Lipid−Protein Interactions of Growth Factor Receptor-Bound Protein 14 in Insulin Receptor Signaling†

Raju V. S. Rajala,*,‡,§ Michael D. Chan,§ and Ammaji Rajala§

Departments of Cell Biology and Ophthalmology, University of Oklahoma Health Sciences Center, and Dean A. McGee Eye Institute, Oklahoma City, Oklahoma 73104

Received July 8, 2005; Revised Manuscript Received September 29, 2005

ABSTRACT: Many retinal degenerative diseases show an early loss of rod cells followed by cone cells. In these degenerations the pathological phenotype is apoptosis. We have previously demonstrated the light-dependent tyrosine phosphorylation of the insulin receptor in the retina, which leads to the activation of anti-apoptotic signaling molecules. The mechanism of the regulation of the insulin receptor in the retina is not known. Yeast two-hybrid screening of a bovine retinal cDNA library with the cytoplasmic domain of the retinal insulin receptor (IRβ) identified a member of the Grb7 (growth factor receptor-bound protein 7) gene family, Grb14. In this report, we describe the unique features of Grb14. Grb14 forms a specific complex with the cytoplasmic domain of IRβ when both are expressed as hybrid proteins in yeast cells. This interaction is strictly dependent upon receptor tyrosine kinase activity. Deletion mutagenesis on Grb14 indicated a phosphorylated insulin receptor interacting (PIR) domain between the PH (pleckstrin homology) and SH2 (Src homology) domains that binds to IRβ. Nuclear import assays in yeast indicated the presence of a functional nuclear localization signal in Grb14 between amino acids 63 and 68 (RRKKD). Subcellular localization of isolated retinas probed with anti-Grb14 antibody further confirmed the presence of Grb14 in nuclear fractions. Analysis using a protein−lipid overlay assay indicated binding of Grb14 and its PH domain to D3 phosphoinositides. In addition, Grb14−phosphoinositide 3,4,5-trisphosphate complexes are detected in lysates prepared from insulin-stimulated retina tissues, whereas Grb14−phosphoinositide 4,5-bisphosphate interactions are observed under non-insulin stimulated conditions. These findings suggest that Grb14 could be a diverse regulator of insulin receptor mediated pathways in the retina.

Insulin receptors (IR) and insulin signaling proteins are widely distributed throughout the central nervous system (CNS) (*1*). Previous experiments have suggested a role for insulin signaling in the regulation of food intake (*2*, *3*) and neuronal growth and differentiation (*4*, *5*). Disregulation of insulin signaling in the CNS has been linked to the pathogenesis of neurodegenerative disorders such as Alzheimer's and Parkinson's disease (*6*, *7*). Cells of bovine and rat retina contain high-affinity receptors for insulin (*1*). However, little research has been done on these receptors since these early reports, probably due to the absence of an identified intracellular target. We have demonstrated that light stimulates tyrosine phosphorylation of the β subunit of the insulin receptor (IRβ)[1] in vivo, which leads to the association of phosphoinositide 3-kinase (PI3K), an anti-apoptotic enzyme with the IRβ (*8*).

The IR serves an important function in guiding retinal photoreceptor axons in *Drosophila* from the retina to the brain during development (*9*). In *Drosophila*, IR also influences the size and number of photoreceptors (*10*). In *Caenorhabditis elegans*, the IR regulates neuronal survival (*11*). The high degree of IR signaling homology between *C. elegans*, *Drosophila*, and humans suggests functional conservation in mammalian retina. Defects in IR signaling in the central nervous system are associated with Alzheimer's disease (*12*−*14*), and lack of insulin receptor activation leads to neurodegeneration in the brain/neuron-specific insulin receptor knockout mice (*15*). Mutation in either IRβ autophosphorylation sites (*9*) or its binding partner Dock (*16*) in *Drosophila* results in a severe photoreceptor axonal misguidance phenotype. Recently, it has been shown that IRS-2 knockout mice lose up to 50% of photoreceptor cells by 2 weeks of age, owing to increased apoptosis (*17*). These studies clearly suggest that the insulin receptor pathway is important for photoreceptor survival and maintenance.

† This work was supported by grants from the National Institutes of Health (EY00871, EY04149, EY12190, and RR17703) and Research to Prevent Blindness Inc. R.V.S.R. is a recipient of a Career Development Award from Research to Prevent Blindness, Inc.

* To whom correspondence should be addressed. Telephone: 405-271-8255. Fax: 405-271-8128. E-mail: raju-rajala@ouhsc.edu.

‡ Department of Cell Biology, University of Oklahoma Health Sciences Center.

§ Department of Ophthalmology, University of Oklahoma Health Sciences Center.

[1] Abbreviations: Grb14, growth factor receptor-bound protein 14; PTB, phosphotyrosine binding; PID, phosphotyrosine interacting domain; PTP1b, protein tyrosine phosphatase 1b; IRS-1, insulin receptor substrate 1; PI3K, phosphoinositide 3-kinase; GST, glutathione *S*-transferase; IRβ, insulin receptor β subunit; RA, homology to Ras-associating domains; PH, pleckstrin homology domains; SH2, Src homology 2 domains; PIR, phosphorylated insulin receptor interacting region; PCR, polymerase chain reaction; SDM, site-directed mutagenesis; NLS, nuclear localization signal; PI-3,4-P_2, phosphoinositide 3,4-bisphosphate; PI-4,5-P_2, phosphoinositide 4,5-bisphosphate; PI-3,4,5-P_3, phosphoinositide 3,4,5-trisphosphate; ROS, rod outer segments.

10.1021/bi0513148 CCC: $30.25
Published on Web 11/05/2005

Insulin receptor signaling provides a trophic signal for transformed retinal neurons in culture (*18*), but the in vivo role of insulin receptor activity in neural tissues is unknown. To identify new binding partners, the cytoplasmic domain of the retinal insulin receptor was used as bait in a two-hybrid screen of a bovine retinal cDNA library in the yeast *Saccharomyces cerevisiae.* One of the proteins identified in this screen was the bovine homologue of human growth factor receptor-bound protein 14 (Grb14) (*19*). Grb14 is a member of an emerging family of noncatalytic adapter proteins that also includes Grb7 and Grb10 (*20*, *21*). Characteristic features of Grb7 family members include a central pleckstrin homology (PH) domain, a C-terminal Src homology 2 (SH2) domain, an N-terminal proline-rich motif, which is similar to a SH3 binding site, and homology to Ras-associating (RA) domain. The presence of these functional domains indicates that Grb7 family members potentially interact with a variety of proteins. Grb14 has been shown to bind to Tek/Tie2, PDGF, EGFR, FGFR, and IR (*22*−*25*). The large number of binding partners for the Grb7 family members suggests that they play key roles as cytoplasmic signaling intermediates. Recently, we demonstrated the presence of a NPXY motif in Grb14 and its interaction with the PTB domain of IRS-1 (*19*). The present study describes novel features of Grb14: the presence of a nuclear localization signal and its localization in the nucleus and its binding to phosphoinositides both in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials. Polyclonal anti-IRβ was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cJun antibody was obtained from Cell Signal (Beverly, MA). Anti-PTP1b antibody was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal anti-opsin antibody (Rho 4D2) was a gift from Dr. Robert Molday (University of British Columbia). Anti-arrestin antibody was a kind gift of Dr. James F. McGinnis (University of Oklahoma Health Sciences Center, Oklahoma City). Anti-glutathione *S*-transferase (GST) antibody and glutathione−Sepharose 4B matrix were obtained from Amersham Biosciences Corp. (Piscataway, NJ). Anti-PI-3,4,5-P_3, anti-PI-4,5-P_2, and anti-PI-3,4-P_2 antibodies and PIP-Strips were obtained from Echelon Research Laboratories, Inc. (Salt Lake City, UT). Human insulin R (rDNA origin) was obtained from Eli Lilly & Co. (Indianapolis, IN). NE-PER nuclear and cytoplasmic extraction reagent was obtained from Pierce (Rockford, IL). All other reagents were of analytical grade from Sigma (St. Louis, MO).

Yeast Two-Hybrid Screen of the Bovine Retinal cDNA Library. The yeast two-hybrid screen was performed in the yeast strain L40 using pLexA-IR, which encodes a constitutively activated insulin receptor β subunit (*26*) against a bovine cDNA library from retina cloned in fusion with the *GAL*4 activation domain in the pGAD10. The details of the identification of Grb14 have been described earlier (*19*). The full-length p85 cDNA was excised from the pGEX-2T vector as a *Bam*HI/*Eco*RI fragment and subcloned into the pAD-GAL-2.1 yeast vector carrying the GAL4 activation domain (*26*).

Nuclear Import Assay. Yeast one-hybrid assay was carried out according to the method described (*27*). The nuclear import vectors were kindly provided by Dr. Citovsky (State University of New York, Stony Brook, NY). Wild-type and NLS mutant Grb14 were cloned into pNIA vectors (*27*).

Filter and Liquid β-Galactosidase Assays. For filter β-galactosidase assays, yeast patches were replica-lifted onto Whatman 40 filter paper and grown for 24 h. After being frozen in liquid nitrogen, these filters were laid over a Whatman 3 filter soaked in Z buffer [50 mM sodium phosphate buffer (pH 7.0), 10 mM KCl, and 1 mM $MgSO_4$ with 0.5% β-mercaptoethanol and 0.2 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal)] (Gibco BRL, Life Technologies). After 2−6 h, the reaction was stopped by incubating the filters in 1 M Na_2CO_3 and drying them at 30 °C in an incubator.

