Phosphorylation of Grb10 Regulates Its Interaction with 14-3-3*

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Grb10 is a member of adapter proteins that are thought to play a role in receptor tyrosine kinase-mediated signal transduction. Grb10 expression levels can influence Akt activity, and Grb10 may act as an adapter involved in the relocation of Akt to the cell membrane. Here we identified 14-3-3 as a binding partner of Grb10 by employing a yeast two-hybrid screen. The 14-3-3-Grb10 interaction requires phosphorylation of Grb10, and only the phosphorylated form of Grb10 co-immunoprecipitates with endogenous 14-3-3. We could identify a putative phosphorylation site in Grb10, which is located in a classical 14-3-3 binding motif, RSVSEN. Mutation of this site in Grb10 diminished binding to 14-3-3. Thus, Grb10 exists in two different states of phosphorylation and complexes with 14-3-3 when phosphorylated on serine 428. We provide evidence that Akt directly binds Grb10 and is able to phosphorylate Grb10 in an in vitro kinase assay. Based on these findings, we propose a regulatory circuitry involving a phosphorylation-regulated complex formation of Grb10 with 14-3-3 and Akt.

Grb10 is a member of adapter proteins that include Grb7 and Grb14 (1). These adapter proteins share similar structural organizations, including an N-terminal proline-rich region, a central segment named the GM region (1) with a PH domain, and a C-terminal Src homology 2 (SH2) domain. Grb10 associates with a variety of growth factors at the cell surface, such as the insulin growth factor receptor (2), and with intracellular protein kinases like Raf1 and MEK1 (3) via its SH2 domain. Serine/threonine phosphorylation of Grb10 in response to insulin (4), as well as tyrosine phosphorylation in response to Tec (5) and Src (6), has been described. Interestingly, previously reported data have shown that the incubation with wortmanin, a PI 3-kinase inhibitor, abolishes Grb10 phosphorylation (4), implicating a kinase downstream of the PI 3-kinase pathway as being responsible for Grb10 phosphorylation.

The finding of four different splicing variants of Grb10 (human Grb10α, β, γ, and δ) may explain the controversy about the specific role of Grb10 as an activator or an inhibitor in particular signal transduction processes (7). Three isoforms (β, γ, and δ) of Grb10 contain an intact PH domain, and one isoform lacks a PH domain (4). Our laboratory has demonstrated that overexpression of a Grb10 isoform with an intact PH domain leads to Akt activation (7), whereas overexpression of Grb10 lacking an intact PH domain abolishes this activation. These data indicated the necessity of the Grb10 PH domain for its stimulatory effect on Akt. In accordance with this hypothesis, overexpression of a Grb10 isoform with an intact PH domain has a positive, stimulatory, and mitogenic effect on platelet-derived growth factor-BB, insulin growth factor-1, and insulin action (8). Expression of a Grb10 isoform lacking the PH domain inhibits insulin-stimulated substrate tyrosine phosphorylation and the activation of PI 3-kinase (9). Therefore, the natural occurrence of different Grb10 isoforms with and without an intact PH domain may provide a mechanism for different regulation of signal transduction processes. However, recent additional studies showed controversial results regarding the role of Grb10 with an intact PH domain in insulin signaling (10–12). The use of different cellular systems may account for these differences.

Interestingly, the fusion of Grb10 to Akt revealed a constitutive active Akt chimera, further suggesting an important role of Grb10 in Akt regulation (7). In addition, a Grb10 mutant, which is constitutively attached to the plasma membrane, induced strong Akt activation and had a potent anti-apoptotic effect in Ba/F3 cells grown without survival factors.2 Together, these data suggest a mechanism of Akt activation through Grb10. Because Akt activation is a multistep process that requires the membrane translocation of the protein, Grb10 may act as a vehicle for Akt that facilitates the membrane localization of Akt. One of the first identified substrates of Akt was the Bcl2 family member Bad (13). Akt phosphorylation of Bad mediates its interaction with 14-3-3, which prevents Bad from binding to Bcl-XL and suppresses apoptosis (14). Another substrate of Akt is FKHL1, a member of the Forkhead family of transcription factors (15). Akt phosphorylates FKHL1 in the presence of survival factors, leading to FKHL1 binding to 14-3-3. Binding to 14-3-3 causes cytosolic sequestration of FKHL1 and prevents the nuclear translocation of FKHL1. Survival factor withdrawal mediates the dephosphorylation and nuclear translocation of FKHL1, where the expression of pro-apoptotic molecules such as the Fas ligand is initiated by FKHL1. In both cases, the pro-apoptotic function of these proteins is suppressed by interaction with 14-3-3, mediating sequestration in an appointed cell compartment.

