

The Adapter Protein ZIP Binds Grb14 and Regulates Its Inhibitory Action on Insulin Signaling by Recruiting Protein Kinase C ζ

Bertrand Cariou,¹ Dominique Perdereau,¹ Katia Cailliau,² Edith Browaeys-Poly,²
Véronique Béréziat,¹ Mireille Vasseur-Cognet,³ Jean Girard,¹
and Anne-Françoise Burnol^{1*}

Département d'Endocrinologie,¹ and Département de Génétique, Développement et Pathologie Moléculaire,³ Institut Cochin, CNRS-INSERM-Université René Descartes, 75674 Paris, and Laboratoire de Biologie du Développement, UE 1033, Université des Sciences et Technologies de Lille, Villeneuve d'Ascq Cedex,² France

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Grb14 is a member of the Grb7 family of adapters and acts as a negative regulator of insulin-mediated signaling. Here we found that the protein kinase C ζ (PKC ζ) interacting protein, ZIP, interacted with Grb14. Coimmunoprecipitation experiments demonstrated that ZIP bound to both Grb14 and PKC ζ , thereby acting as a link in the assembly of a PKC ζ -ZIP-Grb14 heterotrimeric complex. Mapping studies indicated that ZIP interacted through its ZZ zinc finger domain with the phosphorylated insulin receptor interacting region (PIR) of Grb14. PKC ζ phosphorylated Grb14 under in vitro conditions and in CHO-IR cells as demonstrated by in vivo labeling experiments. Furthermore, Grb14 phosphorylation was increased under insulin stimulation, suggesting that the PKC ζ -ZIP-Grb14 complex is involved in insulin signaling. The PIR of Grb14, which also interacts with the catalytic domain of the insulin receptor (IR) and inhibits its activity, was preferentially phosphorylated by PKC ζ . Interestingly, the phosphorylation of Grb14 by PKC ζ increased its inhibitory effect on IR tyrosine kinase activity in vitro. The role of ZIP and Grb14 in insulin signaling was further investigated in vivo in *Xenopus laevis* oocytes. In this model, ZIP potentiated the inhibitory action of Grb14 on insulin-induced oocyte maturation. Importantly, this effect required the recruitment of PKC ζ and the phosphorylation of Grb14, providing in vivo evidences for a regulation of Grb14-inhibitory action by ZIP and PKC ζ . Together, these results suggest that Grb14, ZIP, and PKC ζ participate in a new feedback pathway of insulin signaling.

Molecular adapters are proteins composed of the juxtaposition of various protein-protein or protein-lipid interacting domains and devoided of enzymatic activity. These proteins are essential components of signal transduction pathways. The Grb7 family of adapters, which comprises Grb7, Grb10, and Grb14, is implicated in receptor tyrosine kinase (RTK) signaling (7, 41). Growing evidence is emerging for an inhibitory role of Grb14 and Grb10 in insulin signaling. Grb14 is selectively expressed in insulin-sensitive tissues and upon insulin stimulation interacts in vivo with the insulin receptor (IR). Moreover, the overexpression of Grb14 in the CHO-IR cell line was shown to inhibit insulin-stimulated tyrosine phosphorylation of specific proteins, like IRS-1, and distal effects, like DNA and glycogen synthesis (23, 28). Despite controversial findings (46, 52, 71), overexpression studies of Grb10 isoforms are also consistent with a negative role in insulin signaling (34, 44, 48). The molecular analysis of the interaction between the Grb7 family of proteins and IR recently led to clues on their inhibitory action. These proteins interact in an insulin-dependent manner with the activated tyrosine kinase loop of the IR, and this interaction is mediated by their C-terminal region, containing the phosphorylated IR interacting region (PIR, also known as BPS, between the pleckstrin homology [PH] domain and the Src 2 homology [SH2] domain) and SH2 domains (17,

21, 22, 27, 28, 34). Using in vitro tyrosine kinase assays, it was recently shown that the binding of the PIR inhibits IR tyrosine kinase activity (2, 67).

In addition to the PIR and SH2 domains, members of the Grb7 family of proteins contain several conserved interacting regions, including a central PH domain, and an N-terminal proline-rich motif, which conforms to the consensus sequence of an SH3 binding site. These domains are binding sites for various proteins which are potentially involved in RTK signaling. Many reports have described interactions of the Grb proteins with various RTKs, but fewer studies have identified partners involved in post-receptor signaling steps, most of them concerning Grb10 partners (for a review, see reference 20). Kinases, like the serine/threonine kinases MEK1 and Raf1 or the tyrosine kinases Src and Tec, and the ubiquitin ligase Nedd4 have been shown to interact with the SH2 domain of Grb10 (33, 38, 45, 50). It has also been proposed that cAbl interacts with the proline-rich motif of Grb10 (17). To date, there is only one report of a nonreceptor Grb14 interacting protein, a novel human tankyrase that has been identified in association with the N-terminal domain of Grb14. This tankyrase is an ankyrin repeat-containing protein which is likely to be involved in the subcellular localization of Grb14 (36).

To identify new downstream partners of Grb14, we performed a two-hybrid screen of a rat liver cDNA library using the C-terminal domain of Grb14 as a bait. In this study we showed that the molecular adapter ZIP (protein kinase C ζ [PKC ζ] interacting protein), also known as p62 (55, 60), inter-

* Corresponding author. Mailing address: Institut Cochin, CNRS-INSERM-Université René Descartes, Département d'Endocrinologie, 24 rue du Faubourg Saint-Jacques, 75674 Paris, France. Phone: 33 1 53 73 27 09. Fax: 33 1 53 73 27 03. E-mail: burnol@cochin.inserm.fr.

acted with Grb14. ZIP linked the atypical PKC ζ to Grb14 into a heterotrimeric complex in vivo. In addition, we demonstrated that PKC ζ phosphorylated Grb14 and increased its inhibitory action on IR catalytic activity in vitro. Of functional relevance, these data correlated with the regulation by Grb14 and ZIP of insulin-induced maturation of *Xenopus laevis* oocyte. Together, these findings suggest that Grb14, ZIP, and PKC ζ are involved in a common down-regulation pathway of insulin signaling.

MATERIALS AND METHODS

Reagents and antibodies. The following reagents were purchased from the indicated sources: oligonucleotides, culture media, and G418 (Geneticin) were from Life Technologies; human recombinant PKC ζ was from Upstate Biotechnology Inc.; cell-permeable myristoylated PKC ζ pseudosubstrate inhibitor (Myr-SIYRRGARRWKRL) was from Calbiochem; hygromycin and monoclonal antihemagglutinin (anti-HA) antibodies were from Roche Molecular Biochemicals; monoclonal anti-myc and anti-V5 antibodies were from Invitrogen; monoclonal anti-ZIP (p62) antibodies were from Transduction Laboratories, Inc.; polyclonal anti-PKC ζ and anti-extracellular signal-regulated kinase 2 (anti-ERK2) antibodies were from Santa Cruz; and poly Glu-Tyr (4:1) and all chemicals were from Sigma France. Polyclonal anti-Grb14 antibodies were described previously (28). Polyclonal anti-ZIP antibodies were raised against a glutathione *S*-transferase (GST)-ZIP Δ AID fusion protein and purified on protein A-agarose before use.

Yeast two-hybrid screen. A yeast two-hybrid screen was performed in the *Saccharomyces cerevisiae* strain L40 using on, one hand, pFBL23-Grb14, encoding the C-terminal domain of Grb14 (amino acids 346 to 538) in N-terminal fusion with the LexA binding domain and, on the other hand, an oligo(dT)-primed cDNA library from a rat liver, cloned in fusion with the Gal4 activation domain in the pGAD3S2X plasmid, as previously described (28). The plasmids of the library producing yeast colonies of a His⁺ LacZ⁺ phenotype were isolated, and the specificity of association of their products with Grb14 was tested using pLex-lamin as a negative control. The cDNA inserts of these positive plasmids were sequenced.

5' RACE and cDNA cloning. pGAD-3'ZIP fragment, encompassing amino acids 104 to 440 of rat ZIP, was obtained by the two hybrid screen. To clone the 5' end of the ZIP cDNA, the 5' rapid amplification of cDNA ends (5' RACE) technique was used on a rat liver Marathon Ready premade cDNA library (Clontech), with the Advantage cDNA PCR kit (Clontech). We used a primer corresponding to the 5' sequence of ZIP, upstream from the ATG, according to the published sequence of rat ZIP (GenBank accession no.Y08355) and an antisense primer designed in the cloned insert. We obtained a 940-bp fragment corresponding to the 5' end of the cDNA. This fragment was subcloned by TA cloning into the pTAdv vector (Clontech). An *EcoRI/BamHI* fragment (750 bp long) was then excised from the pTAdv-5'ZIP plasmid and ligated to pGAD-3'ZIP digested by *EcoRI* and *BamHI*, generating pGAD-ZIP (full length).