For liquid β-galactosidase assays (*28*), yeast was grown overnight on appropriate selective media. Cells were pelleted and resuspended in 5 mL of Z buffer, and the OD_{600} was recorded. Eight hundred microliters (either diluted or straight) was transferred to 1.5 mL Eppendorf tubes. Using a Pasteur pipet, 1 drop (∼50 μL) of 0.1% SDS and 2 drops of chloroform were added to each tube, vortexed for 15 s, and allowed to equilibrate at 30 °C for 15 min. Then 160 μL of 2-nitrophenyl β-D-galactopyranoside (ONPG, 4 mg/mL in H_2O) was added and vortexed for 10 s, and the tubes were incubated at 30 °C. The tubes were removed after about 15−20 min (empirically determined by color), and the color was quenched by adding 400 μL of 1 M Na_2CO_3. The cell debris was removed by centrifugation, and the OD of the supernatant was measured at 420 and 550 nm. Results are expressed as Miller units: one unit of β-galactosidase was defined as $1000 \times OD_{420}/(OD_{546} \times \text{min})$.

Retinal Organ Cultures. Retinas were removed from Sprague-Dawley albino rats that were born and raised in dim cyclic light (5 lux; 12 h on, 12 h off) and incubated at 37 °C in DMEM medium (Gibco BRL) in the presence and absence of 1 μM insulin for 0−60 min. At indicated times, retinas were snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

Production of Polyclonal Grb14 Antibody. The peptide TRGCAADRRKKKDLDVLE corresponding to the amino-terminal region from amino acids 57−74 of bovine Grb14 was synthesized and coupled to keyhole limpet hemocyanin to generate polyclonal antibodies in rabbits (*29*). The titer was determined by ELISA, and the antibodies were affinity purified (*29*).

Construction of GST Fusion Proteins and Pull-Down Experiments. The amino acids of bovine Grb14 present in each fusion protein are Grb14 full length (1−540), RAS (86−234), PH (234−341), PH-SH2 (234−341), PIR (342−438), PIR-SH2 (342−540), and SH2 (439−540). The indicated regions in each fusion were amplified by PCR and cloned into either pGEX 2-TK or pGEX-4T-1 or pGEX-4T-2 GST fusion vectors. Grb14 PIR-SH2 and PIR domains were also cloned into pTrcHisA vector. The sequence of each clone was verified by DNA sequencing. All inductions yielded proteins of the expected size as judged by Coomassie staining. Pull-down experiments were carried out as described (*26*) using 5 μg of GST fusion proteins that had been adsorbed onto glutathione−Sepharose 4B matrix. Retina lysates or proteins expressed and purified from bacteria were incubated with either GST or GST fusion proteins at 4 °C for 1.5 h, with continuous stirring. The Sepharose beads were

washed three times in 500 μL of PBS and centrifuged at 5000 rpm for 30−60 s at 4 °C. Bound proteins were eluted by boiling in 2 × SDS sample buffer 5 min prior to 10% SDS−PAGE. After SDS−PAGE, the gels were subjected to Western blot analysis with appropriate antibodies.

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out according to the method described earlier (*26*). The primers used in the site-directed mutagenesis on IRβ are Y960F (sense, tcaaacccagagttcctcagtgccagt; antisense, actggcactgaggaactctgggtttg), Y1146F (sense, atgacgagagacatcttcgagacagattac; antisense, gtaatctgtctcgaagatgtctctcgtcat), Y1150F (sense, atctacgagacagatttctatcggaaaggg; antisense, ccctttccgatagaaatctgtctcgtagat), Y1151F (sense, atctacgagacagattactttcggaaaggg; antisense, ccctttccgaaagtaatctgtctcgtagat), Y1316F (sense, agcatcaaacggacctttgatgaacacatc; antisense, gatgtgttcatcaaaggtccgtttgatgct), Y1322F (sense, gaacacatccccttcacccacataacggg; antisense, cccgttcatgtgggtgaaggggatgtgttc), M1325P (sense, ccctacaccccacccgaacgggggcaagaag; antisense, cttcttgcccccgttcgggtgggtgtaggg), K1018A (sense, gagacccgtgttgcggtggcgacggtcaatgag; antisense, ctcattgaccgtcgccaccgcaacacg), Grb14-SH2 (R466A) (sense, ggagttttcttggtagcggatagtcagagt; antisense, actctgactatccgctaccaagaaaactcc), and Grb14-NLS (RK64,65GE) (sense, gcggcagacaggggagaaaagaaagatctt; antisense, aagatctttcttttctccccctgtctgccgc). After sequencing, IRβ mutants were excised from the sequencing vector and cloned into the pBTM116 vector; the Grb14 mutants were cloned into the GAL 2.1 AD vector. Plasmid DNA was prepared from IRβ and Grb14 mutants and subjected to yeast two-hybrid assays.

Protein−Lipid Overlay Assay. Membrane arrays (PIP-Strips) spotted with 100 pmol of phospholipids were purchased from Echelon Research Laboratories (Salt Lake City, UT) and used for protein−lipid overlay assay by following the manufacturer's instructions. Briefly, membranes were blocked with 3% (w/v) fatty acid-free bovine serum albumin (Sigma) in TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% (w/v) Tween 20] for 1 h at room temperature. Blocked membranes were incubated with 0.5 μg/mL GST fusion proteins overnight at 4 °C with gentle agitation. They were then washed three times with TBST plus 3% fatty acid-free bovine serum albumin, after which the membranes were subjected to Western blot analysis using anti-GST antibody to detect the bound GST fusion proteins.

Preparation of Retinal Section for Immunocytochemistry. Rat eyes were fixed overnight at 4 °C with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). The tissues were rinsed with PBS and immersed in 10%, 20%, and 30% sucrose in PBS. Samples were embedded in OCT compound (Sakura, Tokyo, Japan) under liquid nitrogen and stored at −80 °C. Cryosections (12 μm) of tissue were mounted on slides and air-dried. Retina sections were washed with PBS containing 0.25% Triton X-100 (PBS-Tx) and treated for 10 min with PBS containing 3% Triton X-100. Sections were blocked with 3% normal goat serum in PBS for 30 min at room temperature and incubated with rabbit polyclonal anti-Grb14 or nonimmune rabbit IgG as a control overnight in a moist chamber at 4 °C. After washing, slides were incubated for 1 h with Alexa 488 conjugated goat anti-rabbit IgG at room temperature. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), and the Grb14-stained sections were analyzed using an Olympus laser scanning confocal microscope. Antibody-labeled complexes were examined using Olympus Fluoview Version 4.3 software. For quantitation, all images were captured using identical microscope and camera settings.

Preparation of Tyrosine-Phosphorylated Proteins. Rod outer segments (ROS) prepared as described have an endogenous tyrosine kinase activity (*30*−*32*). Tyrosine-phosphorylated ROS were prepared by incubating ROS for 15 min at 37 °C in a phosphorylation buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 1.0 mM ATP). Nonphospohorylated ROS were prepared by incubating ROS in a similar buffer without ATP. After incubation ROS was solubilized at 4 °C for 60 min in a solubilization buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100, 10% glycerol). Retina lysates were solubilized before being subjected to in vitro phosphorylation. ROS outer segments were prepared from the rat retinas as previously described (*8*).

RESULTS

Identification of Grb14. A bovine retinal cDNA library (3.6×10^6) was screened against the cytoplasmic domain of the retinal insulin receptor in yeast two-hybrid assays and identified Grb14 as one of the interacting proteins. We isolated 60 *HIS3* and LacZ positive clones, and the majorities represent the same protein, Grb14. Both strands of cDNA were completely sequenced using synthetic primers. The Genbank accession number for the retinal Grb14 is AY772475. The longest insert contains 2106 bp with an open reading frame of 1620 bp. This open reading frame is flanked by 189 bp of 5′-untranslated sequence and 297 bp of 3′-untranslated sequence. The ATG initiation codon is found at position 190. The single long open reading frame from the bovine retinal cDNA specifies a protein of 540 amino acids with a predicted molecular mass of 61039 Da. The open reading frame terminates with a TAG stop codon at position 1809. Sequence comparison revealed that bovine retinal Grb14 displays a high degree of similarity with Grb14s from other species at the primary structure level. The predicted retinal Grb14 protein exhibited a 92% identity with human Grb14 (*23*), 84% with rat Grb14 (*25*), and 85% with mouse Grb14 (*24*).

Identification of Bovine Retinal Grb14 Domains Binding to the Retinal Insulin Receptor. Various Grb14 deletion mutants [RA, PH, PH-SH2, PH-SH2 (R466A), PIR, PIR-SH2, PIR-SH2 (R466A), SH2, and SH2 (R466A)] were generated to test their ability to bind to the retinal insulin receptor. RA, PH, and SH2 domains failed to bind receptor, but the PIR-SH2 and PIR domains interacted with the retinal insulin receptor (Table 1), suggesting that the PIR domain in Grb14 binds to the retinal insulin receptor. It is interesting to note that when we expressed PH-SH2 and PH-SH2 (R466A) domains, we failed to observe the interaction with the retinal insulin receptor, suggesting that either these domains failed to express in yeast or could not be targeting to the nucleus where the transcriptional activation takes place. To address these issues, we cloned and expressed the N-terminal SH2 (N-SH2) domain of the p85 subunit of PI3K as a GAL4 fusion in the pGAD-GAL4-2.1 vector. This domain interacts with the retinal insulin receptor in the yeast two-hybrid system (*26*). We fused the PH domain from Grb14 to the N-terminus of the N-SH2 domain of p85 (PH-

Table 1: Interaction of IRβ with Deletion Mutants of Grb14[a]

domain	amino acids	β-gal activity[b]
FL-Grb14	1−540	+++
RA	86−234	−
PH	234−341	−
PH-SH2	234−540	−
PH-SH2 (R466A)	234−540	−
PIR	342−438	+++
PIR-SH2	342−540	+++
PIR-SH2 (R466A)	342−540	+++
SH2	439−540	−
SH2 (R466A)	439−540	−
p85 (N-SH2)		+++
PH-p85 (N-SH2)		−
p85 (N-SH2)-PH		−
RA-PH-p85 (N-SH2)		−
RA-p85 (N-SH2)-PH		−

[a] Yeast two-hybrid assays were carried out as described in Experimental Procedures. [b] Colony color was assayed in three independent transformations. +++, strong interaction; −, no interaction.