The interest in 14-3-3 in recent years has increased intensively because of the important interaction partners of 14-3-3 that are involved in cell cycle regulation (Cdc25), transcrip-

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1 The abbreviations used are: PH, pleckstrin homology; FCS, fetal calf serum; GST, glutathione S-transferase; OA, okadaic acid; PDK, phosphoinositide-dependent kinase; PP, protein phosphatase; PI, phosphatidylinositol; Pipes, 1,4-piperazinediethanesulfonic acid; Ser(P), phosphoryserine; WT, wild type.

2 S. Urschel and J. Duyster, submitted for publication.
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In Vivo Labeling of Cells with 32Pi—In Vivo translation was performed with the TNT-coupled reticulocyte lysate system (Promega, Madison, WI) and [35S]methionine.

MATERIALS AND METHODS

Cell Lines, Expression Constructs, and Transfections—293, COS1, COS7, HeLa, and U2OS cells were maintained in Dulbecco’s modified Eagle’s medium with 10% FCS. Ba/F3 cells were incubated in RPMI medium containing 10% FCS and murine interleukin-3.

The murine Grb10 cDNA (pRK5 Grb10) was kindly provided by Renato Baserga of the Kimmel Cancer Institute, Philadelphia, PA. The human Grb10 cDNA (pBEX Grb10 gamma) was a kind gift from Feng Liu of the Department of Pharmacology, University of Texas Health Science Center, San Antonio, TX. To obtain a FLAG-tagged murine Grb10, the cDNA was cloned into pCMVTag from Stratagene. For mutagenesis of the Grb10 Ser-76 and Ser-428, the serine was changed to alanine by site-directed mutagenesis using appropriate primers.

The 14-3-3 cDNA was isolated from the yeast DNA and cloned into pCMV-Myc tagged from Stratagene. To obtain higher expression levels, we cloned the Myc-tagged 14-3-3 cDNA into a pcDNA 3.1 vector (Invitrogen).

The hemagglutinin-tagged protein kinase B-Akt construct (pCMV6 HA-Akt) was kindly gifted from Thomas Franke, Department of Pharmacology, Columbia University, New York, NY. 293 cells were transiently transfected with M-[1-2,3-dioleoyloxy]propyl]-N,N,N-trimethyl-ammonium methyl-sulfate (DOTAP liposomal transfection reagent; Roche Applied Science). COS1 and COS7 cells were transiently transfected and harvested, and immunoprecipitation experiments were performed. The bound fractions were washed with phosphatase buffer (Pipes, pH 6) and incubated for the indicated time points at 30 °C with 0.4 units phosphatase (Sigma) with or without phosphatase inhibitors (NaF, ortho-vanadate, and glycerol phosphate). Cells were incubated with OA (Roche, Mannheim, Germany) for the indicated time points at the described concentrations (Fig. 1, C and D).

COS7 cells were incubated for 30 min in Dulbecco’s modified Eagle’s medium lacking FCS containing 500 nM wortmannin (Sigma). Untreated cells were incubated in media containing the same concentration of Me2SO.

RESULTS

The Serine/Threonine Kinase Akt Interacts Directly with the Adapter Protein Grb10 and Phosphorylates Grb10 in Vitro—First we wanted to determine whether the described immunoprecipitation between Grb10 and Akt is a direct interaction between the two proteins (7). We performed GST pull-downs with in vitro translated, [35S]labeled Grb10 and purified GST-Akt protein (Fig. 1A). Both, Grb10WT and Grb10PAK showed a specific and direct interaction with Akt (Fig. 1A).

We noted previously a mobility shift of Grb10 in SDS gels in Mo7e and K562 cells expressing the c-kit receptor or Bcr-Abl (7), respectively. Other groups reported tyrosine phosphorylation of Grb10 as an effect of Tec (5), as well as serine phosphorylation in response to insulin stimulation (4). Additional data revealed that incubation with wortmannin, an inhibitor of the PI 3-kinase/Akt pathway, abolished phosphorylation of Grb10, implicating a kinase downstream of this pathway (4, 7). Because of their direct association, we assumed that Akt itself may be the kinase phosphorylating Grb10. In vitro kinase assays were performed with GST-Grb10 and with kinase active and inactive Akt. Bad, a known substrate of Akt, was used as a positive control (15). The autoradiogram showed that GST-Grb10 incorporated [32P] in the presence of kinase-active Akt. In this assay, the phosphorylation intensity was comparable with the phosphorylation of Bad (Fig. 1B). In the presence of kinase-inactive Akt, no [32P] signal could be detected. Comassie staining of the gel demonstrated equal levels of GST-Grb10 (Fig. 1B, bottom section). Thus, we identified Akt as a kinase able to phosphorylate Grb10 in an in vitro kinase assay.

