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Articles

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[†]

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

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

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ABSTRACT: Recently we have shown that insulin fails to induce the phosphorylation of IRS-1 in the retina [Rajala et al. (2004) *Biochemistry 43*, 5637−5650], even though there is widespread expression of IRS-1 throughout the retina. These results suggest the expression of tissue-specific regulators in the retina. Yeast two-hybrid screening of a bovine retinal cDNA library with the cytoplasmic domain of retinal insulin receptor identified a novel member of the Grb7 gene family, Grb14. Phosphorylation prediction software indicated 6 out of 18 tyrosine residues were most likely to be phosphorylated. Out of six tyrosine phosphorylation sites, one of the tyrosine residues in Grb14 is present in a conserved sequence motif, FXNPXY. The NPXY motifs are recognized by proteins containing a domain known as phosphotyrosine-binding (PTB) or phosphotyrosine-interacting domain (PID). The biological function of the PTB domain is to drive recruitment of signaling adapters such as IRS-1 or Shc to NPXpY (pY stands for phosphotyrosine) on activated receptor tyrosine kinases. We have made a novel finding that the PTB domain of IRS-1 binds to the NPXY motif of Grb14 in a phosphorylation-independent manner. In addition, Grb14−IRS-1 complexes are detected in lysates prepared from retina tissues. We suggest that the Grb14 NPXY motif could be acting as a dominant negative for IRS-1 functions in the retina, and this hypothesis is consistent with the recent study that Grb14-deficient mice exhibit enhanced IRS-1 phosphorylation and activation of protein kinase B. This is the first report describing the presence of the NPXY motif in Grb14 and binding of the PTB domain of IRS-1 in a phosphorylation-independent manner.

Cells of bovine and rat retina contain high-affinity receptors for insulin (*1*−*4*). However, little research has been done on these receptors since these early reports probably due the absence of an identified intracellular target. We have demonstrated that light stimulates tyrosine phosphorylation of the β-subunit of insulin receptor (IRβ)[1] in vivo, which

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* To whom correspondence should be addressed. Raju V. S. Rajala Ph.D., 608 Stanton L. Young Blvd., Room #409, Oklahoma City, OK 73104. Telephone: 405-271-8255. Fax: 405-271-8128. E-mail: rajurajala@ouhsc.edu.

[‡] Department of Cell Biology.

[§] Department of Ophthalmology.

[1] Abbreviations: Grb14, growth factor receptor-bound protein 14; PTB, phosphotyrosine-binding; PID, phosphotyrosine-interacting domain; IRS-1, insulin receptor substrate-1; pY, phosphotyrosine; Dab, disabled; PI3K, phosphoinositide 3-kinase; GST, glutathione *S*-transferase; IRβ, insulin receptor beta subunit; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDM, site-directed mutagenesis.

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leads to the direct association of phosphoinositide 3-kinase (PI3K), an antiapoptotic enzyme activity with the IRβ (*5*).

There is widespread expression of IRS-1 throughout the retina, and IRS-1 is found in regions of the retina abundant in insulin receptors, including outer segments (*6*). We (*7*) and others (*8*) have shown independently that insulin treatment fails to result in phosphorylation of IRS-1, whereas IRS-2 interacts with the p85 subunit of PI3K in response to insulin (*7*). Also, insulin-induced activation of Akt was completely inhibited by the PI3K inhibitor LY294002, suggesting that insulin receptor/PI3K/Akt signaling is operative in the retina independent of IRS-1, but dependent on IRS-2. (*7*). Inactivation of the IRS-1 gene in the mouse by a homologous recombination approach did not result in any dramatic pathologic phenotype, suggesting the possible existence of alternative signaling pathways (*9*, *10*). However, it has been shown recently that IRS-2 knockout mice lose up to 50% of photoreceptors by 2 weeks of age, owing to increased apoptosis (*11*). These studies clearly suggest a tissue-specific regulation of IRS proteins in the retina.

