Cells were then stimulated with insulin, and the effect of insulin on IR levels was determined. In the absence of the inhibitors, treatment of cells with insulin for 4 h resulted in a decrease in IR protein levels (Fig. 4). The inhibitory effect of insulin on IR was significantly blocked by pretreatment of cells with MG-132 (Fig. 4). Chloroquine treatment appeared to also partially prevent insulin-induced IR degradation (Fig. 4). However, statistical analysis revealed that the protective effect of chloroquine on IR levels is not significant. These results suggest that the insulin-induced IR degradation is primarily mediated by the proteasomal pathway.

Finally, to determine whether the IR is targeted to the proteasome by ubiquitination, we examined whether the IR is ubiquitinated in cells. Western blot of immunoprecipitated IR

Fig. 2. Effect of proteasomal inhibitor MG-132 on IR protein levels. A: HeLa/IR or HeLa/IR/ΔGrb10 cells were treated with or without 10 μM MG-132 for 4 h in growth medium. Cell lysates were analyzed by Western blot using antibodies for IR (top) and tubulin (bottom). B: fluorescence of the protein bands from A was quantitated with a Licor/Odyssey scanner and software. The graph represents the average fold change in IR protein levels with MG-132 treatment compared with no treatment in each cell line (n = 4; *P < 0.05, **P < 0.01 by ANOVA).

Fig. 3. Insulin stimulation decreases IR protein levels. A: serum-starved HeLa/IR cells or HeLa/IR/ΔGrb10 cells were stimulated with 100 nM insulin (Ins) for the times indicated. Equal amounts of proteins were separated by SDS-PAGE and analyzed by Western blot using antibodies for IR and β-tubulin. B: fluorescence of the protein bands from A was quantitated with a Licor/Odyssey scanner and software. The graph represents the relative IR protein levels with insulin treatment compared with no treatment in each cell line (n = 4; *P < 0.05, **P < 0.01 by ANOVA).
with anti-ubiquitin antibody revealed that the IR was ubiquitin-terminated in insulin-stimulated cells (Fig. 5, top). The multiple bands in the high-molecular-weight smear recognized by the anti-ubiquitin antibody indicate that the IR is most likely polyubiquitinated in response to insulin stimulation in HeLa/IR cells. In contrast, insulin-stimulated ubiquitination of the IR was greatly reduced and delayed in the HeLa/IR/ΔGrb10 cells compared with controls, suggesting that Grb10 may mediate insulin-stimulated ubiquitination of the IR (Fig. 5, top).

**DISCUSSION**

Despite the massive body of information on the regulation of insulin signaling, little is known on whether regulation of this signaling pathway occurs at the level of IR protein expression. Earlier works with 125I-insulin binding assays showed that prolonged insulin stimulation resulted in a decrease in 125I-insulin binding, and this effect was attributed to the internalization and subsequent degradation of IR (7, 8). However, it is still unclear how insulin-stimulated degradation of IR is regulated and what interacting proteins are involved.

In this study, we found that IR protein levels are increased in cells in which the expression levels of the adaptor protein, Grb10, is suppressed by RNAi (Fig. 1A). From this observation and previous studies that indicate a role for Grb10 in regulating the degradation of the IGF-1 and the VEGF-2 receptors, we hypothesized that Grb10 regulates IR protein levels on insulin stimulation (12, 17). Consistent with this, IR protein levels are greatly increased in cells in which the expression of endogenous Grb10 is suppressed by RNAi (Fig. 1A). In addition, overexpression of Grb10 led to reduced IR expression levels (Fig. 1B). Our results are consistent with the recent findings of Vecchione et al. (17), who reported that Grb10 and its interacting partner, the ubiquitin ligase Nedd4, regulate ligand-induced ubiquitination and stability of the IGF-1 receptor. However, although our finding showed that Grb10 plays a role in regulating IR protein levels, receptor-specific mechanisms may regulate the expression levels of the IR, IGF-I receptor, or the VEGF-2 receptor. In contrast to the decrease seen in VEGF-2 receptor levels, for example, overexpressed Nedd4 had no effect on IR levels, suggesting that Nedd4 may not target the IR to the proteasome (12).

From studies on several components of the insulin-signaling pathway, it is becoming increasingly clear that the ubiquitin-proteasomal pathway plays a major role in negatively regulating insulin signaling. For instance, both IRS-1 and IRS-2 have been shown to be degraded through the ubiquitin-proteasomal pathway in a tissue- and cell-specific manner mediated by the suppressor of cytokine signaling proteins (13, 14, 16). Although ligand-induced IR ubiquitination has been shown to be mediated by the interaction with the adapter protein, APS, and possibly the ubiquitin ligase, c-Chi, it remains to be demonstrated whether insulin-stimulated ubiquitination of IR targets it for degradation through the proteasomal pathway (1). In the present study, we found that treatment of HeLa/IR cells with the proteasomal inhibitor MG-132 reduced the effect of prolonged insulin stimulation on IR degradation (Fig. 4A, top). In addition, we found that the protective effect of MG-132 was diminished in cells in which Grb10 expression is suppressed (Fig. 2, top). These findings suggest a potential role for Grb10 to target IR to the proteasomal degradation pathway. Furthermore, although prolonged insulin treatment in HeLa/IR cells resulted in ubiquitination of the IR, this effect was inhibited and delayed in cells in which Grb10 expression is suppressed (Fig. 5, top). Thus these results indicate that Grb10 mediates the ubiquitination of the IR, which ultimately targets it to the proteasomal degradation pathway.

Hyperinsulinemia is often a precursor to type 2 diabetes, and understanding the mechanism of its effects on insulin signaling may provide a means to prevent its detrimental consequences. In this study, we provide evidence that prolonged insulin treatment results in the Grb10-mediated entry of IR to proteasomal degradation pathway. Future studies with Grb10 knockout or transgenic mice may help to determine whether this also
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occurs in vivo. If so, Grb10 may prove to be a useful therapeutic target to alleviate the harmful effects of hyperinsulinemia.

GRANTS

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REFERENCES