Phosphorylation is often regulated by a balance of kinases and phosphatases. Okadaic acid is a potent inhibitor of the protein phosphatases P1 and P2A. Okadaic acid causes a 2.5–3-fold higher phosphorylation of many proteins (27), because the balance shifts in favor of phosphorylation. To inves-
tigate whether Grb10 phosphorylation is affected by phosphatase inhibitors, we treated COS1 cells expressing Grb10 with OA and harvested the cells after different time points (Fig. 1C). Western blot analysis showed that after 40 min of incubation with OA, Grb10 started to undergo a mobility shift (Fig. 1C, lane labeled +40min). It is known that OA inhibits PP1 in the micromolar range and PP2A in the nanomolar range (27). We transfected COS1 cells transiently expressing Grb10 with OA and harvested at the indicated time points. D, cells expressing Grb10 or Bad (as a control) were incubated with different concentrations of OA to identify the phosphatase responsible for Grb10 dephosphorylation. E, HeLa cells that had been serum-starved, stimulated with FCS, or treated with okadaic acid were incubated with 32P, for 2 h in phosphate-free Dulbecco’s modified Eagle’s medium. Thereafter, immunoprecipitations (IP) using either Grb10 antiserum (Upstate Biotechnology) or control antiserum were performed from the respective lysates. An autoradiogram of the precipitated fractions is shown. F, a phospho-specific Grb10 antibody (α-P-Grb10) was used to detect phosphorylated Grb10 (P-Grb10) in immunoprecipitates (IP) from either serum-starved or FCS-stimulated HeLa cells using polyclonal Grb10 antiserum (Upstate Biotechnology). G, Western blot analysis of HeLa lysates treated as indicated was performed using the phospho-specific Grb10 antiserum. Addition of the cognate phosphopeptide was performed to demonstrate specificity. The anti-Grb10 antibody (α Grb10) (Upstate Biotechnology) detects the levels of Grb10 expression in the respective lysates. IB, immunoblot.

As depicted in Fig. 1E, phosphorylation of Grb10 was detectable in FCS-stimulated cells and at a reinforced level in cells treated with OA, whereas no phosphorylation was observed in serum-starved cells. The control precipitates did not give rise to unspecified signals. To further substantiate Grb10 phosphorylation, we generated a polyclonal phospho-specific Grb10 antibody raised against a phosphopeptide of Grb10. To test for specificity, we immunoprecipitated Grb10 from lysates derived from HeLa cells that were either serum-starved or stimulated with FCS and processed the respective blot for Western blot analysis. The phospho-Grb10 antiserum detected Grb10 only in the precipitate from FCS-stimulated cells, whereas no signal was observed in the serum-starved sample (Fig. 1F, top). Equal amounts of precipitated Grb10 were detectable in the respective lysates. IB, immunoblot.
detectable in serum-starved cells. The specific phospho-Grb10 signal was abolished upon the addition of the relevant phosphopeptide (Fig. 1G, middle). Of note, an up-shift of Grb10 upon FCS stimulation or treatment with OA was not observed with the endogenous protein as compared with the overexpressed FLAG-tagged Grb10 protein.

A Yeast Two-hybrid Screen Identified 14-3-3 as a New Binding Partner of Grb10—From our data we hypothesized that Grb10 exists in two different states of phosphorylation that may regulate its interaction with other signaling molecules. To identify such binding proteins, we performed a yeast two-hybrid screen of a K562 cDNA library with a Grb10-LexA fusion protein as bait. In two independent screens the strongest interaction partner of Grb10 was identified to be the ε isoform of 14-3-3.