Plasmid constructions. ZIP α , ZIP β , ZIP Δ AID+ZZ (Δ 1-163), ZIP Δ AID (Δ 1-103) ZIPZZ (104-163), ZIPAID+ZZ (1-163) were amplified from the pGAD-ZIP plasmid using primers to introduce an *EcoRI* site in the 5' end and an *XhoI* site in the 3' end and then cloned in frame in a pGEX4T2 vector (Amersham Pharmacia Biotech) to produce GST-ZIP fusion proteins. ZIP α and ZIP β were also cloned in pcDNA6/V5-HisC (Invitrogen) for expression of a V5 epitope-tagged version. All constructs were verified by DNA sequence analysis. pECE-myc-Grb14, pGEX-Grb7, pGEX-Grb10, and all pGEX-Grb14 constructs were described previously (2, 28).

Cell culture and preparation of lysates. Chinese hamster ovary cells stably overexpressing IR (CHO-IR) or IR and Grb14 (CHO-IR-Grb14) were grown in Ham F-12 medium supplemented with 1 mM glutamine, penicillin (100 U/ml), streptomycin (100 μ g/ml), and 10% fetal bovine serum, in the presence of G418 (75 μ g/ml) (CHO-IR) and with the addition of hygromycin (100 μ g/ml) (CHO-IR-Grb14) (28). COS cells were grown in Dulbecco's modified Eagle's medium, supplemented with 1 mM glutamine, penicillin (100 U/ml), streptomycin (100 μ g/ml), and 10% fetal bovine serum. Confluent cells were transiently transfected using Lipofectamine (Invitrogen) as recommended by the manufacturer. Cells were serum starved for 24 h and stimulated or not with insulin (10^{-7} M) for 10 min at 37°C. They were then 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, pepstatin [1 μ g/ml], leupeptin [2 μ g/ml], aprotinin [5 μ g/ml], 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate) with gentle rotation at 4°C for 30 min. Cell lysates were then cleared by a 15,000 \times g centrifugation at 4°C for 15 min.

Production of GST fusion proteins. Purified GST fusion proteins were produced as described previously (29). Fusion proteins were quantified by Coomassie blue staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. For in vitro phosphorylation experiments and *Xenopus* oocyte microinjections, the fusion proteins were eluted from the glutathione-Sepharose beads with 50 mM Tris-HCl, pH 8, containing 10 mM reduced glutathione, and quantified as described above.

In vitro interaction assays. Cell lysates (250 μ g) were incubated overnight at 4°C with 3 μ g of immobilized GST fusion proteins. For in vitro interaction with PKC ζ , 2.5 ng of human recombinant PKC ζ was incubated during 1 h at 4°C in 500 μ l of lysis buffer with 1.5 μ g of immobilized GST fusion proteins. After extensive washing, bound proteins were eluted by heating in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose. We verified by Ponceau red staining of the nitrocellulose membranes that equal amounts of GST fusions were used. Membranes were then subjected to immunodetection with the indicated antibodies. Immunoreactive bands were revealed using an ECL detection kit (Amersham Pharmacia Biotech).

Immunoprecipitation and Western blot analysis. For coimmunoprecipitation experiments, cells were transiently transfected with the indicated expression plasmids. Beginning at 48 h after transfection, cells were serum starved for 24 h and stimulated or not with insulin (10^{-7} M) for 10 min at 37°C. Cell lysates were prepared as described above. After a preclearing step, 500 μ g of cell lysates was incubated overnight at 4°C with the indicated antibodies in the presence of 100 μ l of protein A-agarose. The immunoprecipitates were washed three times in lysis buffer, subjected to SDS-PAGE, and immunodetected with the indicated antibodies. Immunoreactive bands were revealed as explained above.

For in vivo studies in rats, liver proteins were extracted in lysis buffer, and then lysates were solubilized with gentle rotation for 90 min at 4°C and cleared by a 15,000 \times g centrifugation at 4°C for 15 min. Protein concentrations were determined using a bicinchoninic acid protein assay (Pierce). Immunoprecipitations were performed as described above.

In vitro phosphorylation of Grb14 by PKC ζ . The PKC ζ kinase assays were performed essentially as recommended by the manufacturer (Upstate Biotechnology Inc.). Human recombinant PKC ζ (50 ng) was preincubated with the indicated amounts of eluted GST fusion proteins during 10 min at room temperature in assay dilution buffer II (20 mM MOPS [pH 7.2], 25 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM calcium chloride). The kinase reaction (final reaction mixture volume, 60 μ l) was initiated by the addition of 5 μ g of phosphatidylserine, 7.5 mM MgCl₂, 50 μ M ATP, and 5 μ Ci of [γ -³²P]ATP. After a 30-min incubation at 30°C, the reaction was stopped by the addition of Laemmli sample buffer and the samples were boiled for 5 min. Samples were then subjected to SDS-10% PAGE, and the bands corresponding to phosphorylated proteins were detected by autoradiography. For in vitro IR tyrosine kinase assays with GST-PIR-SH2, and for microinjection of phosphorylated GST-PIR into *Xenopus* oocytes, 2 μ g of GST fusion proteins was incubated with 50 ng of PKC ζ as described above. Various amounts of GST fusion proteins were obtained by dilution with assay dilution buffer II.

Metabolic labeling. Confluent CHO-IR cells were transiently transfected with myc-Grb14, HA-PKC ζ , and pcDNA6 (empty vector) as indicated. Beginning at 24 h after transfection, cells were serum starved for 16 h. Cells were labeled for 3 h in the same medium containing 100 μ Ci/ml of [³²P]orthophosphate (Amersham Pharmacia Biotech) and then stimulated or not with insulin (10^{-7} M) for 10 min at 37°C. When indicated, 20 μ M PKC ζ pseudosubstrate inhibitor was added 30 min before insulin stimulation. Cells were washed twice in KRBH buffer, pH 7.4 (107 mM NaCl, 5 mM KCl, 3 mM CaCl₂, 1 mM MgSO₄, 20 mM HEPES, 10 mM glucose, 1% bovine serum albumin, 7 mM NaHCO₃) and scraped into lysis buffer. Lysates were cleared by a 15,000 \times g centrifugation at 4°C for 15 min. The supernatant fractions were immunoprecipitated with anti-Grb14 polyclonal antibodies. Immunocomplexes were washed three times with lysis buffer, boiled in Laemmli sample buffer, separated by SDS-10% PAGE, and transferred to nitrocellulose. Phosphorylated Grb14 was detected with a PhosphorImager (Molecular Dynamics) and quantified (ImageQuant). The nitrocellulose membranes were then immunodetected using anti-myc antibodies.

IR tyrosine kinase activity in vitro assays. IRs were partially purified by affinity chromatography on wheat germ agglutinin (WGA), essentially as described previously (2). WGA-purified receptors were incubated for 1 h at room temperature in the presence or in the absence of 100 nM insulin, in 30 mM HEPES buffer (pH 7.6), supplemented with 30 mM NaCl, 0.1% Triton X-100, and 0.03% bovine serum albumin. The autophosphorylation of receptors was initiated by the addition of phosphorylation buffer (8 mM MgCl₂, 4 mM MnCl₂, 20 μ M ATP, and 5 μ Ci of [γ -³²P]ATP per sample) for 30 min at room temperature, in the presence of variable amounts of GST-PIR-SH2 of Grb14, previously phosphorylated by PKC ζ or not. The tyrosine kinase assay was performed using 15 μ g of