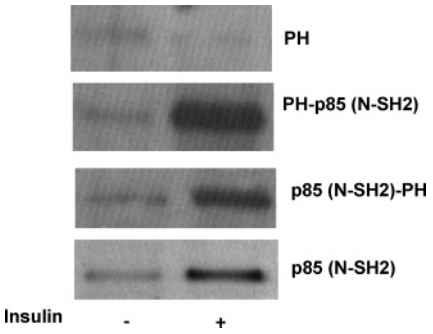

FIGURE 1: Retinal Grb14 interacting with the activated IRβ. Rat retinas were dissected and incubated at 37 °C in DMEM medium in the presence and absence of insulin. After incubation, the retinas were lysed and subjected to GST pull-down assay (125 μg) with GST-PH, GST-PH-p85 (N-SH2), GST-p85 (NSH-2)-PH, or GST-p85 (N-SH2) followed by Western blot analysis of the bound proteins with anti-IRβ antibody.

p85); the PH-p85 domain failed to interact with the retinal insulin receptor (Table 1). Fusion of the PH domain to the C-terminal end of p85 also did not allow interaction with the retinal insulin receptor. To confirm the expression of PH-p85, p85-PH, p85, and PH domains, we expressed these proteins as GST fusion in bacteria. The purified proteins were incubated with insulin-stimulated and nonstimulated retinas, lysed, and subjected to GST pull-down assay followed by Western blot analysis using anti-IRβ antibody. GST-PH fusion protein failed to pull down IRβ from either insulin-stimulated or nonstimulated rat retinas, while the p85 N-SH2, PH-p85, and p85-PH fusion proteins pulled down IRβ from insulin-stimulated rat retinas (Figure 1). To confirm the integrity of the PH domain, we eluted the proteins from glutathione beads with 10 mM glutathione and subjected the purified proteins to the protein−lipid overlay assay as described in Experimental Procedures. The results indicate that PH, PH-p85, and p85-PH domains bind to phosphoinositides (not shown) in a similar way as described in Figure 5. These experiments clearly indicate that the absence of interaction of PH-SH2 domains with retinal IRβ in yeast is due to the mistargeting of the PH domain to the membrane. The above experiments also suggest that the sequence 5′ to the PH domain could properly target the Grb14 to the nucleus.

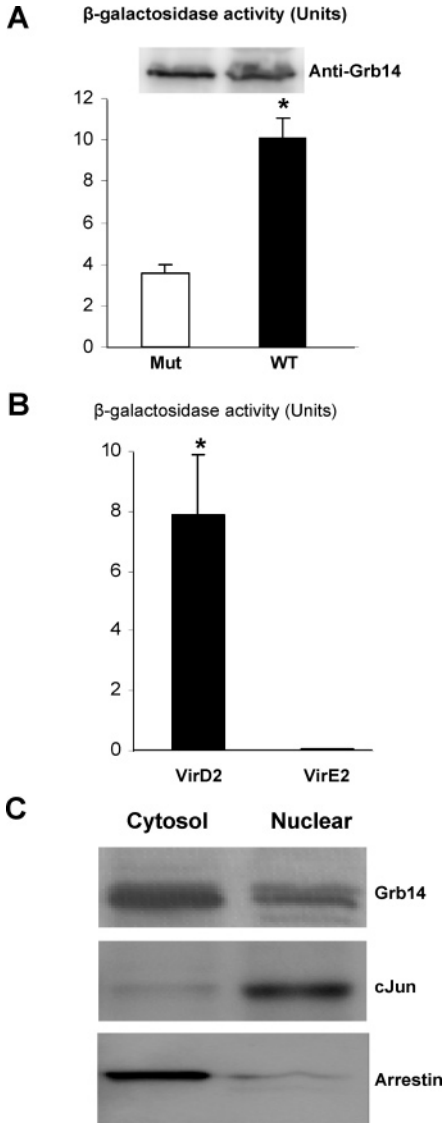

FIGURE 2: Nuclear import assay to identify the functional nuclear localization signal in Grb14. Wild-type and mutant (substitution of RK with GE in the NLS sequence) Grb14 cDNAs were cloned into the pNIA vector, and one-hybrid assay was carried out as described in Experimental Procedures. Yeast cells were subjected to liquid β-galactosidase assays (A). To ensure the protein expression of wild-type and mutant Grb14, yeast cultures were subjected to Western blot analysis with anti-Grb14 antibody (A, inset). Positive (VirD2) and negative (VirE2) control vectors were transformed in yeast, and the cells were subjected to liquid β-galactosidase assays (B). Data are the means ± SD. The asterisk indicates that the difference between wild-type and mutant Grb14 is significant at $P < 0.004$ (A) and the difference between VirD2 and VirE2 is significant at $P < 0.021$ (B). All experiments were done in triplicate. Cytosolic and nuclear fractions of the retina were subjected to Western blot analysis with anti-Grb14, anti-cJun, and anti-arrestin antibodies (C).

To identify the target signal, we fused the RA domain to the N-terminal end of both PH-p85 and p85-PH domains and carried out a yeast two-hybrid assay. Both these fusions failed to interact with the retinal insulin receptors (Table 1). This experiment suggests that Grb14 may contain a nuclear localization signal upstream to the RA domain.

Identification of the Nuclear Localization Signal (NLS) in Grb14. To identify the NLS in Grb14, we analyzed the Grb14 protein sequence using the NLS prediction program (http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl), which

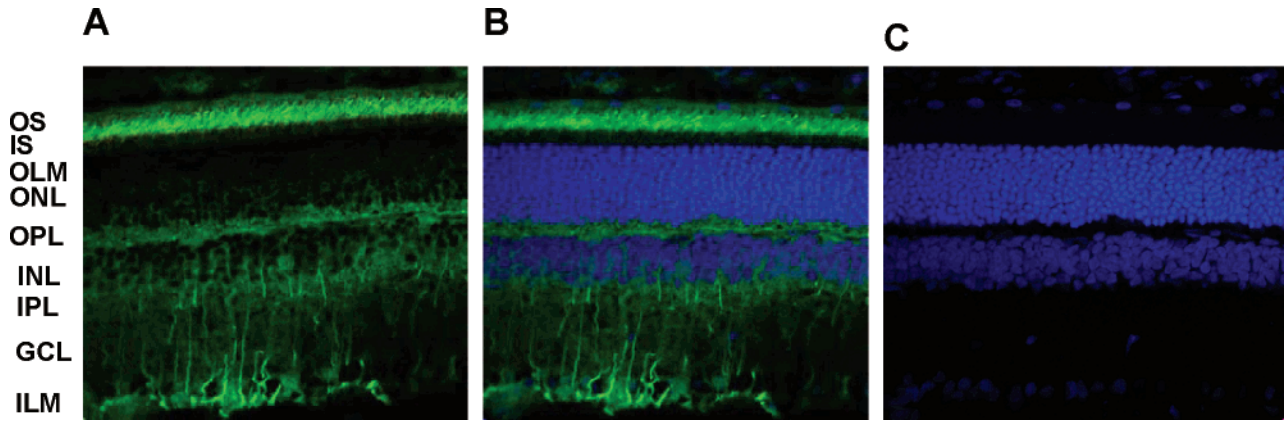

FIGURE 3: Immunocytochemical analysis of Grb14 in rat retina. Rat retina frozen sections were subjected to immunocytochemistry. (A) Incubation with anti-Grb14. (B) Incubation with Grb14 and DAPI. (C) Incubation with Grb14 blocking peptide. Key: OS, photoreceptor outer segments; IS, photoreceptor inner segments; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform cell layer; INL, inner nuclear layer; IPL, inner plexiform layers; GCL, ganglion cell layer; ILM, inner limiting membrane.