To confirm the interaction between Grb10 and 14-3-3 in vitro, we performed GST pull-down assays. We expressed Grb10WT in COS1 cells and were able to precipitate Grb10 with GST-14-3-3 (Fig. 2A). Next, we transiently expressed FLAG-tagged Grb10 and Myc-tagged 14-3-3 in COS1 cells (Fig. 2B) and 293 cells (Fig. 2C) and performed co-immunoprecipitations in both directions. Western blotting revealed a specific interaction between Grb10 and 14-3-3 in both cell lines.

**Grb10-14-3-3 Interaction Requires Phosphorylation of Grb10—**14-3-3 proteins specifically recognize phosphorylated serine and threonine residues (29) but interact with unphosphorylated proteins as well (21). To investigate whether this interaction is phosphorylation-dependent, we treated Grb10-14-3-3 co-immunoprecipitates with phosphatase. Transiently expressed FLAG-tagged Grb10 and Myc-tagged 14-3-3 in 293 cells were precipitated with an anti-Myc antibody. The precipitated proteins were treated with and without an unspecific phosphatase or with phosphatase together with a specific phosphatase inhibitor (Fig. 3A). The untreated co-immunoprecipitation showed a strong association between Grb10 and 14-3-3 (Fig. 3A, top section, lane labeled 2). Incubation with the un-specific phosphatase resulted in a greatly diminished interaction between Grb10 and 14-3-3 (Fig. 3A, top section, lanes labeled +/5min and +/-10min). The presence of phosphatase inhibitors could partially restore the interaction (Fig. 3A, top section, lane labeled +/5min/+Inhibitor). The second section from the top in Fig. 3A shows equal amounts of precipitated 14-3-3. The center section of Fig. 3A shows the control immunoprecipitation (IP), and the two bottom sections show similar expression levels of Grb10 and 14-3-3 in the cell lysates. These data indicate a requirement of Grb10 phosphorylation for 14-3-3 interaction.

To emphasize the necessity of Grb10 phosphorylation for 14-3-3 interaction, we treated cells with OA and performed co-immunoprecipitations. Co-immunoprecipitation experiments revealed a greatly enhanced association between 14-3-3 and the hyperphosphorylated form of Grb10 in the presence of OA compared with the interaction between 14-3-3 and Grb10 in untreated cells (Fig. 3B, top section).

Next, we were interested as to whether endogenous 14-3-3 associates with Grb10. Therefore, we stably expressed FLAG-tagged Grb10 or empty vector as a control in Ba/F3 cells. We treated the cells with or without OA and performed co-immunoprecipitation experiments (Fig. 3C). Only the hyperphosphorylated form of Grb10 was able to form a complex with endogenous 14-3-3, whereas we could not detect an interaction between Grb10 and endogenous 14-3-3 in untreated cells. The binding specificity was confirmed by the vector control reaction (Fig. 3C, two lanes labeled PCMV2). To ascertain the interaction between the endogenous proteins, we stimulated serum starved HeLa cells with serum in the presence of OA and performed co-immunoprecipitations (Fig. 3D). The experiment showed an interaction between endogenous 14-3-3 and endogenous Grb10 only in serum-stimulated cells (Fig. 3D, left sections, two right lanes). Accordingly, 14-3-3 and Grb10 could be coprecipitated from murine lung tissue when a polyclonal antibody was used to immunoprecipitate Grb10 from mouse lung (Fig. 3E, top section).

**Interaction of Grb10 and 14-3-3 Is Wortmannin-sensitive—**The phosphorylation of Grb10 is PI-3 kinase-dependent (4, 7). Therefore, we tested whether the inhibition of Grb10 phosphorylation through the PI-3 kinase inhibitor wortmannin is able to abolish the association between Grb10 and 14-3-3. We transiently co-expressed Grb10 and 14-3-3 and treated the cells with wortmannin. The cells were harvested, and co-immunoprecipitation experiments were performed. Wortmannin treatment reduced the association between Grb10 and 14-3-3 as compared with the Me2SO (DMSO) control (Fig. 4, top section).

**Identification of Serine 428 in Grb10 as the 14-3-3 Recognition Site—**14-3-3 proteins recognize conserved motifs containing phosphoserine such as Arg-X-Arg-X-Ser(P)-X-Pro, Arg-Ser-X-Ser(P)-X-Pro, and Arg-X-Ser(P). We found two potential 14-3-3 binding sites in Grb10, namely serine 76 within a RXRSXP motif and serine 428 within a RSVSEN motif (Fig. 5A). Both residues were substituted with alanine by site-directed mutagenesis, and the ability of both mutants to bind 14-3-3 was examined. We transfected Grb10 WT, Grb10 S76A, and Grb10 S428A in COS1 cells and performed GST binding studies with GST-14-3-3 (Fig. 5B). Our data indicate that mu-
tation at serine 428 impairs the ability of 14-3-3 to bind Grb10 (Fig. 5B, top section, lane labeled Grb10 S428A). The mutation of serine 76 to alanine did not affect 14-3-3 binding to Grb10 (Fig. 5B, top section, lane labeled Grb10 S76A). As a control, we incubated the Grb10 cell lysates with GST (Fig. 5B, middle section). The cell lysates used in this experiment are shown in Fig. 5B, bottom section.