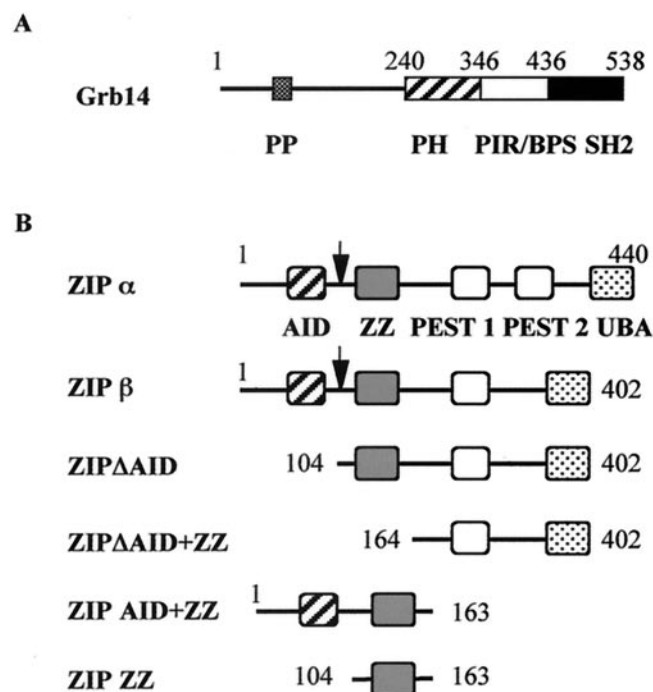


FIG. 1. Schematic representation of Grb14 and ZIP proteins. (A) Grb14. Shown are the proline-rich motif (PP); the PH domain; the PIR (28), also called BPS, between the PH and SH2 domain (22); and the SH2 domain. (B) ZIP and various ZIP constructs. The arrow indicates the length of the ZIP protein isolated in the two-hybrid screen. Shown are the AID (47), ZZ, PEST sequences (PEST2 is lacking in the ZIP β isoform), and ubiquitin-associated (UBA) domain (70).

the synthetic substrate poly Glu-Tyr (4:1). After 30 min the reaction was stopped by spotting the mixture onto Whatman ET31 filter paper squares (2 by 2 cm), which were treated as described previously (2). Results were expressed as a percentage of the maximal insulin-stimulated kinase activity measured in the absence of added fusion proteins.

Xenopus oocyte studies. After anesthesia with MS 222 (1 g/liter; Sandoz), *Xenopus laevis* ovarian fragments were surgically removed and placed in modified OR2 medium lacking potassium (82.5 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM Na₂HPO₄, 5 mM HEPES, adjusted to pH 7.4), supplemented with streptomycin-penicillin (each at 50 μ g/ml; Eurobio), tetracycline (50 μ g/ml; Sigma), sodium pyruvate (225 μ g/ml; Sigma), and soybean trypsin inhibitor (30 μ g/ml; Sigma). Stage VI oocytes were harvested by using 1-h treatment with collagenase A (1 mg/ml; Boehringer Mannheim). Complete defolliculation of the oocytes was achieved by manual dissection. The oocytes were kept at 19°C in the modified OR2 medium. Microinjections of eluted GST fusion proteins were performed in the equatorial region of the oocyte. Germinal vesicle breakdown (GVBD) was determined by the appearance of a white spot at the center of the animal pole. For ERK2 phosphorylation analysis, oocytes were lysed in ice-cold homogenization buffer (60 mM β -glycerophosphate, 15 mM nitrophenylphosphate, 15 mM EGTA, 15 mM MgCl₂, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM NaF, leupeptin [10 μ g/ml], aprotinin [10 μ g/ml], soybean trypsin inhibitor [10 μ g/ml], 1 mM phenylmethylsulfonyl fluoride, 100 μ M benzamide, 25 mM MOPS [pH 7.2]). Electrophoresis was performed on 15% modified polyacrylamide gels (30% acrylamide and 0.2% bisacrylamide). Western blot analysis was performed as described above.

RESULTS

Cloning of ZIP by interaction with Grb14 in a yeast two-hybrid screen. A rat liver cDNA library was screened using the C-terminal domain of Grb14, including the PIR and SH2 domains as a bait (amino acids 346 to 538) (Fig. 1A). The rat used

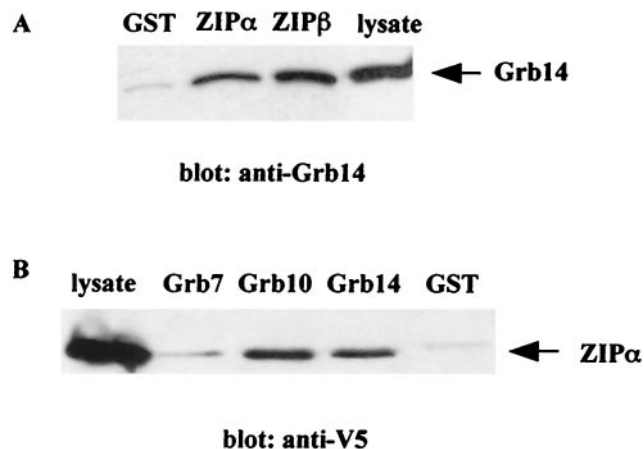


FIG. 2. Interaction of Grb14 with ZIP in vitro. (A) ZIP isoforms expressed as GST-fusion proteins or GST control were immobilized on glutathione-Sepharose beads and incubated with lysates of CHO-IR-Grb14 cells. Bound proteins were immunodetected with anti-Grb14 polyclonal antibodies. (B) Grb7 family members expressed as immobilized GST-fusion proteins were incubated with lysates of COS cells transiently transfected with V5 tagged ZIP α . Bound proteins were detected by immunodetection with anti-V5 monoclonal antibodies. These blots are representative of 3 different experiments.

for the library construction had been starved for 48 h and refed with a high-carbohydrate diet, to favor the identification of genes involved in insulin-stimulated metabolism. From 2×10^6 tested colonies, eight clones contained plasmids encoding proteins interacting with Grb14, and not with a nonspecific control like lamin. Sequence analysis of the cDNAs inserted in these plasmids revealed that all encoded the same protein, although they can be divided into two groups, differing by a deletion of 114 bp in the 3' end. The longest insert was 1.6 kb and encoded the last 337 amino acids of the protein ZIP (amino acids 104 to 440). ZIP was previously isolated by its interaction with the atypical PKC ζ (55, 60). ZIP also interacts with several proteins like p56^{lck} (26), RIP (62), TRAF6 (61), COUP-TFII (40), and Kv β 2 (18). The full-length rat ZIP cDNAs were cloned using the 5'-RACE technique. The full-length 440-amino-acid ZIP protein was called ZIP α . The shorter form, encoded by the cDNA with 114 bp deleted, carried a deletion of amino acids 349 to 387 and was called ZIP β . This variant is different from the previously reported isoform ZIP2 (deletion of amino acids 222 to 248) (18). ZIP is a molecular adapter, characterized by the succession of several conserved protein domains (Fig. 1B): an N-terminal atypical PKC-interacting domain (AID), a cysteine-rich sequence called ZZ zinc finger domain, two central PEST domains, which are likely to be implicated in protein stability, and a C-terminal ubiquitin-associated domain potentially involved in ubiquitination-mediated protein degradation (55, 61, 70). The ZIP β isoform lacked the second PEST domain.

ZIP forms a heteromultimeric complex with Grb14 and PKC ζ . The Grb14-ZIP interaction observed in yeast was first confirmed in vitro using GST pull down assays. Immobilized GST-ZIP α and GST-ZIP β were incubated with CHO-IR-Grb14 cell lysates, and retained proteins were analyzed on anti-Grb14 immunoblots. As shown in Fig. 2A, Grb14 was similarly retained by GST-ZIP α and GST-ZIP β . GST alone

did not bind to Grb14. Based on the observation that PIR and SH2 domains are conserved between the members of Grb7 family (8, 28), we compared their respective binding abilities to that of ZIP α . A series of constructs of each Grb7 family protein fused to GST were immobilized on glutathione-Sepharose and used to pull down ZIP α from lysates of transiently transfected COS cells. As shown in Fig. 2B, Grb10 and Grb14 bound to ZIP α , whereas no interaction was detected with Grb7.

To verify that the interaction between Grb14 and ZIP also occurs in intact mammalian cells, CHO-IR cells were transiently transfected with myc-Grb14 and V5-ZIP α . At 48 h after transfection, cells were stimulated or not with insulin, and cell lysates were immunoprecipitated with anti-Grb14 polyclonal antibodies. The anti-Grb14 immunoprecipitates were analyzed with anti-V5 antibodies. As shown in Fig. 3A (upper blots, lanes 4 and 5), V5-ZIP α coprecipitated with myc-Grb14 under basal conditions as well as after insulin stimulation. Similar results were obtained when using the V5-ZIP β expression plasmid (data not shown).