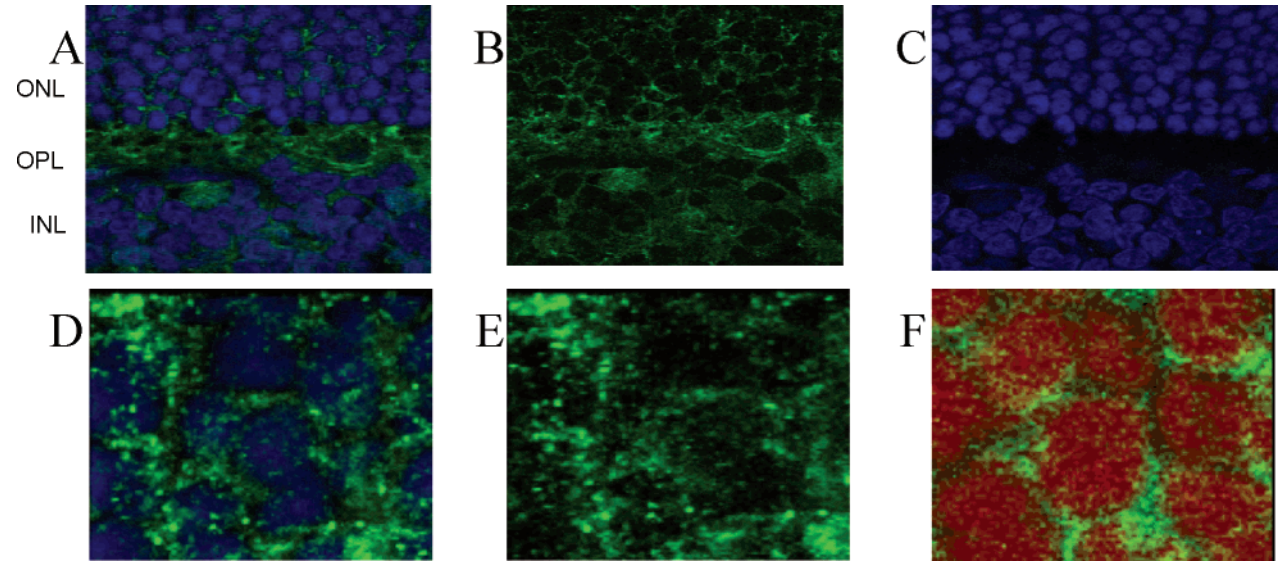

FIGURE 4: Immunolocalization of Grb14 in the nuclear layer of rat retina. Frozen sections of rat retina were subjected to immunocytochemistry using anti-Grb14, and its immunolocalization was examined in the outer and inner nuclear layers. The sections were stained with DAPI for visualization of nuclei (A) and Grb14 (A, B). The Grb14 immunorectivity was neutralized with Grb14 blocking peptide (C). Merge of Grb14 and DAPI (D) and Grb14 staining in the enlarged area (E). A single optical section through nuclei was scanned, and the analysis indicates the perinuclear and nuclear localization of Grb14 (F). Grb14 is in green, and nuclei are in red (F). Key: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer.

indicated a putative NLS (RRKKD) between amino acids 63 and 68. To determine whether the NLS is functional, we carried out nuclear import assays. The basic strategy of this experiment is based on expression in yeast cells of a triple-fusion protein comprising bacterial LexA, yeast Gal4p activation domain (Gal4AD), and the tested protein encoded by a cDNA subcloned in-frame downstream of Gal4AD. If the tested protein contains a functional NLS, the fusion product will enter the yeast nucleus. Following the nuclear import, the LexA domain will target the fusion protein to the LexA operator sites of the reporter LacZ gene contained in the L40 yeast strain. Gal4AD then activates the expression of LacZ, resulting in β-galactosidase activity. In the absence of a NLS, the fusion protein is unable to reach the cell nucleus and does not activate the reporter gene. The wild-type Grb14 and mutant Grb14 in which the arginine and lysine (RRKKD) residues in the NLS sequence were mutated to glycine and glutamic acid (RGEKKD) were subcloned in-frame downstream of Gal4AD for yeast one-hybrid assays. Filter (data not shown) and liquid β-galactosidase assays indicate that wild-type Grb14 activates the reporter gene, whereas mutant Grb14 does not (Figure 2A). To verify the expression of wild-type and mutant Grb14, we lysed the yeast cells and examined the expression of Grb14 with anti-Grb14 antibody. Grb14 expression was evident in both the wild type and mutant (Figure 2A, inset). These results suggest that Grb14 has an active NLS sequence in its amino-terminal end. Controls (Figure 2B) include VirD2 protein which carries a NLS sequence while VirE2 protein does not (*27*).

Expression of Grb14 in Cytosolic and Nuclear Fractions from Rat Retina. Rat retina lysates were subjected to cytosolic and nuclear fractionation. SDS−PAGE and Western blot analysis with anti-Grb14 antibody indicated the presence of Grb14 in these fractions (Figure 2C). To ensure the purity of nuclear fractions, nuclear protein cJun and cytosolic protein arrestin localization was examined in the cytosolic and nuclear fractions of the retina. Enrichment of cJun in the nuclear fraction and arrestin in the cytosolic fraction

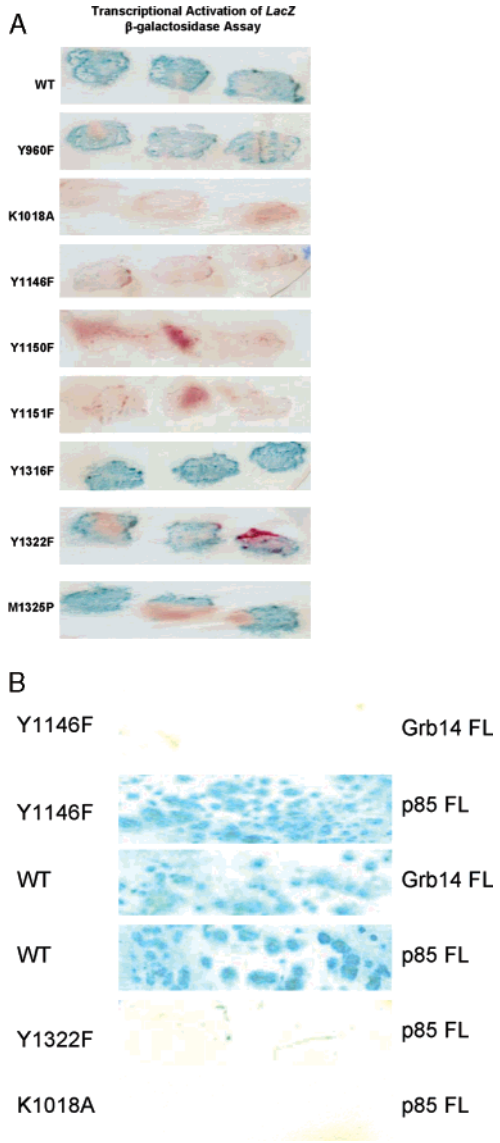

FIGURE 5: Site-directed mutagenesis of selected tyrosine residues in retinal IRβ and its interaction with Grb14. The entire cytoplasmic domain of the retinal insulin receptor and its mutants were coexpressed along with Grb14 (A) or p85 (B) and carried out in yeast two-hybrid assays as described in Experimental Procedures. Transformants were assayed for β-galactosidase assay by colony color. All experiments were done in triplicate.

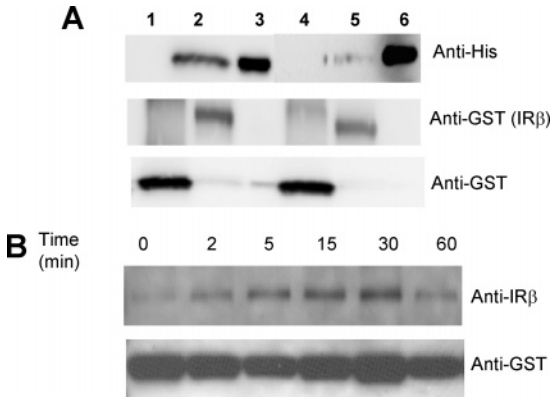

FIGURE 6: Interaction of Grb14 with activated IRβ. GST and GST-IRβ fusion proteins were incubated with the PIR-SH2 and PIR domain followed by GST pull-down assay with anti-His antibody (A). The blot was also stripped and reprobed with anti-GST antibody (B). Retinas were cultured in DMEM in the presence of insulin (1 μM) at different time points. After insulin stimulation, retinal lysates were subjected to GST pull-down assay with the GST-PIR-SH2 domain of Grb14 followed by Western blot analysis with anti-IRβ antibody (C). The blot was stripped and reprobed with anti-GST antibody to ensure an equal amount of fusion in each pull down (D).

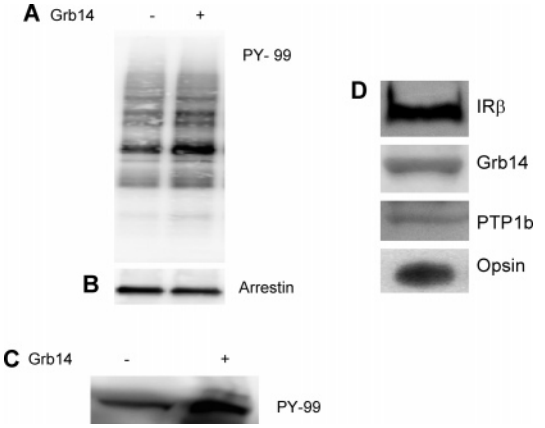

FIGURE 7: Grb14 enhances the tyrosine phosphorylation of ROS and the insulin receptor in vitro. Bovine ROS was phosphorylated in the absence and presence of Grb14 followed by Western blot analysis with anti-phosphotyrosine (PY-99) antibody (A). The blot was stripped and reprobed with anti-arrestin antibody to ensure an equal amount of protein in each lane (B). Retinal lysates were phosphorylated in vitro in the presence and absence of recombinant GST-Grb14 (1 μg). After phosphorylation the retinal insulin receptor was immunoprecipitated with anti-IRβ antibody followed by Western blot analysis with anti-PY99 antibody (C). ROS proteins were subjected to Western blot analysis with anti-IRβ, anti-Grb14, anti-PTP1b, and anti-opsin antibodies (D).

further attested to the purity of nuclear fractions (Figure 2C).