To further explore these possibilities, the cytoplasmic domain of 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). Grb14 is a member of an emerging family of noncatalytic adapter proteins that also includes Grb7 and Grb10 (*12*, *13*). Characteristic features of Grb7 family members include a central pleckstrin homology (PH) domain, a C-terminal Src homology 2 (SH2) domain, and an N-terminal proline-rich motif, suggesting a putative SH3 binding site. The presence of these functional domains indicates that Grb7 family members have the potential to interact with a variety of proteins. Grb14 has been shown to bind to Tek/Tie2, PDGF, EGFR, FGFR, and IR (*14*−*17*). Insulin-stimulated tyrosine phosphorylation of IRS-1, Shc, and Dok has shown to be decreased in Grb14-overexpressing cells (*17*, *18*). The molecular mechanism behind the decrease in the phosphorylation of IRS-1, Shc, and Dok is not known. In proteins such as IRS-1 and Shc, the PTB domain binds in a phosphotyrosine-dependent fashion to growth factor receptors (*19*). The present study describes a novel feature of Grb14, the presence of a NPXY motif, and demonstrates its interaction with the PTB domain of IRS-1.

EXPERIMENTAL PROCEDURES

Materials. Polyclonal anti-IRS-1 antibody was obtained from Upstate, Inc. (Lake Placid, NY). Polyclonal anti-IRβ antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-His antibody was obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-glutathione *S*-transferase (GST) antibody and glutathione-Sepharose 4B matrix were obtained from Amersham Biosciences Corp. (Piscataway, NJ). Ni-NTA Superflow resin was obtained from Qiagen, Inc. (Valencia, CA). Human recombinant-insulin receptor kinase-GST fusion was obtained from Calbiochem (San Diego, CA). We have generated a polyclonal anti-peptide antibody to the amino terminal end of bovine Grb14 (NH2-(GC) TRGCAADRRKKKDLDVLE-COOH 57−74). All other reagents were of analytical grade from Sigma (St. Louis, MO).

Plasmid Constructs. Cloning of retinal insulin receptor cytoplasmic domain was described earlier (*7*). The cDNA encoding the cytoplasmic domain of rat insulin receptor (amino acids 941−1343) was cloned into a pLexA yeast two-hybrid vector. The cDNA fragments encoding amino acids 144−316 of IRS-1 (sense, cgcggatccgcgacttgagctatgacacgggc; antisense, ccggaattctggttcccacccaccatact) and amino acids 591−786 (sense, cgcggatccgcctagatgaatacactctc; antisense, ccggaattcgaggccttcactgcctcc) from IRS-2 were amplified from first strand cDNA reverse-transcribed from rat (IRS-1) and mouse (IRS-2) retinal RNA. The PIR-SH2 domain (amino acids 342−540) was amplified from Grb14 cDNA (sense, tggatcccatatggcatgcagctgtaccag; antisense, gtcgacctaaagtgcaatcctagc). The PCR products were verified by DNA sequencing, digested with BamHI and EcoRI, and cloned into either pTrcHisC (IRS-1) (Invitrogen Life Technologies, Carlsbad, CA) or pGEX-3X (IRS-1 and IRS-2) vectors. The cloning of the GST-p85 N-SH2 (amino acids 314−446) vector was described previously (*20*). The PIR-SH2 domain was cloned into the pGEX-4T-2 vector (Amersham Biosciences Corp. (Piscataway, NJ).

The 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 (*7*) against a bovine retinal cDNA library (3.6×10^6) cloned in frame fusion with the *GAL*4 activation domain in the pGAD10. The bovine retinal cDNA library was kindly provided by Dr. Wolfgang Baehr, University of Utah, Salt Lake City, UT. After transformation by the lithium acetate procedure (*7*), yeast were plated on a tryptophan-leucine-histidine-deficient medium plus 5 mM aminotriazole. Colonies growing in the absence of histidine were subsequently tested for β-galactosidase activity (*7*). The plasmids of the library producing yeast colonies of a $His^+/LacZ^+$ phenotype were isolated, and the cDNA inserts of these positive plasmids were sequenced.