**Grb10 Exists in Two Different States of Phosphorylation**, i.e. in the Unphosphorylated Form It May Primarily Associate with Akt, and in the Phosphorylated Form It Interacts with 14-3-3—From our data we propose a hypothetical model in which Grb10 exists in two different states of phosphorylation (Fig. 6B). In the unphosphorylated state, Grb10 interacts with Akt. The Grb10-Akt complex translocates to the plasma membrane, where PDK1/2 phosphorylates Akt. The activated Akt phosphorylates Grb10, the Grb10-Akt complex dissociates, and the interaction with 14-3-3 occurs. The phosphorylation of Grb10 is the requirement for binding to 14-3-3. PP2A dephosphorylates Grb10, mediating dissociation of the Grb10-14-3-3 complex and leading to interaction with Akt.

To further elucidate this hypothesis, we transfected COS1 cells with Akt, with or without 14-3-3, and with Grb10 WT and Grb10 S428A, the 14-3-3 binding mutant. Western blot analysis with the indicated antibodies revealed that Grb10 WT mediates Akt activation, as we reported previously (7) (Fig. 6A, top section, second lane from the left). However, co-expression of Grb10 and 14-3-3 resulted in a reduced Akt activation (Fig. 6A, top section, second lane from the right). Interestingly, the expression of the Grb10 S428A mutant, which is unable to interact with 14-3-3, seems to result in an enhanced activation of Akt (Fig. 6A, top section, far right lane). This could be explained by the fact that the Grb10

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**Fig. 3. Phosphorylation of Grb10 mediates association with 14-3-3.** A, 293 cells were transfected with Grb10 and 14-3-3. After 48 h the cells were harvested, and co-immunoprecipitation (IP) experiments were performed. The immunoprecipitated beads were treated with phosphatase for the indicated time periods. The cell extracts used for the precipitations are shown at the bottom. RαM, rabbit anti-mouse control. B and C, treatment with OA induced the complex formation between Grb10 and 14-3-3 in cells transiently expressing Grb10 and 14-3-3 (B) and in Ba/F3 cells stable expressing Grb10 and endogenous 14-3-3 (C). D, serum stimulation and OA treatment induced complex formation between endogenous 14-3-3 and Grb10. Stimulation with serum and 1 μM OA was carried out for 1 h in serum-starved HeLa cells. Anti-Grb10 immunoprecipitations were analyzed by immunoblotting with an anti-14-3-3 antibody (top) and an anti-Grb10 antibody (bottom). E, proteins were extracted from mouse lung by homogenization. The cell suspensions were incubated with a Grb10 antibody or with a control antibody. The anti-Grb10 immunoprecipitation was analyzed by immunoblotting with an anti-14-3-3 antibody.
molecule interacts either with Akt or with 14-3-3 in a competing fashion.

**DISCUSSION**

By performing a yeast two-hybrid screen we identified the H9280 form of 14-3-3 as a new interaction partner of Grb10. We show that Grb10 binds 14-3-3 in vitro and in vivo. It has been shown that Grb10 is phosphorylated on serine/threonine residues (4) and that incubation with the specific PI 3-kinase inhibitor wortmannin abolished Grb10 phosphorylation (4). These data suggested that a kinase downstream of the PI 3-kinase pathway is responsible for Grb10 phosphorylation.