Immunocytochemical Localization of Grb14 in the Nuclear Layer of Rat Retina. Frozen sections were stained with Grb14 (Figure 3A) and DAPI for outer and inner nuclear layers of rat retina (Figure 3B,C). In the sections incubated with antibody against Grb14, immunoreactive product is present in the rod outer segments and several other retinal layers (Figure 3A,B). Rod outer segments and Muller cells were heavily stained with Grb14 antibody (Figure 3A,B). The Grb14 immunoreaction was completely abolished by neutralizing the primary antibody before incubation with the peptide (TRGCAADRRKKKDLDVLE, amino-terminal region from amino acids 57−74 of bovine Grb14) it was raised against (Figure 3C). To ensure the labeling of Grb14 in the nuclear layer, confocal microscopy was used. At high magnification the results indicate the presence of Grb14 immunoreactivity in the outer and inner nuclear layers of the retina (Figure 4A,B). The immunostaining of Grb14 was completely blocked using the peptide from which the antibody was generated (Figure 4C). Control sections incubated with normal nonimmune rabbit IgG did not show any reaction (data not shown). The staining pattern does not rule out whether the localization of Grb14 is nuclear or perinuclear. To examine this possibility, a region in the nuclear layer was selected and examined for the presence of Grb14. The nuclear layer was positive for the Grb14 immunoreactivity (Figure 4D,E). The merged image of Grb14 and DAPI immunostaining clearly indicates increased Grb14 immunoreactivity around the nucleus, suggestive of a perinuclear localization and also some immunoreactivity within the nucleus (Figure 4D). To further confirm the perinuclear and

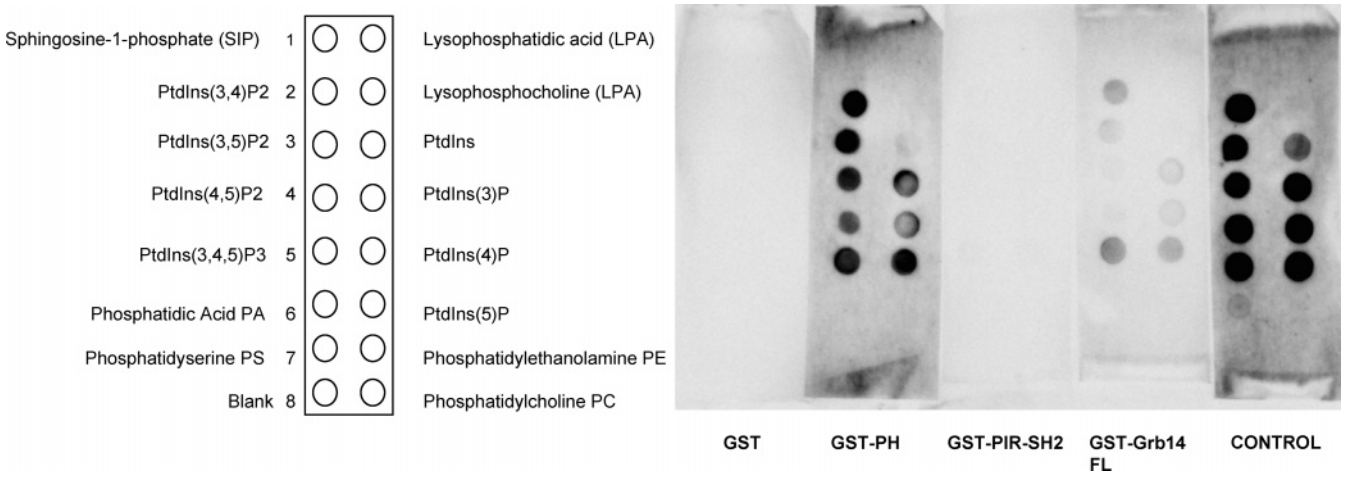

FIGURE 8: Phospholipid binding specificity of the Grb14 PH domain. An equal amount of purified proteins containing various Grb14 constructs (PH, PIR-SH2, and Grb14 full length) was incubated with nitrocellulose strips that had been spotted with various phospholipids. The bound proteins were detected by Western blotting using anti-GST antibody. The control experiment was carried out with the fusion protein supplied by Echelon Research Laboratories, Inc.

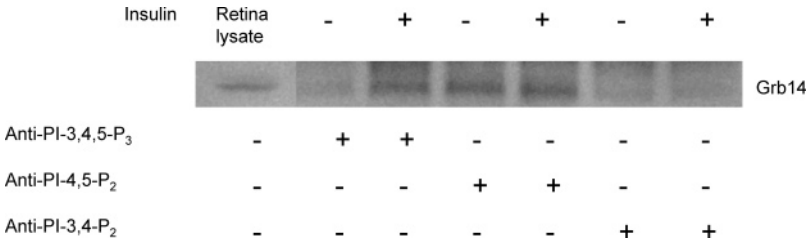

FIGURE 9: Binding interaction between phosphoinositides and Grb14. Insulin-treated and untreated retinas were lysed and subjected to immunoprecipitation with anti-PI-3,4,5-P_3, anti-PI-4,5-P_2, and anti-PI-3,4-P_2 antibodies followed by Western blotting analysis with anti-Grb14 antibody.

nuclear localization of Grb14, single optical sections through nuclei were scanned, and the results confirm the perinuclear and nuclear localization of Grb14 (Figure 4F).

Identification of Grb14 Binding Sites on the Retinal Insulin Receptor. Wild-type and mutant (Y960F, Y1146F, Y1150F, Y1151F, Y1316F, Y1316F, Y1322F, M1325F, and K1018A) IRβs were coexpressed along with wild-type full-length Grb14, and yeast two-hybrid assays were carried out as described in Experimental Procedures. Three independent yeast patches were subjected to LacZ assays. Grb14 formed a specific complex with the cytoplasmic domain of the insulin receptor when both were expressed as hybrid proteins in yeast cells. This interaction is strictly dependent upon receptor tyrosine kinase activity since Grb14 shows no interaction with a kinase-inactive receptor hybrid containing a mutated ATP-binding site (K1018A) (Figure 5A). Substitution of Y960F did not affect the binding of Grb14 to IRβ, whereas substitutions of Y1146F, Y1150F, and Y1151F abolished binding. In addition, substitution of Y1316F, Y1322F, and M1325P did not affect the binding of Grb14 to IRβ. These results indicate that Grb14 binds to IRβ on tyrosine residues 1146, 1150, and 1151 (Figure 5A). It has been shown previously that substitution of Y1146F blocked only 50% of either full-length rat liver Grb14 or the isolated PIR domain (*25*). In the present study, substitution of Y1146F completely blocks the binding of full-length bovine retinal Grb14 to IRβ. To confirm whether the generated mutant Y1146F is functional in the yeast two-hybrid assay, we have carried out interaction studies with the p85 subunit of PI3K whose binding site on IRβ is Y1322 (*26*). Under conditions where we do not observe the interaction of Y1146F with Grb14, we do observe the interaction of this mutant with the p85 (Figure 5B). However, substitution of Y1322F or K1018A in IRβ abolished p85 binding to the IRβ (Figure 5B). These results clearly demonstrate that the Y1146F mutation, while abolishing interaction with Grb14, does not affect the ability of the expressed IRβ to interact with a different binding partner. Wild-type retinal IRβ interacts with both full-length Grb14 and p85 (Figure 5B). These results suggest that Y1146 may be the binding site for Grb14 on IRβ.

Interaction of IRβ with PIR and PIR-SH2 Domains of Grb14. The cDNA encoding the PIR and PIR-SH2 domains was amplified by PCR from Grb14 cDNA, cloned into the plasmid pTrcHisA vector, and expressed in bacteria as His-tagged proteins that were purified by Ni^+-NTA column chromatography (data not shown). Purified PIR-SH2 and PIR fusion proteins were incubated with purified GST and GST-IRβ. Glutathione−Sepharose 4B matrix was added to the above mixture and subjected to GST pull-down assay followed by Western blotting analysis with anti-His antibody. GST protein failed to pull-down His-PIR-SH2 and His-PIR, while the GST-IRβ pulled down His-PIR-SH2 and His-PIR (Figure 6A). These results suggest a direct interaction between IRβ and Grb14 in the cell-free system.

Interaction of IRβ with the PIR-SH2 Domain of Grb14 in Retinal Organ Culture. Retinas were stimulated in culture with insulin (1 μM) at various time points (0, 2, 5, 15, 30, and 60 min). GST pull-down experiments were carried out employing GST-PIR-SH2 fusion proteins, and the bound proteins were subjected to Western blot analysis with anti-IRβ antibody. The GST-PIR-SH2 domain was capable of

bringing down IRβ from insulin-stimulated retinas (Figure 6B). These results further demonstrate the interaction of Grb14 with activated IRβ. The blot was stripped and reprobed with anti-GST antibody to examine the fusion protein in each lane.

Grb14 Enhances the Tyrosine Phosphorylation of ROS Proteins and the Insulin Receptor in Vitro. Increased phosphorylation of several ROS proteins was observed in the presence of Grb14 (Figure 7A). This increase is not due to increased protein loading as the arrestin blot shows an equal amount of protein in each lane (Figure 7B). Further, we have observed an increase in the insulin receptor phosphorylation in the presence of recombinant Grb14 (Figure 7C). The increase in ROS and insulin receptor phosphorylation could be due to the inhibition of protein tyrosine phosphatase activity. Therefore, we looked for the expression of PTP1b in ROS, and the results indicate the presence of PTP1b in the ROS (Figure 7D). Western blotting analysis of ROS proteins with anti-IRβ, anti-Grb14, anti-PTP1b, and anti-opsin (outer segment marker) indicates the presence of all of these proteins in ROS. These results suggest that Grb14, IRβ, and PTP1b are expressed in the same cells (Figure 7D).