Previous studies have shown that ZIP interacts with PKC ζ and behaves as a link between PKC ζ and other proteins, such as RIP (62) or Kv β 2 (18), in ternary complexes. Therefore, we next investigated whether ZIP could simultaneously interact with Grb14 and PKC ζ . CHO-IR cells were transfected with myc-Grb14, V5-ZIP α , and HA-PKC ζ , and cell extracts were immunoprecipitated with anti-Grb14 antibodies. The immunoprecipitates were then analyzed with either anti-V5 or anti-HA antibodies. The results of Fig. 3A (lanes 6 and 7) indicate that V5-ZIP α and HA-PKC ζ coprecipitated with myc-Grb14, in the basal state as well as after insulin stimulation. To further demonstrate that ZIP is the link that connects PKC ζ to Grb14, CHO-IR cells were transfected with only myc-Grb14 and HA-PKC ζ . Interestingly, HA-PKC ζ was immunoprecipitated with the myc-Grb14 antibodies. However, the amount of PKC ζ immunoprecipitated was lower than in the presence of coexpressed V5-ZIP α (middle blots [compare lanes 8 and 9 to lanes 6 and 7]). As ZIP is expressed in CHO cells (data not shown), endogenous ZIP was likely to mediate the interaction between Grb14 and PKC ζ . To verify this hypothesis, we tested direct *in vitro* interaction between PKC ζ and Grb14. Immobilized GST-ZIP α and GST-Grb14 were incubated with recombinant PKC ζ , and retained proteins were analyzed on anti-PKC ζ immunoblots. As shown in Fig. 3B, PKC ζ was retained by GST-ZIP α as expected, whereas it did not interact with GST-Grb14 or GST alone. All together, these results indicate that ZIP forms a bridge between Grb14 and PKC ζ .

Next, we attempted to confirm that these interactions also occur under more-physiological conditions. Since Grb14 was not expressed in various cell lines, like CHO or COS cells (data not shown), we investigated whether endogenous ZIP and PKC ζ can form a complex with ectopically expressed Grb14. COS cells were transiently transfected with myc-Grb14. Under these conditions, the level of expression of Grb14 protein was about fourfold higher than that in rat liver (data not shown). Cell extracts were immunoprecipitated with anti-Grb14 antibodies, and the immunoprecipitates were analyzed with either monoclonal anti-ZIP or polyclonal anti-PKC ζ antibodies. As reported in Fig. 3C, endogenous ZIP and PKC ζ coprecipitated

with myc-Grb14, whereas no interaction was detected with nonspecific rabbit immunoglobulin G.

Grb14 is specifically expressed in insulin-sensitive tissues (28). Then, we used rat liver extracts to further demonstrate the heterotrimeric complex formation *in vivo*, in a physiological target of insulin. In agreement with the data obtained with transfected cell lines, we were able to detect a ternary complex between endogenous Grb14, ZIP, and PKC ζ in a coimmunoprecipitation experiment using anti-Grb14 antibodies (Fig. 3D). This result suggests that the constitution of the heterotrimeric complex may be physiologically relevant.

Mapping of regions involved in the Grb14-ZIP association.

In order to determine which region of ZIP is required for the interaction with Grb14, a series of ZIP constructs fused to GST (Fig. 1B) was used to pull down Grb14 from lysates of CHO-IR-Grb14 cells. As shown in Fig. 4A, deletion of the N terminus of ZIP, containing the AID and ZZ domains, suppressed Grb14 interaction (lanes 1, 2, and 6). The complementary construct, amino acids 1 to 163 of ZIP, retained Grb14, but to a lower extent than wild-type (WT) ZIP (compare lanes 2 and 3). This suggests that the presence of the following domains in C terminus of the ZZ domain is required for a correct folding of the protein, and thus for a full binding activity. The Δ AID construct, containing the ZZ domain and the C terminus of the protein, bound Grb14, but also with a lower affinity than WT ZIP (compare lanes 2 and 5). This decreased binding could be explained by a role of the AID domain, ZIP amino acids 1 to 103, in Grb14 binding. However, this is unlikely since (i) a similar amount of Grb14 was retained by the AID-ZZ and the ZZ domains (lanes 3 and 4); (ii) the clones isolated in the two-hybrid screen did not contain the AID domain (Fig. 1B), suggesting that it is dispensable for the ZIP-Grb14 interaction in yeast; and (iii) similar amounts of WT ZIP and ZIP Δ AID were retained in anti-Grb14 immunoprecipitation experiments performed in mammalian cells (data not shown). Thus, more probably, the presence of the GST in N terminus of the ZIP Δ AID construct had altered the protein folding and partially masked the ZZ interaction domain. Although we cannot exclude from these experiments the possibility that other domains of ZIP participate in the interaction with Grb14, these results are in favor of a role of the ZZ domain (amino acids 104 to 163) in Grb14 binding.

By the same approach, using a series of Grb14 deletion constructs fused to GST, we investigated the role of the different domains of Grb14 in its binding to ZIP. Figure 4B clearly shows that the strongest binding was detected with the PIR domain alone. By contrast, the SH2 domain was unable to bind to ZIP α . The PIR-SH2 domain and the full-length Grb14 bound to ZIP to a lower extent than did the PIR alone. This decreased binding could indicate that the presence of the other domains modifies the conformation of the PIR and reduces its binding affinity to ZIP. Collectively, these results suggest that the Grb14-ZIP binding is mediated by the PIR-ZZ interaction.

PKC ζ phosphorylates Grb14 *in vitro* and in intact cells.

Previous studies reported that ZIP allows PKC ζ to be associated with specific effectors in signaling complexes and to phosphorylate them (18). Thus, to determine whether PKC ζ was able to phosphorylate Grb14, we performed *in vitro* phosphorylation assays using human recombinant PKC ζ and GST-Grb14 fusion protein. Incubation of PKC ζ with increasing