Grb14 NPXY (199−202) Motif Cloning and Phosphorylation. The Grb14 NPXY (amino acids 194−233) motif was amplified from Grb14 cDNA employing the following primers (sense, 5′-gga tcc gag ttc ttt aaa aac cca atg; antisense, 3′-gaa ttc tat gtg ctt gaa ctt aga aac. All PCR products were sequenced, and the insert was subcloned into the bacterial expression plasmid pGEX-2TK. To investigate the role of phosphorylation in the binding between the NPXY motif of Grb14 and the PTB domain of IRS-1, we made use of a system in which mammalian polypeptides are expressed and inducibly phosphorylated in bacteria (*20*). The pGEX-Grb14 NPXY plasmid was co-transformed with plasmid-encoding inducible tyrosine kinase (TK) gene, *elk* tyrosine kinase, according to the method described earlier (*20*).

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out according to the method described earlier (*7*). The primers used in the site-directed mutagenesis are as follows: Grb14 NPXY-F197S (sense, gagttctctaaaaacccaatgtat; antisense, atacattgggtttttagagaactc); Grb14 NPXY-Y202F (sense, aaaaacccaatgttttttttcca; antisense, tggaaaaaaaaacattgggttttt). The cDNAs of all mutants were sequenced after PCR, and the NPXY mutants were excised from the sequencing vector as BamHI/EcoRI fragments and cloned into the pGEX-2TK vector. The proteins were expressed in bacteria after inducing with 0.1 mM IPTG.

Tyrosine Kinase Assay. A GST-fusion protein corresponding in sequence to residues R657−L760 of IRS-1 (*7*) was used as a substrate for insulin receptor kinase. This fragment contains potential phosphorylation sites, which are the in vivo binding sites for p85 following tyrosine phosphorylation (*21*). The phosphorylation reaction was performed essentially as described (*20*) in a total volume of 25 μL of 50 mM Tris-HCl buffer (pH 7.0), 50 mM $MgCl_2$, 5mM $MnCl_2$, 50 mM Na_3VO_4, 7 μg/mL *p*-nitrophenyl phosphate, and insulin receptor cytoplasmic domain (1 μg). The reaction was initiated by adding 2.5 μL [γ^{32}P]ATP to reach a final concentration of 200 μM, and the reaction was terminated by adding 10 μL of 50% (v/v) acetic acid. Twenty-five microliters of assay mixture was spotted onto phosphocellulose filter paper disks (1.5 cm × 1.5 cm), which were immersed in a solution containing 0.75% phosphoric acid (v/v). The filter paper disks containing the bound phosphorylated peptide were washed three times with phosphoric acid and rinsed in acetone. Radioactivity was quantified in 5 mL of liquid scintillation cocktail and counted (Ready Safe Liquid Scintillation Cocktail and Liquid Scintillation Counter, Beckman, Fullerton, CA).

SDS−PAGE and Western Blot Analysis. Proteins were resolved by SDS−PAGE and transferred onto nitrocellulose membranes, and the blots were washed two times for 10 min with TTBS (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1% Tween-20) and blocked with either 10% bovine serum albumin or nonfat dry milk powder (Bio-Rad) in TTBS overnight at 4 °C. Blots were then incubated with anti-His (1:1000), anti-PY (1:1000), anti-Grb14 (1:500), or anti-GST (1:5000) antibodies overnight at 4 °C. Following primary antibody incubations, immunoblots were incubated with HRP-linked secondary antibodies (either anti-rabbit, anti-mouse or anti-goat) and developed by ECL according to the manufacturer's instructions.

RESULTS

Insulin Receptor Pull-Down by GST-IRS-1, GST-IRS-2 and GST-p85 N-SH2 Domains. IRS-1 (PTB domain), IRS-2 (novel domain) (*22*), and p85 (N-SH2 domain) were cloned and expressed as GST fusion proteins. All these domains were capable of bringing down insulin receptor from insulin-stimulated retina lysates (Figure 1). These results suggest that these domains could recognize the specific phosphorylation sites on the insulin receptor. The absence of IRS-1 phosphorylation in response to insulin treatment (*7*, *8*) is not due to the lack of Y960 phosphorylation in the insulin receptor as GST-IRS-1 brings down the insulin receptor in a phosphorylation-dependent manner (Figure 1).