The PH Domain Mediates Grb14 Interaction with Phosphoinositides. To evaluate the binding specificity of Grb14 PH domain to various phospholipids, we employed a protein−lipid overlay assay using membranes that had been spotted with an equal amount (100 pmol) of different phospholipids. The purified GST fusion proteins containing Grb14 or GST-PH or GST-PIR-SH2 were incubated with the membrane strips as described under Experimental Procedures. After washing, the bound GST fusion proteins were detected by Western blotting with anti-GST antibody. As expected, the GST and GST-PIR-SH2 domain did not bind to any of the phospholipids (Figure 7) while GST-Grb14 showed weak binding with various affinities to PtdIns(3,4)-P_2, PtdIns(3,5)P_2, PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, and phosphatidic acid. Similarly, the PH domain alone bound to these phosphatidylinositol phosphates, in addition to PtdIns-(4,5)P_2 and PtdIns(3,4,5)P_3, and exhibited a stronger binding than the full-length Grb14 (Figure 8). Together, these results indicate that Grb14 preferentially interacts with D3 and D5 phosphoinositides via its PH domain. These results also suggest that Grb14 is a phospholipid binding protein.

Association between Grb14 and Phosphoinositides in Vivo. To evaluate the in vivo association of the Grb14 PH domain to various phospholipids in retina, we employed retinal organ cultures that were either stimulated or nonstimulated with insulin. Retinas thus treated were lysed and subjected to immunoprecipitation with anti-PI-3,4,5-P_3, anti-PI-4,5-P_2, or anti-PI-3,4-P_2 followed by Western blotting analysis with anti-Grb14 antibody. Increased Grb14 was recovered from anti-PI-3,4,5-P_3 immunoprecipitates from insulin-stimulated retinas compared to non-insulin-stimulated conditions (Figure 9). Grb14 was also recovered from anti-PI-4,5-P_2 immunoprecipitates, although there was no difference between insulin-stimulated and nonstimulated conditions (Figure 9). Grb14 recovery was absent in anti-PI-3,4-P_2 immunoprecipitates (Figure 9).

DISCUSSION

Cells of retina contain high-affinity receptors for insulin (*1*, *33*−*35*). We have demonstrated that light stimulates tyrosine phosphorylation of the IRβ in vivo, which leads to the association of PI3K with the IRβ (*8*). The light effect was localized to photoreceptor neurons and is independent of insulin secretion (*8*). Ligand-independent activation of the insulin receptor has been reported previously (*36*−*39*). Two compounds capable of stimulating insulin receptor autophosphorylation by acting on the cytoplasmic domain have been reported (*36*, *37*). One of these (L783,281) modestly elevates insulin receptor autophosphorylation in the absence of insulin (*40*), whereas the other (TLK16988) potentiates receptor autophosphorylation in the presence of insulin, suggesting two different mechanisms of action (*39*). Structural and biochemical evidence for an autoinhibitory role for tyrosine 984 in the insulin receptor has been reported (*41*). Substitution of tyrosine 984 in the β sheet−αC cleft with alanine resulted in the increase of basal level of insulin receptor phosphorylation. On the basis of these results, it has been proposed that compounds that bind in the β sheet−αC cleft and displace tyrosine 984 should partially activate the insulin receptor (*41*). Further, regulation of insulin receptor kinase activity by phosphatidylinositol in the absence of insulin has also been reported (*38*). These studies suggest that the cytoplasmic domain of the insulin receptor can be autophosphorylated independent of insulin. To identify regulators of the insulin receptor, we used a yeast two-hybrid system to screen a bovine retinal cDNA library for binding partners to the cytoplasmic domain of the retinal insulin receptor and identified Grb14, a member of the Grb7 family (*23*).

Grb14 was originally cloned as a growth factor receptor-binding protein by interaction with EGFR, using the CORT techniques (cloning of receptor targets) (*23*). However, the association between EGFR and Grb14 could not be detected in intact mammalian cells, even upon transient co-overexpression. In vitro interaction observed between Grb14 and platelet-derived growth factor receptors (PDGFR) could not be confirmed in transformed cell lines either (*23*). Grb14 has been shown to interact with the insulin receptor, fibroblast growth factor, and Tek/Tie2 receptors (*22*, *24*, *25*). Grb14 binding to insulin and FGF receptors has been demonstrated in cell lines overexpressing the proteins, and the association is dependent on ligand stimulation (*24*, *25*).

We cloned the 41 kDa cytoplasmic region of the retinal insulin receptor (IRβ) and used the yeast two-hybrid assay of protein−protein interaction to further study the interaction between Grb14 and the cytoplasmic region of the retinal insulin receptor. Grb14 formed a specific complex with the cytoplasmic domain of the insulin receptor when both were expressed as hybrid proteins in yeast cells. This interaction is strictly dependent upon receptor tyrosine kinase activity since Grb14 shows no interaction with a kinase-inactive receptor hybrid containing a mutated ATP-binding site (K1018A). Deletion analysis on Grb14 indicated that the PIR domain in Grb14 is the minimum domain required for interaction with the retinal insulin receptor, in agreement with previous studies (*23*, *25*). Retinal Grb14 binds to the phosphorylated activation loop of the insulin receptor. Mutations of the tyrosyl residue of this loop (Y1146F, Y1150F, and Y1151F) completely block the interaction, and

this inhibition is quite evident from the in vitro IRS-1 peptide phosphorylation by the purified insulin receptor in the presence of the Grb14 PIR domain (*19*). Rat Grb14, as well as Grb10, binds to the phosphorylated activation loop of the insulin receptor, and mutation of the tyrosyl residues of this loop (Tyr1146, Tyr1150, and Tyr1151) decreases the interaction (*21*, *25*). It has been shown previously that substitution of Y1146F decreases the binding of either full-length Grb14 or the isolated PIR domain (*25*). In the present study we did not observe any interaction between bovine retinal Grb14 and Y1146F mutant IRβ. The observed differences could be species specific. Absence of interaction between bovine retinal Grb14 and the Y1150F and Y1151F mutants of IRβs is in agreement with the previously published studies on Grb14 and Grb10 (*21*, *25*). The results suggest that Y1146 may be the binding site for Grb14 on IRβ. Further studies, however, are needed to elucidate the binding site of Grb14 on IRβ.

It is interesting to note that PH-SH2 and PH-SH2 (R416A) failed to interact with IRβ in the yeast two-hybrid system. Our results clearly indicate that the lack of interaction was due to the mistargeting of protein due to the PH domain at the N-terminal end of the fusion and not to an expression problem since the expressed protein was found to interact with IRβ and phosphoinositides in vitro. This observation is supported by earlier studies showing that IRS proteins were never isolated in yeast two-hybrid assays as IRβ interacting proteins in which the PH domain is situated in the N-terminus of the protein (*42*). Grb7, Grb10, and Grb14 have been successfully identified using the yeast two-hybrid approach when the PH domain was situated in the middle region of the protein between amino acids 234 and 341 (*20*, *21*, *23*). In addition to the PH domain, Grb14 has a putative nuclear localization signal (NLS) between amino acids 63 and 68 (RRKKD), and substitution of R64G and K65E in Grb14 failed to localize the protein to the yeast nucleus as shown by the yeast one-hybrid nuclear import assay (Figure 2). Western blotting analysis of nuclear fractions isolated from rat retinas clearly indicates the presence of Grb14 in nuclear fractions, and the presence of the NLS signal in Grb14 could have some regulatory role. Our immunocytochemical studies also confirm the nuclear and perinuclear localization of Grb14 in the outer and inner nuclear layers of the retina. Grb14 has previously been shown to co-reside with tankyrase and tankyrase 2 in a subcellular fraction enriched in Golgi vesicles and endosomes in DU145 prostate carcinoma cells (*43*). A small fraction of the cellular tankyrase pool localizes to telomeres (*44*); the majority of tankyrase resides in the cytoplasm in a perinuclear localization (*45*, *46*). Further studies, however, are required to examine whether Grb14 interacts with tankyrase in the retinal cells. Recently, we have identified a NPXY motif in Grb14 and also demonstrated its interaction with the phosphotyrosine binding (PTB) domain of IRS-1 in a phosphorylation-independent manner (*19*). IRS-1 is known to translocate to the nucleus in response to vsrc and IGF-1 stimulus and plays a specialized role in ribosomal RNA synthesis and/or processing (*47*). This nuclear translocation is inhibited by deletion of the PTB domain of IRS-1 (*47*). We and others have shown that, in the retina, insulin failed to induce the phosphorylation of IRS-1 (*26*, *48*). It is tempting to speculate that Grb14 may also regulate the translocation of IRS-1 to the nucleus in the retina, and such a possibility cannot be ruled out.

We have immunolocalized the expression of Grb14 to rod photoreceptors and Muller cells and to some extent in other retinal cell layers using custom-made polyclonal Grb14 antibodies. Previously, we have reported the immunolocalization of the insulin receptor in the outer and inner segments of photoreceptor cells using polyclonal IRβ antibodies (*34*). Our studies also indicate the expression of Grb14, IRβ, and PTP1b in ROS. For IR-Grb14 colocalization studies we have tried several combinations of commercially available antibodies (monoclonal versus polyclonal); however, we failed to observe reproducible results. Results described in this report on the immunolocalization of Grb14 in the ROS and our previous studies on the immuolocalization of the insulin receptor in ROS (*8;34*) may at least suggest that they are present in the same cells.