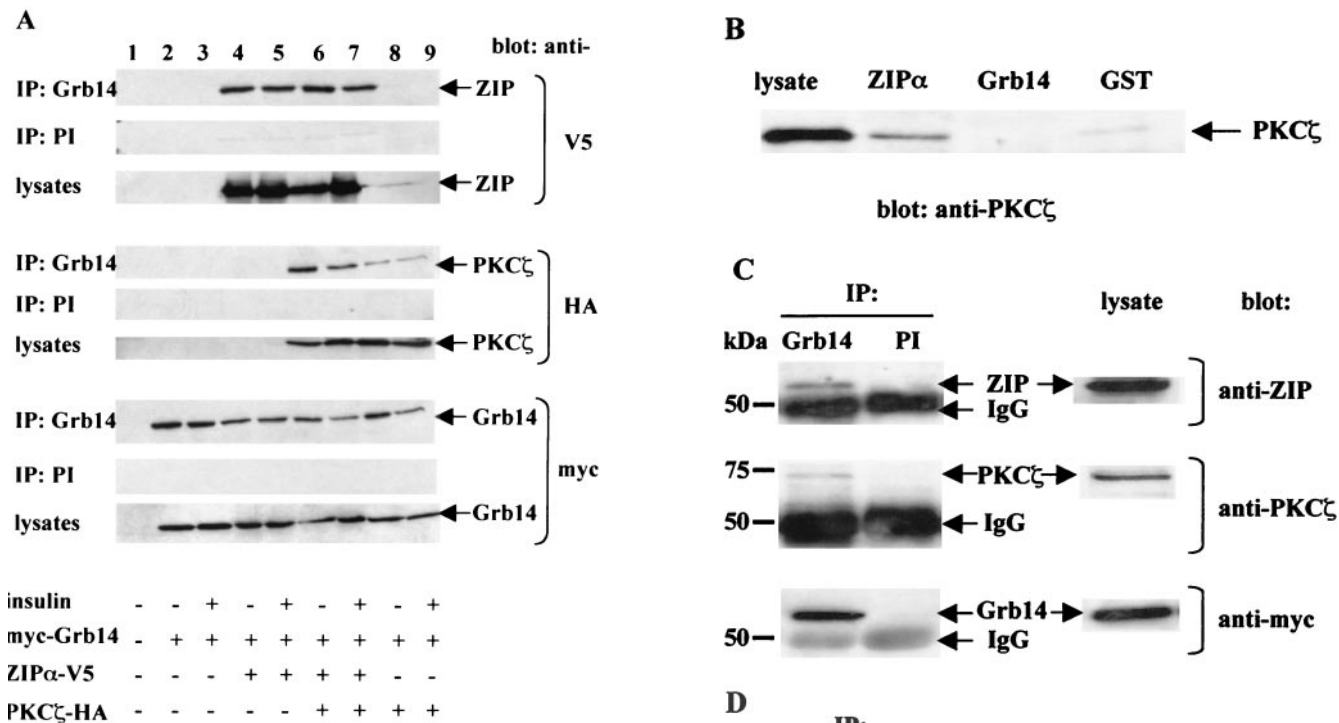


FIG. 3. ZIP forms a ternary complex with Grb14 and PKC ζ in vivo. (A) CHO-IR cells were transiently transfected as indicated with 1 μ g of either pECE myc-Grb14, pcDNA6 V5-ZIP α , or pcDNA3 HA-PKC ζ . pcDNA6 empty plasmid was used to normalize total DNA amount to 3 μ g. At 48 h after transfection, cells were stimulated or not with 10^{-7} M insulin. Cell extracts were immunoprecipitated with polyclonal anti-Grb14 antibodies. Control immunoprecipitations using rabbit preimmune serum were simultaneously performed (PI). The immunoprecipitates were separated by SDS-PAGE and immunoblotted with monoclonal anti-HA, anti-V5, or anti-myc antibodies. Lysates of the same cells were separated by SDS-PAGE and immunoblotted with the same antibodies to confirm equal expressions levels of proteins. (B) Grb14 does not interact with PKC ζ in vitro. ZIP α and Grb14 expressed as GST fusion proteins were incubated with human recombinant PKC ζ . Bound PKC ζ was detected by Western blotting using anti-PKC ζ antibodies. (C) Lysates from COS cells transiently transfected with myc-Grb14 were immunoprecipitated with polyclonal anti-Grb14 antibodies. Control immunoprecipitation using rabbit preimmune serum was simultaneously performed. The immunoprecipitates were separated by SDS-PAGE and immunoblotted as indicated with monoclonal anti-ZIP antibodies, anti-myc antibodies, or polyclonal anti-PKC ζ antibodies. Lysates of the same cells were analyzed in parallel. (D) Rat liver extracts were immunoprecipitated with polyclonal anti-Grb14 antibodies cross-linked to protein A-agarose. Control immunoprecipitation using rabbit preimmune serum was simultaneously performed. Immunoprecipitates were analyzed by sequential immunodetection using polyclonal anti-ZIP and anti-PKC ζ antibodies. In this figure the blots are representative of two or three independent experiments, and lysates and preimmune controls were analyzed on the same gels as experimental samples.

amounts of GST-Grb14 resulted in an increased phosphorylation of GST-Grb14, whereas control GST was not phosphorylated (Fig. 5, lanes 3, 4, and 7). The addition of GST-ZIP α or GST-ZIP β to the reaction did not modify the level of Grb14

phosphorylation (data not shown), suggesting that under in vitro conditions, ZIP was dispensable for Grb14 phosphorylation by PKC ζ . To determine if a particular domain of Grb14 was preferentially phosphorylated by PKC ζ , similar experiments were performed using various GST-Grb14 constructs. The C-terminal domain of Grb14, including the PIR and SH2 domains (amino acids 346 to 538), was more intensively phosphorylated than the N-terminal domain (amino acids 1 to 346) (Fig. 5, lanes 5 and 6). Interestingly, the PIR was a much better substrate for PKC ζ than the SH2 domain (Fig. 5, lanes 8 to 10). In order to assess the phosphorylation state of Grb14 in intact cells, we performed in vivo metabolic labeling experiments. First, CHO-IR cells were transiently transfected with myc-Grb14, labeled with [32 P]orthophosphate, and stimulated or not with insulin for 10 min. Consistent with previous results (8, 58), Grb14 was phosphorylated under basal conditions. Treatment of the cells with insulin induced a 60% increase in Grb14 level of phosphorylation (Fig. 6). The addition of cell-permeable myristoylated PKC ζ pseudosubstrate (Myr-SIYRRGARRWRKL), which specifically inhibits PKC ζ enzyme (65), decreased insulin-stimulated Grb14 phosphorylation,



FIG. 4. Mapping of domains involved in the Grb14-ZIP interaction. (A) ZIP α and various ZIP constructs expressed as GST fusion proteins were incubated with lysates of CHO-IR-Grb14 cells. Bound proteins were immunodetected with anti-Grb14 antibodies. (B) Various Grb14 constructs expressed as GST fusion proteins were incubated with lysates of COS cells transiently transfected with V5-ZIP α . Bound proteins were immunodetected using anti-V5 antibodies. Ponceau red staining of the proteins after transfer confirmed that equivalent amounts of GST fusions were loaded. These blots are representative of three different experiments.

restoring phosphorylation to the level observed in the absence of insulin. Cotransfection of CHO-IR cells with HA-PKC ζ resulted in a 60% increase in insulin-stimulated Grb14 phosphorylation. By contrast, in the absence of insulin stimulation, the overexpression of PKC ζ did not increase Grb14 phosphorylation (data not shown). Together, these results support the

hypothesis that PKC ζ may account for the *in vivo* insulin-induced Grb14 phosphorylation.

The phosphorylation of Grb14 by PKC ζ increases its inhibitory effect on *in vitro* IR tyrosine kinase activity. The PIR of Grb14 mediates both the binding to the IR and the inhibitory action of the protein on the catalytic activity of the receptor (2, 28). As the PIR was the main domain of Grb14 phosphorylated by PKC ζ , we hypothesized that this phosphorylation could modify the inhibitory action of Grb14 on IR catalytic activity. IR tyrosine kinase activity was then investigated *in vitro* in the presence of increasing amounts of GST-PIR-SH2, which was or was not previously phosphorylated by PKC ζ . As shown in Fig. 7, the dose-response curve of IR kinase inhibition by phosphorylated GST-PIR-SH2 was shifted to the left compared to that of inhibition by the nonphosphorylated protein, reflecting an increased sensitivity of the IR to its inhibitory effect. Fifty percent inhibition of IR tyrosine kinase activity was observed for 40 ng of phosphorylated GST-PIR-SH2 versus 160 ng for the nonphosphorylated form. When performing the IR kinase assay, PKC ζ was added together with phosphorylated GST-PIR-SH2. As serine-threonine phosphorylation of IR has been described to inhibit IR tyrosine kinase activity (4, 68, 69), we tested the ability of PKC ζ to modify the IR catalytic activity under the same conditions. However, when added alone, the same amounts of PKC ζ did not modify IR tyrosine kinase activity. The displacement of the dose-response curve was then attributable to the phosphorylation of the PIR-SH2 domain. Thus, these results clearly show that phosphorylation of Grb14 by PKC ζ increases its inhibitory action on IR catalytic activity.

ZIP potentiates the Grb14 inhibitory action on insulin-induced reinitiation of meiosis in *X. laevis* oocytes. *Xenopus* oocyte, physiologically arrested at the G₂ stage of the first meiotic prophase, is a suitable system for investigating the functional roles of signaling molecules initiated by several extracellular

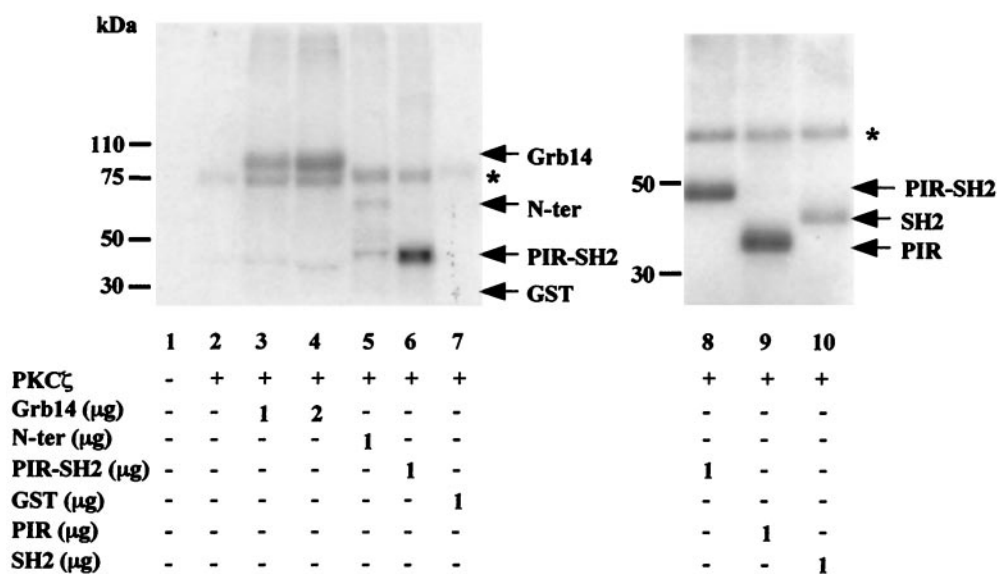


FIG. 5. PKC ζ phosphorylates Grb14 *in vitro*. Phosphorylation assays were performed *in vitro* using human recombinant PKC ζ as described in Material and Methods. GST fusion proteins were used as substrate as indicated. Phosphorylated proteins were subjected to SDS-PAGE and analyzed by autoradiography. The asterisk indicates autophosphorylated PKC ζ . These blots are representative of four different experiments.