We observed that the Grb10-H18528 interaction was diminished after wortmannin treatment, providing evidence that the interaction between Grb10 and 14-3-3 requires the phosphorylation of Grb10. Treatment with wortmannin inhibits the downstream target of PI 3-kinase Akt. In this study, we were able to show a direct interaction between Grb10 and Akt using purified proteins in an in vitro binding assay. Moreover, we demonstrated that Akt is capable of phosphorylating Grb10 in an in vitro kinase assay. Thus, Akt may be the kinase responsible for Grb10 phosphorylation and regulation of the Grb10/H18528 complex. The regulation of 14-3-3 interactions with signaling molecules by phosphorylation has been shown previously by other groups. For example, the interaction of 14-3-3 and PDK1 requires phosphorylation of PDK1 (30). Muslin et al. identified a motif for 14-3-3 interactions (Arg-Ser-X-Ser(P)-X-Pro) (19). Recent studies with phosphopeptide libraries revealed that there exist additional preferred motifs for 14-3-3 binding, Arg-X-Arg-X-Ser(P)-X-Pro or Arg-X-Ser(P)-X-Pro (18). Grb10 contains two potential 14-3-3 binding motifs in its amino acid sequence RXSSXP and RSVSEN (76). The serine 76 (S76 in panel A) within the RXSSXP 14-3-3 binding motif and serine 428 (S428 in panel A) within the RSVSEN motif were mutated to alanine. GST pull-down assays with GST-14-3-3 and cells expressing both mutants were performed. Comparable amounts of cell lysates were used in the assay (bottom).

FIG. 4. Grb10 interaction with 14-3-3 is wortmannin-sensitive. 293 cells transiently expressing Grb10 and 14-3-3 were incubated with wortmannin for 30 min. Anti-Myc immunoprecipitates (IP) were analyzed by immunoblotting with an anti-FLAG antibody (top) and an anti-Myc antibody (bottom). Rm, rabbit anti-mouse control.

FIG. 5. Identification of the 14-3-3 binding motif in Grb10. A, schematic illustration of Grb10. The localization of the potential 14-3-3 binding motifs are indicated as RXSSXP and RSVSEN. B, serine 76 (S76 in panel A) within the RXSSXP 14-3-3 binding motif and serine 428 (S428 in panel A) within the RSVSEN motif were mutated to alanine. GST pull-down assays with GST-14-3-3 and cells expressing both mutants were performed. Comparable amounts of cell lysates were used in the assay (bottom).

FIG. 6. Hypothetical model of a regulatory circuitry involving Grb10, 14-3-3, and Akt. A, COS1 cells were transiently transfected with Akt and Grb10 WT or the Grb10 S428A mutant and with (+) or without (−) 14-3-3 as indicated. Cells were serum-starved, and the cell lysates were analyzed by Western blot analysis with the antibodies indicated. B, in the unphosphorylated state Grb10 preferentially interacts with Akt. Upon receptor stimulation the complex translocates to the plasma membrane, where PDK phosphorylates and activates Akt. The activated Akt phosphorylates Grb10, which then leads to its interaction with 14-3-3. After dephosphorylation, Grb10 may interact with Akt again.
plex formation between endogenous 14-3-3 and Grb10 was only detectable after stimulation with serum and OA in various cell lines. There exist two explanations for the absent interaction between endogenous Grb10 and 14-3-3 in unstimulated or serum-starved cells. First, the level of Grb10 phosphorylation is too faint and, therefore, the affinity of 14-3-3 to Grb10 is too low. The second explanation involves the subcellular localization of the target protein. Without stimulation the two proteins may exist in two different cell compartments and, therefore, the two proteins could not associate. The fact that GST-14-3-3 pulls down overexpressed Grb10 WT but not the Grb10 S428A mutant from cell lysates argues for the first explanation.

We have previously identified the serine/threonine kinase Akt as a binding partner of the adapter protein Grb10 (7). This interaction mediates the activation of Akt. These data differ from two recent studies by Wick et al. (12) and Langlais et al. (11), who demonstrated that human Grb10 or the Grb10 construct used, we transiently transfected Akt activity that the differences in these studies are due to the cell line stably expressing the insulin receptor. To rule out the possibility that the three proteins Akt, 14-3-3, and Grb10. In a hypothetical circuitry as depicted in Fig. 6B, the translocation of Grb10 back to the cytosol. Here, PP2A could dephosphorylate Grb10 to allow a new interaction with Akt. We observed that the Grb10A428S mutant, which failed to interact with 14-3-3, enhances the activation of Akt as compared with Grb10WT. Therefore, it may also be true that Akt and 14-3-3 compete for the interaction with Grb10. In this scenario, 14-3-3 associates with Grb10 to prohibit the association of Grb10 with Akt.

In summary, our data show that Grb10 interacts specifically with 14-3-3 in a phosphorylation-regulated manner. This interaction may represent an important part of a regulatory circuitry as depicted in Fig. 6B.