Several studies have shown that Grb14 may not directly associate with IRβ but could indirectly regulate either IRβ or downstream targets of IRβ. Consistent with this hypothesis, Grb14 has recently been shown to regulate the localization of 3-phosphoinositide-dependent kinase 1 (*49*). In the present study Grb14 enhances the tyrosine phosphorylation of the insulin receptor, and this increase could be due to the inhibition of a protein tyrosine phosphatase. Consistent with this hypothesis, PTP1b is expressed in ROS. Bereziat et al. (*50*) reported that Grb14 overexpression reduces the association of the insulin receptor and PTP1b in vivo and that addition of recombinant Grb14 reduces dephosphorylation of the insulin receptor by this enzyme in vitro. Further, ablation of Grb14 is shown to result in reduced insulin-stimulated insulin receptor tyrosine phosphorylation in the liver due to the activation of PTP1b (*51*). These studies suggest that Grb14 may regulate the insulin receptor pathway in a tissue-specific manner.

The PH domain is a conserved protein module in many signaling and cytoskeletal proteins, which mediates interaction with various phosphoinositides and in some cases other proteins in signal transduction (*52*−*55*). We showed here that Grb14 can interact with phosphatidylinositol phosphates through its PH domain. Interaction of the Grb7 PH domain with phosphoinositides (*56*, *57*) regulates cell migration (*57*). Interaction of phospholipids with PH domain-containing proteins has been suggested to facilitate protein localization to the plasma membrane and/or induce conformational changes of the target proteins (*58*, *59*). Analysis using protein−lipid overlay assays indicated binding of Grb14 and its PH domain to D3 phosphoinositides. Our studies also indicate under insulin stimulated conditions that retinal Grb14 associates with PI-3,4,5-P_3, whereas association between Grb14 and PI-4,5-P_2 is insulin-independent. This differential binding could be due to changes in the relative amounts of phosphoinositides in the retina and suggests that there could be more PI-4,5-P_2 than PI-3,4,5-P_3 under nonstimulated conditions. Previous studies using radiolabeled inositol have shown that the enzymes for PI-4,5-P_2 synthesis are present in bovine ROS (*60*). Further, light adaptation of bovine retinas in situ stimulates phosphatidylinositol (PI) synthesis in rod outer segments (*60*). These studies indicate an active PI cycle in rod outer segments that can provide substrate PI-4,5-P_2 for phosphorylation by PI3K. Further, our studies also indicate the rod outer segment localization of Grb14. All of these observations suggest that Grb14 could also be

light-activated. Phosphatidylinositol is known to activate the insulin receptor (*38*), and binding of Grb14 to phosphoinositides may suggest a role for Grb14 in modulating the phospholipid levels in the regulation of insulin receptor signaling. Studies are underway in our laboratory to test this hypothesis.

ACKNOWLEDGMENT

The authors acknowledge Dr. Robert E. Anderson for support and valuable suggestions in this study. We thank Dr. Michael H. Elliott for reading the manuscript and for helpful suggestions. The technical assistance of Mr. Mark E. McClellan is highly appreciated. We thank Mr. Todd Wuest for help in confocal imaging.

REFERENCES

1. Havrankova, J., Roth, J., and Brownstein, M. (1978) Insulin receptors are widely distributed in the central nervous system of the rat, *Nature 272*, 827−829.
2. Baskin, D. G., Figlewicz, L. D., Seeley, R. J., Woods, S. C., Porte, D., Jr., and Schwartz, M. W. (1999) Insulin and leptin: dual adiposity signals to the brain for the regulation of food intake and body weight, *Brain Res. 848*, 114−123.
3. Schwartz, M. W., Sipols, A. J., Marks, J. L., Sanacora, G., White, J. D., Scheurink, A., Kahn, S. E., Baskin, D. G., Woods, S. C., Figlewicz, D. P., and. (1992) Inhibition of hypothalamic neuropeptide Y gene expression by insulin, *Endocrinology 130*, 3608−3616.
4. Heidenreich, K. A. (1993) Insulin and IGF-I receptor signaling in cultured neurons, *Ann. N.Y. Acad. Sci 692*, 72−88.
5. Robinson, L. J., Leitner, W., Draznin, B., and Heidenreich, K. A. (1994) Evidence that p21ras mediates the neurotrophic effects of insulin and insulin-like growth factor I in chick forebrain neurons, *Endocrinology 135*, 2568−2573.
6. Takahashi, M., Yamada, T., Tooyama, I., Moroo, I., Kimura, H., Yamamoto, T., and Okada, H. (1996) Insulin receptor mRNA in the substantia nigra in Parkinson's disease, *Neurosci. Lett. 204*, 201−204.
7. Frolich, L., Blum-Degen, D., Bernstein, H. G., Engelsberger, S., Humrich, J., Laufer, S., Muschner, D., Thalheimer, A., Turk, A., Hoyer, S., Zochling, R., Boissl, K. W., Jellinger, K., and Riederer, P. (1998) Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease, *J. Neural Transm. 105*, 423−438.
8. Rajala, R. V., McClellan, M. E., Ash, J. D., and Anderson, R. E. (2002) In vivo regulation of phosphoinositide 3-kinase in retina through light-induced tyrosine phosphorylation of the insulin receptor beta-subunit, *J. Biol. Chem. 277*, 43319−43326.
9. Song, J., Wu, L., Chen, Z., Kohanski, R. A., and Pick, L. (2003) Axons guided by insulin receptor in *Drosophila* visual system, *Science 300*, 502−505.
10. Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., and Hafen, E. (2001) An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control, *Curr. Biol. 11*, 213−221.
11. Wolkow, C. A., Kimura, K. D., Lee, M. S., and Ruvkun, G. (2000) Regulation of *C. elegans* life-span by insulinlike signaling in the nervous system, *Science 290*, 147−150.
12. Steen, E., Terry, B. M., Rivera, E. J., Cannon, J. L., Neely, T. R., Tavares, R., Xu, X. J., Wands, J. R., and de la Monte, S. M. (2005) Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease—is this type 3 diabetes?, *J. Alzheimer's Dis. 7*, 63−80.
13. de la Monte, S. M., and Wands, J. R. (2005) Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease, *J. Alzheimer's Dis. 7*, 45−61.
14. de la Monte, S. M., and Wands, J. R. (2004) Alzheimer-associated neuronal thread protein mediated cell death is linked to impaired insulin signaling, *J. Alzheimer's Dis. 6*, 231−242.
15. Schubert, M., Gautam, D., Surjo, D., Ueki, K., Baudler, S., Schubert, D., Kondo, T., Alber, J., Galldiks, N., Kustermann, E., Arndt, S., Jacobs, A. H., Krone, W., Kahn, C. R., and Bruning, J. C. (2004) Role for neuronal insulin resistance in neurodegenerative diseases, *Proc. Natl. Acad. Sci. U.S.A. 101*, 3100−3105.
16. Garrity, P. A., Rao, Y., Salecker, I., McGlade, J., Pawson, T., and Zipursky, S. L. (1996) *Drosophila* photoreceptor axon guidance and targeting requires the dreadlocks SH2/SH3 adapter protein, *Cell 85*, 639−650.
17. Yi, X., Schubert, M., Peachey, N. S., Suzuma, K., Burks, D. J., Kushner, J. A., Suzuma, I., Cahill, C., Flint, C. L., Dow, M. A., Leshan, R. L., King, G. L., and White, M. F. (2005) Insulin receptor substrate 2 is essential for maturation and survival of photoreceptor cells, *J. Neurosci. 25*, 1240−1248.
18. Barber, A. J., Nakamura, M., Wolpert, E. B., Reiter, C. E., Seigel, G. M., Antonetti, D. A., and Gardner, T. W. (2001) Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol 3-kinase/Akt-mediated mechanism that reduces the activation of caspase-3, *J. Biol. Chem. 276*, 32814−32821.
19. Rajala, R. V., and Chan, M. D. (2005) Identification of a NPXY motif in growth factor receptor-bound protein 14 (Grb14) and its interaction with the phosphotyrosine-binding (PTB) domain of IRS-1, *Biochemistry 44*, 7929−7935.
20. Han, D. C., Shen, T. L., and Guan, J. L. (2001) The Grb7 family proteins: structure, interactions with other signaling molecules and potential cellular functions, *Oncogene 20*, 6315−6321.
21. He, W., Rose, D. W., Olefsky, J. M., and Gustafson, T. A. (1998) Grb10 interacts differentially with the insulin receptor, insulin-like growth factor I receptor, and epidermal growth factor receptor via the Grb10 Src homology 2 (SH2) domain and a second novel domain located between the pleckstrin homology and SH2 domains, *J. Biol. Chem. 273*, 6860−6867.
22. Jones, N., Master, Z., Jones, J., Bouchard, D., Gunji, Y., Sasaki, H., Daly, R., Alitalo, K., and Dumont, D. J. (1999) Identification of Tek/Tie2 binding partners. Binding to a multifunctional docking site mediates cell survival and migration, *J. Biol. Chem. 274*, 30896−30905.
23. Daly, R. J., Sanderson, G. M., Janes, P. W., and Sutherland, R. L. (1996) Cloning and characterization of GRB14, a novel member of the GRB7 gene family, *J. Biol. Chem. 271*, 12502−12510.
24. Reilly, J. F., Mickey, G., and Maher, P. A. (2000) Association of fibroblast growth factor receptor 1 with the adaptor protein Grb14. Characterization of a new receptor binding partner, *J. Biol. Chem. 275*, 7771−7778.
25. Kasus-Jacobi, A., Perdereau, D., Auzan, C., Clauser, E., Van Obberghen, E., Mauvais-Jarvis, F., Girard, J., and Burnol, A. F. (1998) Identification of the rat adapter Grb14 as an inhibitor of insulin actions, *J. Biol. Chem. 273*, 26026−26035.
26. Rajala, R. V., McClellan, M. E., Chan, M. D., Tsiokas, L., and Anderson, R. E. (2004) Interaction of the retinal insulin receptor beta-subunit with the p85 subunit of phosphoinositide 3-kinase, *Biochemistry 43*, 5637−5650.
27. Rhee, Y., Gurel, F., Gafni, Y., Dingwall, C., and Citovsky, V. (2000) A genetic system for detection of protein nuclear import and export, *Nat. Biotechnol. 18*, 433−437.
28. Breeden, L., and Nasmyth, K. (1985) Regulation of the yeast HO gene, *Cold Spring Harbor Symp. Quant. Biol. 50*, 643−650.
29. Qi, Q., Rajala, R. V., Anderson, W., Jiang, C., Rozwadowski, K., Selvaraj, G., Sharma, R., and Datla, R. (2000) Molecular cloning, genomic organization, and biochemical characterization of myristoyl-CoA:protein N-myristoyltransferase from *Arabidopsis thaliana*, *J. Biol. Chem. 275*, 9673−9683.
30. Bell, M. W., Desai, N., Guo, X. X., and Ghalayini, A. J. (2000) Tyrosine phosphorylation of the alpha subunit of transducin and its association with Src in photoreceptor rod outer segments, *J. Neurochem. 75*, 2006−2019.
31. Bell, M. W., Alvarez, K., and Ghalayini, A. J. (1999) Association of the tyrosine phosphatase SHP-2 with transducin-alpha and a 97-kDa tyrosine-phosphorylated protein in photoreceptor rod outer segments, *J. Neurochem. 73*, 2331−2340.
32. Rajala, R. V., and Anderson, R. E. (2001) Interaction of the insulin receptor beta-subunit with phosphatidylinositol 3-kinase in bovine ROS, *Invest. Ophthalmol. Visual Sci. 42*, 3110−3117.
33. Waldbillig, R. J., Fletcher, R. T., Chader, G. J., Rajagopalan, S., Rodrigues, M., and LeRoith, D. (1987) Retinal insulin receptors. 1. Structural heterogeneity and functional characterization, *Exp. Eye Res. 45*, 823−835.
34. Yu, X., Rajala, R. V., McGinnis, J. F., Li, F., Anderson, R. E., Yan, X., Li, S., Elias, R. V., Knapp, R. R., Zhou, X., and Cao, W. (2004) Involvement of insulin/phosphoinositide 3-kinase/Akt signal pathway in 17 beta-estradiol-mediated neuroprotection, *J. Biol. Chem. 279*, 13086−13094.