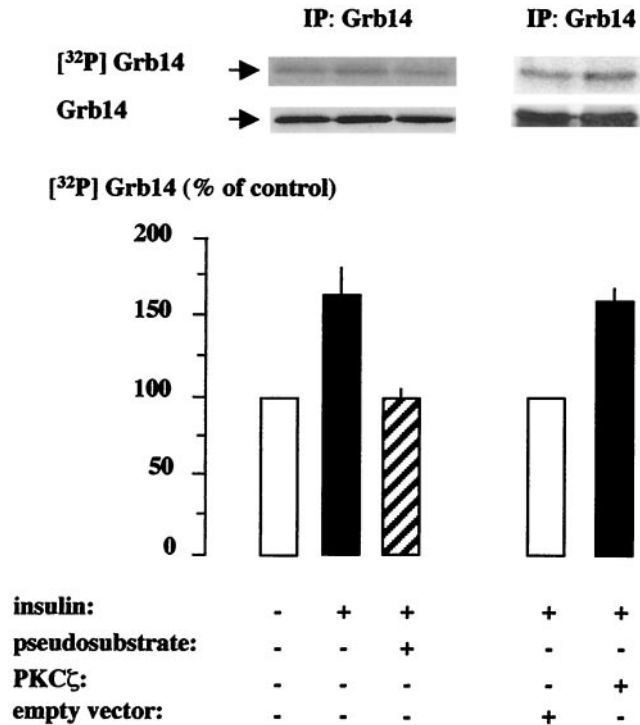


FIG. 6. Grb14 phosphorylation in intact cells. CHO-IR cells transiently transfected with myc-Grb14 and labeled with [³²P]orthophosphate were stimulated (+) or not (-) with insulin as indicated. Cells were either cotransfected with HA-PKC ζ or empty vector (right part) or treated 30 min with 20 μ M of myristoylated PKC ζ (Myr-SIYRRG ARRWRKL) pseudosubstrate prior to insulin stimulation (left part). Cell lysates were immunoprecipitated with anti-Grb14 antibodies, separated by SDS-PAGE, transferred to nitrocellulose membrane, and quantified with a PhosphorImager (upper blot). Anti-myc immunoblots were then performed to confirm that equal amounts of Grb14 were immunoprecipitated (lower blot). Results are expressed as a percentage of their respective control, in the absence of insulin (left part) or in the absence of HA-PKC ζ (right part), and represent the means \pm standard errors of the means (error bars) of three (right part) or four (left part) independent experiments.

inducers like progesterone or insulin (15, 37, 42). The binding of these hormones to their receptors stimulates the entry of oocytes into the M phase, leading to a GVBD, an event that can be easily monitored and used as an indicator of reinitiation of meiosis. The effect of Grb14 and its domains on insulin-induced oocyte maturation was studied by microinjection of GST-fusion proteins 1 h prior to stimulation with insulin (10⁻⁶ M). As shown in Fig. 8A, microinjection of 100 ng of GST-Grb14 totally blocked the maturation induced by insulin. Injection of the same amount of GST had no effect on insulin-induced GVBD. In addition, this inhibitory action of Grb14 was specific for the insulin transduction pathway, since it was not observed when the oocytes were stimulated with progesterone (data not shown). Interestingly, the PIR-SH2 and the PIR-GST fusions were sufficient to inhibit the reinitiation of meiosis, whereas the GST-SH2 fusion had no effect. The microinjection of 100 ng of GST-ZIP α , (or GST-ZIP β [data not shown]) did not alter the insulin-induced reinitiation of oocyte meiosis.

Numerous studies have shown the importance of p21^{ras}-

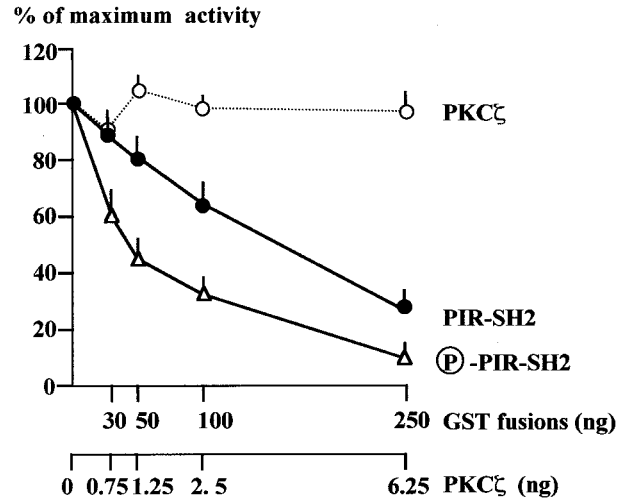


FIG. 7. The phosphorylation of PIR-SH2 of Grb14 increases its inhibitory effect on IR catalytic activity. Tyrosine kinase activity of WGA-purified IR was measured using a synthetic substrate (poly Glu-Tyr 4:1), in the presence of increasing amounts of GST-PIR-SH2 previously phosphorylated or not by PKC ζ , or in the presence of PKC ζ alone. Results are expressed as a percentage of the maximal insulin effect measured in the absence of GST fusion proteins, and are the means \pm standard errors of the means of three to five different experiments.

mitogen-activated protein (MAP) kinase cascade in insulin-induced GVBD (19, 54), and MAP kinase activation appears to be necessary and sufficient to induce oocyte maturation (19). Thus, to further investigate the molecular mechanism of Grb14 inhibitory action in *X. laevis* oocytes, we studied its effect on MAP kinase activation. Insulin-induced ERK2 phosphorylation was detected by immunoblot analysis using anti-ERK2 antibodies (Fig. 8B). A shift to a higher electrophoretic mobility of the 42-kDa protein, indicative of the presence of ERK2 phosphorylated form, was observed in oocytes stimulated with insulin but did not occur in oocytes previously microinjected with GST-Grb14. As expected, GST-ZIP α and GST did not alter ERK phosphorylation. These data support the notion that Grb14 inhibits the IR catalytic activity and subsequent activation of MAP kinase during insulin stimulation (2).

The study of the effect of decreasing amounts of Grb14 indicated that at least 50 ng of GST-Grb14 was required to inhibit insulin-induced GVBD (Fig. 9). Coinjection of 100 ng of GST-ZIP α with low amounts of GST-Grb14, which were unable to inhibit the action of insulin, restored the inhibition of insulin-induced GVBD (Fig. 9). Similar results were obtained when using ZIP β (data not shown). Thus, these results suggest that ZIP potentiates the inhibitory action of Grb14 on oocyte maturation.

ZIP must interact with PKC ζ to potentiate the action of Grb14. To further explore the mechanism by which ZIP regulates the action of Grb14, we used ZIP deletion constructs expressed as GST-fusion proteins: ZIP Δ AID+ZZ, from which both Grb14 and PKC ζ interacting domains were deleted, and ZIP Δ AID, which could not bind to PKC ζ but retained its Grb14 binding ability. As shown previously, coinjection of ZIP α with 10 ng of GST-Grb14 inhibited insulin-induced

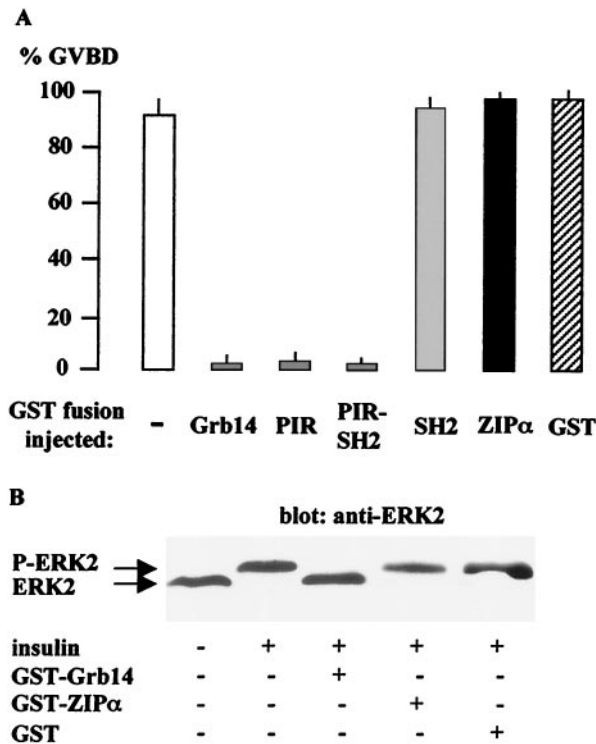


FIG. 8. Grb14 inhibits insulin-induced *X. laevis* oocyte maturation. Oocytes were microinjected with 100 ng of the indicated GST fusion proteins 1 h before insulin stimulation and analyzed 20 h later for the appearance of GVBD. (A) Effect of the injection of various GST fusions on the insulin induction of oocyte maturation. Results are expressed as a percentage of GVBD. Data represent the means + standard errors of the means (error bars) of two to three animals, each concerning 20 oocytes. (B) Western blot analysis of oocyte lysates using anti-ERK2 antibodies. The blot is representative of three different experiments.