35. Reiter, C. E., and Gardner, T. W. (2003) Functions of insulin and insulin receptor signaling in retina: possible implications for diabetic retinopathy, *Prog. Retinal Eye Res. 22*, 545−562.
36. Zhang, B., Salituro, G., Szalkowski, D., Li, Z., Zhang, Y., Royo, I., Vilella, D., Diez, M. T., Pelaez, F., Ruby, C., Kendall, R. L., Mao, X., Griffin, P., Calaycay, J., Zierath, J. R., Heck, J. V., Smith, R. G., and Moller, D. E. (1999) Discovery of a small molecule insulin mimetic with antidiabetic activity in mice, *Science 284*, 974−977.
37. Manchem, V. P., Goldfine, I. D., Kohanski, R. A., Cristobal, C. P., Lum, R. T., Schow, S. R., Shi, S., Spevak, W. R., Laborde, E., Toavs, D. K., Villar, H. O., Wick, M. M., and Kozlowski, M. R. (2001) A novel small molecule that directly sensitizes the insulin receptor in vitro and in vivo, *Diabetes 50*, 824−830.
38. Sweet, L. J., Dudley, D. T., Pessin, J. E., and Spector, A. A. (1987) Phospholipid activation of the insulin receptor kinase: regulation by phosphatidylinositol, *FASEB J. 1*, 55−59.
39. Qureshi, S. A., Ding, V., Li, Z., Szalkowski, D., Biazzo-Ashnault, D. E., Xie, D., Saperstein, R., Brady, E., Huskey, S., Shen, X., Liu, K., Xu, L., Salituro, G. M., Heck, J. V., Moller, D. E., Jones, A. B., and Zhang, B. B. (2000) Activation of insulin signal transduction pathway and anti-diabetic activity of small molecule insulin receptor activators, *J. Biol. Chem. 275*, 36590−36595.
40. Li, M., Youngren, J. F., Manchem, V. P., Kozlowski, M., Zhang, B. B., Maddux, B. A., and Goldfine, I. D. (2001) Small molecule insulin receptor activators potentiate insulin action in insulin-resistant cells, *Diabetes 50*, 2323−2328.
41. Li, S., Covino, N. D., Stein, E. G., Till, J. H., and Hubbard, S. R. (2003) Structural and biochemical evidence for an autoinhibitory role for tyrosine 984 in the juxtamembrane region of the insulin receptor, *J. Biol. Chem. 278*, 26007−26014.
42. White, M. F. (1998) The IRS-signalling system: a network of docking proteins that mediate insulin action, *Mol. Cell. Biochem. 182*, 3−11.
43. Lyons, R. J., Deane, R., Lynch, D. K., Ye, Z. S., Sanderson, G. M., Eyre, H. J., Sutherland, G. R., and Daly, R. J. (2001) Identification of a novel human tankyrase through its interaction with the adaptor protein Grb14, *J. Biol. Chem. 276*, 17172−17180.
44. Smith, S., Giriat, I., Schmitt, A., and de Lange, T. (1998) Tankyrase, a poly(ADP-ribose) polymerase at human telomeres, *Science 282*, 1484−1487.
45. Chi, N. W., and Lodish, H. F. (2000) Tankyrase is a Golgi-associated mitogen-activated protein kinase substrate that interacts with IRAP in GLUT4 vesicles, *J. Biol. Chem. 275*, 38437−38444.
46. Smith, S., and de Lange, T. (1999) Cell cycle dependent localization of the telomeric PARP, tankyrase, to nuclear pore complexes and centrosomes, *J. Cell Sci. 112* (Part 21), 3649−3656.
47. Tu, X., Batta, P., Innocent, N., Prisco, M., Casaburi, I., Belletti, B., and Baserga, R. (2002) Nuclear translocation of insulin receptor substrate-1 by oncogenes and Igf-I. Effect on ribosomal RNA synthesis, *J. Biol. Chem. 277*, 44357−44365.
48. Reiter, C. E., Sandirasegarane, L., Wolpert, E. B., Klinger, M., Simpson, I. A., Barber, A. J., Antonetti, D. A., Kester, M., and Gardner, T. W. (2003) Characterization of insulin signaling in rat retina in vivo and ex vivo, *Am. J. Physiol. Endocrinol. Metab. 285*, E763−E774.
49. King, C. C., and Newton, A. C. (2004) The adaptor protein Grb14 regulates the localization of 3-phosphoinositide-dependent kinase-1, *J. Biol. Chem. 279*, 37518−37527.
50. Bereziat, V., Kasus-Jacobi, A., Perdereau, D., Cariou, B., Girard, J., and Burnol, A. F. (2002) Inhibition of insulin receptor catalytic activity by the molecular adapter Grb14, *J. Biol. Chem. 277*, 4845−4852.
51. Cooney, G. J., Lyons, R. J., Crew, A. J., Jensen, T. E., Molero, J. C., Mitchell, C. J., Biden, T. J., Ormandy, C. J., James, D. E., and Daly, R. J. (2004) Improved glucose homeostasis and enhanced insulin signalling in Grb14-deficient mice, *EMBO J. 23*, 582−593.
52. Kavran, J. M., Klein, D. E., Lee, A., Falasca, M., Isakoff, S. J., Skolnik, E. Y., and Lemmon, M. A. (1998) Specificity and promiscuity in phosphoinositide binding by pleckstrin homology domains, *J. Biol. Chem. 273*, 30497−30508.
53. Tsukada, S., Simon, M. I., Witte, O. N., and Katz, A. (1994) Binding of beta gamma subunits of heterotrimeric G proteins to the PH domain of Bruton tyrosine kinase, *Proc. Natl. Acad. Sci. U.S.A. 91*, 11256−11260.
54. Yao, L., Kawakami, Y., and Kawakami, T. (1994) The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C, *Proc. Natl. Acad. Sci. U.S.A. 91*, 9175−9179.
55. Yao, L., Janmey, P., Frigeri, L. G., Han, W., Fujita, J., Kawakami, Y., Apgar, J. R., and Kawakami, T. (1999) Pleckstrin homology domains interact with filamentous actin, *J. Biol. Chem. 274*, 19752−19761.
56. Li, H., Sanchez-Torres, J., Del Carpio, A. F., Nogales-Gonzalez, A., Molina-Ortiz, P., Moreno, M. J., Torok, K., and Villalobo, A. (2005) The adaptor Grb7 is a novel calmodulin-binding protein: functional implications of the interaction of calmodulin with Grb7, *Oncogene 24*, 4206−4219.
57. Shen, T. L., Han, D. C., and Guan, J. L. (2002) Association of Grb7 with phosphoinositides and its role in the regulation of cell migration, *J. Biol. Chem. 277*, 29069−29077.
58. Lemmon, M. A., and Ferguson, K. M. (2000) Signal-dependent membrane targeting by pleckstrin homology (PH) domains, *Biochem. J. 350* (Part 1), 1−18.
59. Toker, A., and Newton, A. C. (2000) Cellular signaling: pivoting around PDK-1, *Cell 103*, 185−188.
60. Ghalayini, A. J., and Anderson, R. E. (1995) Light adaptation of bovine retinas in situ stimulates phosphatidylinositol synthesis in rod outer segments in vitro, *Curr. Eye Res. 14*, 1025−1029.

BI0513148