GVBD. By contrast, the coinjection of ZIP Δ AID+ZZ did not allow the blocking of insulin-induced reinitiation of meiosis (Fig. 10). Interestingly, ZIP Δ AID was also unable to potentiate the inhibitory effect of Grb14. These data suggest that the ZIP-Grb14 interaction was not sufficient to mediate the functional action of ZIP, and that ZIP must associate with both Grb14 and PKC ζ to potentiate Grb14 inhibitory action on oocyte *Xenopus* maturation. Similar results were obtained when using the PIR of Grb14 instead of full-length protein (Fig. 10), whereas the SH2 domain had no effect (data not shown). Ten nanograms of GST-PIR was not sufficient to block the insulin-induced reinitiation of oocyte meiosis. Coinjected ZIP potentiated the inhibitory action of PIR, whereas ZIP Δ AID+ZZ and ZIP Δ AID were ineffective. These results reinforce the central role played by the PIR in Grb14 function.

We have shown that phosphorylation of Grb14 increased its inhibitory effect on *in vitro* IR tyrosine kinase activity (Fig. 7). We reasoned that if the role of ZIP is to allow Grb14 to be phosphorylated by PKC ζ , previous phosphorylation of the GST-PIR fusion would increase its inhibitory effect on the action of insulin in the *Xenopus* oocyte model. We thus tested the effect of decreasing amounts of GST-PIR, prephosphorylated or not, on insulin-induced oocyte maturation. As shown in Fig. 11, the injection of 12.5 ng of GST-PIR did not inhibit

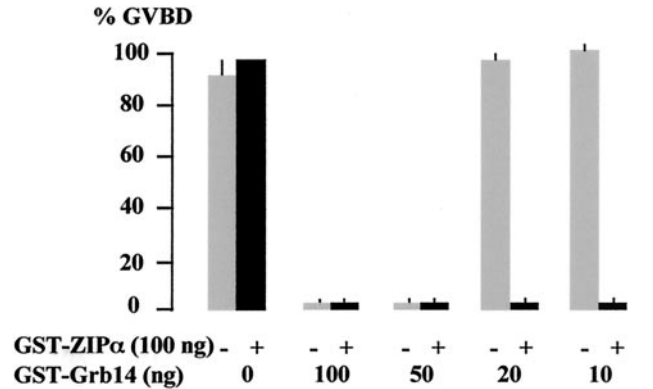


FIG. 9. ZIP potentiates Grb14 inhibition of insulin-induced oocyte maturation. Oocytes were microinjected with decreasing amounts of GST-Grb14 as indicated, together with (filled bars) or without (grey bars) 100 ng of GST-ZIP α . Oocyte maturation in response to insulin was monitored as in Fig. 8. Data are the means + standard errors of the means (error bars) of three animals, each concerning 20 oocytes.

the effect of insulin. However, if the protein was prephosphorylated by recombinant PKC ζ , the injection of 12.5 ng, as well as 7.5 ng, totally inhibited the effect of insulin. As a control, microinjection of the same amounts of activated PKC ζ in the absence of GST-PIR did not modify insulin-induced oocyte maturation. These results confirm *in vivo* that the inhibitory action of Grb14 is increased when the protein is phosphorylated.

All together, these data are consistent with the notion that ZIP acts as an adapter, linking PKC ζ to Grb14 into a new signaling pathway.

DISCUSSION

The atypical protein kinase C isoforms (aPKCs), which include PKC ζ and PKC λ , are implicated in multiple intracellular pathways. They regulate critical cellular functions such as cell proliferation and apoptosis (3, 12, 30, 49), and they are also important mediators of physiological actions of insulin such as translocation of the insulin-responsive glucose transporter GLUT4 and enhancement of glucose transport in adipocytes and skeletal muscle (1, 6, 16, 65). Accumulating studies support the existence of adapter proteins coordinating physical association between PKC and substrates and thus acting as catalysts for specific reactions (47, 63). These proteins are key components for the determination of the selectivity of signaling events. Some of these effectors can modulate aPKC catalytic activity, like lambda interacting protein and Par-6 (partitioning-defective 6), acting as activators (10, 56), or Par-4 (prostate androgen response-4), acting as an inhibitor (9). Atypical PKC isotype-specific interacting protein (ASIP) binds the aPKC catalytic domain (24). Interestingly, overexpression of ASIP in 3T3-L1 adipocytes inhibits insulin-induced glucose uptake (31). However, the mechanism by which ASIP inhibits PKC λ effect in GLUT4 translocation remains unclear. Another aPKC-interacting protein, ZIP, binds selectively and constitutively to the V1 portion of their regulatory domain and does not modulate their catalytic activity (55, 60). ZIP contains a number of conserved motifs, suggesting a role as a scaffold

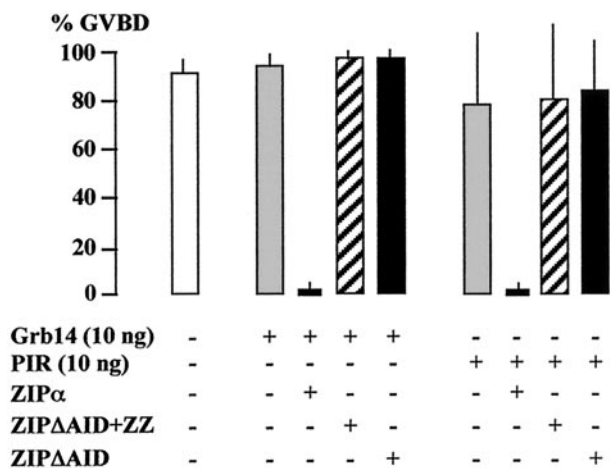


FIG. 10. Importance of Grb14-ZIP-PKCζ interaction in ZIP modulation of Grb14 action. Oocytes were coinjected with GST-Grb14 or GST-PIR in the presence of 100 ng of various GST-ZIP constructs, as indicated. Oocyte maturation in response to insulin was monitored as in Fig. 8. Data are the means + standard errors of the means (error bars) of three animals, each concerning 20 oocytes.

protein linking aPKCs to other proteins implicated in various signaling pathways. ZIP has been shown to recruit the proteins RIP and TRAF6, connecting PKCζ to the TNF-α and IL-1 receptors, respectively (61, 62). These interactions appear to be of functional relevance since they stimulate NF-κB activity. In addition, it was recently reported that through its association with the NGF receptor TrkA, the ZIP-TRAF6 complex is implicated in the NGF-induced NF-κB activation (72). ZIP acts also as a link between PKCζ and the neuronal potassium channel Kvβ2 (18).

In this study, we identified ZIP as a new downstream partner of Grb14. Although we isolated two isoforms, ZIPα and ZIPβ, we did not find any functional difference between them. They bound similarly to Grb14, and both of them potentiated the inhibitory role of Grb14 on insulin induced *Xenopus* oocyte maturation. Results of mapping studies indicated that the ZZ zinc finger domain of ZIP, which includes a novel atypical zinc finger motif (55), is required for the interaction with Grb14. This domain was also recently shown to interact with the death domain kinase RIP (62). All together, these results suggest that the ZZ zinc finger domain of ZIP is a new protein-protein interacting region. The PKCζ binding domain of ZIP, termed AID, is located N terminal of the ZZ zinc finger domain, indicating that ZIP can bind simultaneously Grb14 and PKCζ through two distinct but proximal modules. Using coimmunoprecipitation experiments in transfected CHO-IR and COS cells, we confirmed that these interactions are not antagonistic, resulting in a ternary complex including Grb14, ZIP, and PKCζ. Importantly, Grb14, ZIP, and PKCζ form a heterotrimer also in vivo in rat liver, demonstrating that endogenous proteins interact under physiological conditions. By contrast with previous studies reporting a ligand induction of ZIP multimeric complexes (61, 62, 72), we were unable to detect any effect of insulin on the formation of this heterotrimer (Fig. 3A and data not shown). Nevertheless, the functional regulation by insulin of the Grb14-ZIP-PKCζ complex may involve other

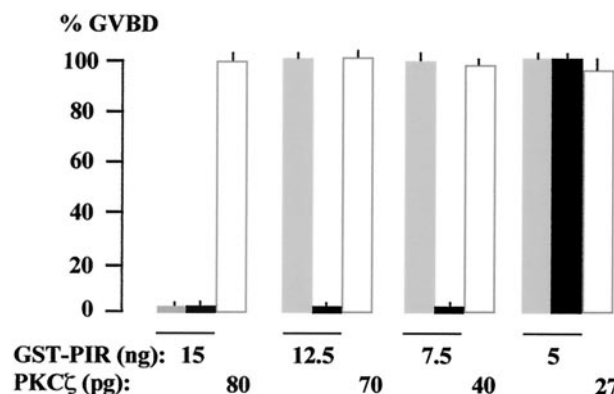


FIG. 11. PIR phosphorylation increases its inhibition of insulin-induced oocyte maturation. Oocytes were either microinjected with the indicated amounts of GST-PIR, which was (black bars) or was not (grey bars) previously phosphorylated by recombinant PKCζ as described in Materials and Methods, or microinjected with PKCζ alone (white bars). Oocyte maturation in response to insulin was monitored as in Fig. 8. Data are the means + standard errors of the means (error bars) of three animals, each concerning 20 oocytes.

modifications, like phosphorylations or conformational changes in response to the hormone. In this regard, we reported that Grb14 undergoes insulin-stimulated phosphorylation during labeling experiments in intact cells. Since we previously reported that Grb14 was not phosphorylated on tyrosine residues under insulin stimulation in CHO-IR-Grb14 cells (28), Grb14 is likely to be phosphorylated on serine/threonine residues. This is in agreement with previous studies reporting that Grb14 was phosphorylated in vivo mainly on serine residues (8, 58). In these studies, stimulation with PDGF (8) or fibroblast growth factor 2 (58) increased Grb14 phosphorylation to an extent similar to that after insulin stimulation. Other members of the Grb7 family of proteins are also phosphorylated on serine/threonine residues (20). More particularly, it was shown that insulin increased Grb10 serine phosphorylation, and it can be underlined that the effect of insulin on Grb10 and on Grb14 phosphorylation is of similar amplitude (13). The serine/threonine kinases involved in Grb7 protein phosphorylation are still unidentified (20), but they do not appear to be either cyclic AMP- or calcium-stimulated kinases (53) or classical or novel PKC isozymes (13, 53). Insulin-induced Grb10 phosphorylation was blocked by wortmannin, suggesting that insulin stimulates a kinase acting in a phosphatidylinositol 3-kinase dependent manner (13). Since insulin is known to stimulate PKCζ catalytic activity (5, 64, 65), and since ZIP anchors PKCζ and Grb14 into a heterotrimeric complex, we hypothesized that Grb14 could be a substrate for the kinase. We thus tested whether PKCζ could be implicated in insulin-stimulated Grb14 phosphorylation. In intact cells, insulin-stimulated Grb14 phosphorylation is decreased by a PKCζ pseudosubstrate, which inhibits PKCζ enzymatic activity. Furthermore, the overexpression of PKCζ increased insulin-induced Grb14 phosphorylation. Together, these data suggest that the serine/threonine kinase PKCζ is a potential candidate for in vivo insulin-induced Grb14 phosphorylation. However, a role for other kinases cannot be excluded, and further experiments are needed to clarify this point. More

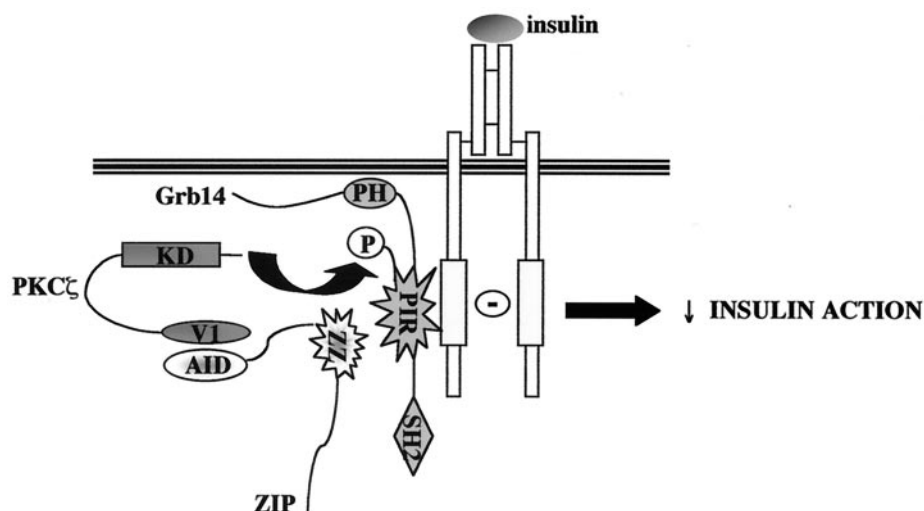


FIG. 12. A proposed model for the role of ZIP as an adapter connecting PKC ζ to Grb14. ZIP interacts with the V1 domain of PKC ζ through its AID domain, and with the PIR of Grb14 through its ZZ zinc finger domain, allowing the formation of a heterotrimeric complex. Under insulin stimulation, the PIR binds to the activated IR regulatory tyrosine kinase loop. PIR phosphorylation by PKC ζ leads to an increased Grb14 inhibitory action on IR catalytic activity and insulin action.

particularly, the identity of kinases responsible for the high basal phosphorylation state of these proteins is still unclear.

Of functional relevance, we provided evidence that phosphorylation of Grb14 increased its inhibitory effect on IR tyrosine kinase activity. Interestingly, among the various domains of Grb14, the PIR, which is responsible for both the specific interaction with the activated IR tyrosine kinase loop and the inhibition of the IR catalytic activity (2, 28), was preferentially phosphorylated by PKC ζ in vitro. Although this domain does not contain a conventional phosphorylation motif for PKC, it exhibits 4 serine residues followed in +1 by a hydrophobic amino acid, which has been shown to be present in all bona fide PKC phosphorylation sites (51). In addition, PKC ζ was already described to phosphorylate IKK β in a non-consensus motif (32). The identification of the Grb14-phosphorylated sites and the mechanism whereby Grb14 phosphorylation increases its inhibitory effect deserve further investigation. However, a simple hypothesis could be that this increased activity reflects an increased binding affinity of the phosphorylated PIR for the IR kinase loop.

Xenopus oocyte, which endogenously expresses insulin-signaling molecules, provides a widely used experimental model to study in vivo insulin action (15, 25, 59, 66). We show here that injection of Grb14 into *Xenopus* oocyte specifically blocks insulin-induced reinitiation of meiosis. These data correlate with Grb14 inhibition of distal insulin effects, such as DNA or glycogen synthesis (28). By contrast, injection of ZIP alone did not alter oocyte maturation. Since PKC ζ is a critical step for the mitogenic signal in oocytes (3, 11), this result seems to confirm that ZIP does not regulate PKC ζ activity in vivo. On the other hand, ZIP was able to potentiate Grb14 inhibitory action. This effect required the recruitment of PKC ζ and could be mimicked by previous Grb14 phosphorylation. These observations provide in vivo evidence that ZIP acts as a functional scaffold protein connecting PKC ζ to Grb14, allowing its phosphorylation. Interestingly, the PIR expressed alone was

sufficient to mediate both the inhibitory effect of Grb14 on insulin-induced reinitiation of meiosis and the potentiation by ZIP of this inhibitory effect. This clearly demonstrates that the interaction of the PIR with ZIP does not antagonize the regulation of its phosphorylation by PKC ζ in vivo. These data also correlate with the observation that ZIP binding to Grb14 did not alter the insulin-induced Grb14-IR interaction (data not shown). The PIR of Grb14 appears thus to be the functional core of the protein in the regulation of insulin signaling. Considering our in vitro and in vivo findings, a model emerges whereby ZIP acts as a physical link in the assembly of a PKC ζ -ZIP-Grb14 complex, targeting the PKC ζ activity to Grb14 (Fig. 12). The constitution of this complex has major functional consequences, since it potentiates the inhibitory effect of Grb14 on insulin signaling.

PKC ζ is known as a positive effector of the insulin signal transduction pathways, implicated in insulin-induced glucose transport, GLUT4 translocation, and protein synthesis (1, 16, 43, 64, 65). However, recent studies reported that PKC ζ can also play an inhibitory role in insulin signal transduction, by the inhibition of Akt (14, 39) or by the serine phosphorylation of IRS-1 (35, 57). Serine phosphorylation of IRS-1 by PKC ζ participates in a negative feedback control mechanism induced by insulin, which promotes the dissociation of the IR-IRS-1 complex and leads to the termination of insulin signal (35, 57). Interestingly, the present work suggests that by enhancing the inhibitory activity of Grb14, PKC ζ can use an additional way to down-regulate insulin signaling.

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