Identification of Grb14. Bovine retinal cDNA library 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 have isolated 60 *HIS3* and LacZ positive clones, and the majority of the clones represent the protein Grb14. The Genbank accession number for retinal Grb14 is AY772475.

Grb14 Is an Inhibitor of Insulin Receptor Kinase Activity. Phosphorylation of the IRS-1 peptide resulted in the incorporation of about 0.4 pmol phosphate/mol of IRS-1 peptide at 60 min and did not reach plateau level after 60 min (Figure 2parts A and B). This stoichiometry was inhibited approximately 8-fold (0.05 pmol/mol of peptide) in the presence of the PIR-SH2 domain (Figure 2 A.B). Similar inhibition of IRS-1 peptide phosphorylation was observed with the PIR domain alone (data not shown). These results suggest that the PIR domain of Grb14 inhibits the insulin receptor kinase activity. However, this experiment does not address whether Grb14 contains inhibitory or phosphatase activity.

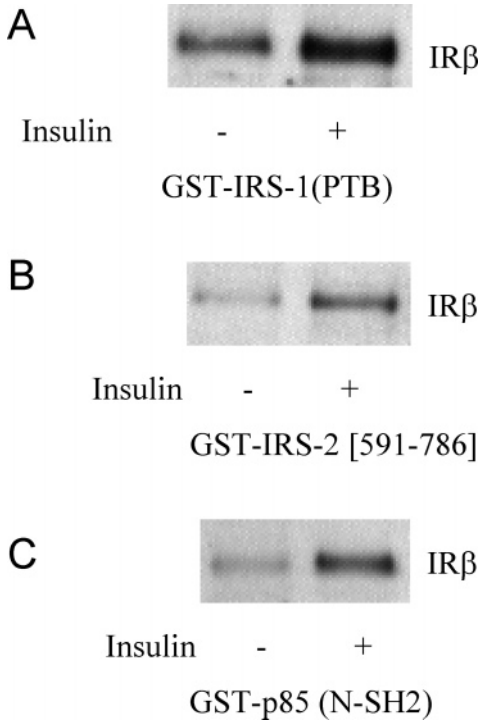

FIGURE 1: Characterization of domains capable of interacting with specific phosphorylation sites in insulin receptor. Rat retinas were stimulated in culture with insulin followed by GST pull-down assays with IRS-1 (PTB), IRS-2 (novel domain), and p85 (N-SH2 domain). The bound proteins were subjected to Western blot analysis with anti-IRβ antibody.

Lack of Phosphatase Activity Associated with Grb14. Insulin receptor kinase assays were carried out with insulin receptor cytoplasmic domain employing IRS-1 peptide as substrate. The experiment was carried out in duplicate, and at indicated time points, aliquots were removed and the radioactivity in the substrate peptide was measured. After 20 min, one tube was supplemented with GST and the other with GST-PIR-SH2, and the reaction continued up to 60 min. The PIR-SH2 domain blocks the incorporation of radioactivity in the IRS-1 peptide from 20−60 min (Figure 2C). These results suggest that the PIR-SH2 domain of Grb14 does not contain phosphatase activity; otherwise, we would expect to see a decrease in the incorporated radioactivity after 20 min.

Tyrosine Phosphorylation Sites on Grb14. The phosphorylation site prediction program (*23*) (http://www.cbs.dtu/services/NetPhos/) was used to indicate which tyrosine residues within the retinal Grb14 sequence are most likely to be phosphorylated. This program examines the sequence context of each tyrosine residue (i.e., the four residues before and after each tyrosine) and assigns an output score between 0 and 1. Scores above 0.5 are possible phosphorylation sites, and the higher the score, the more likely a particular site will be phosphorylated. This approach predicts phosphorylation sites in independent sequences with a sensitivity of

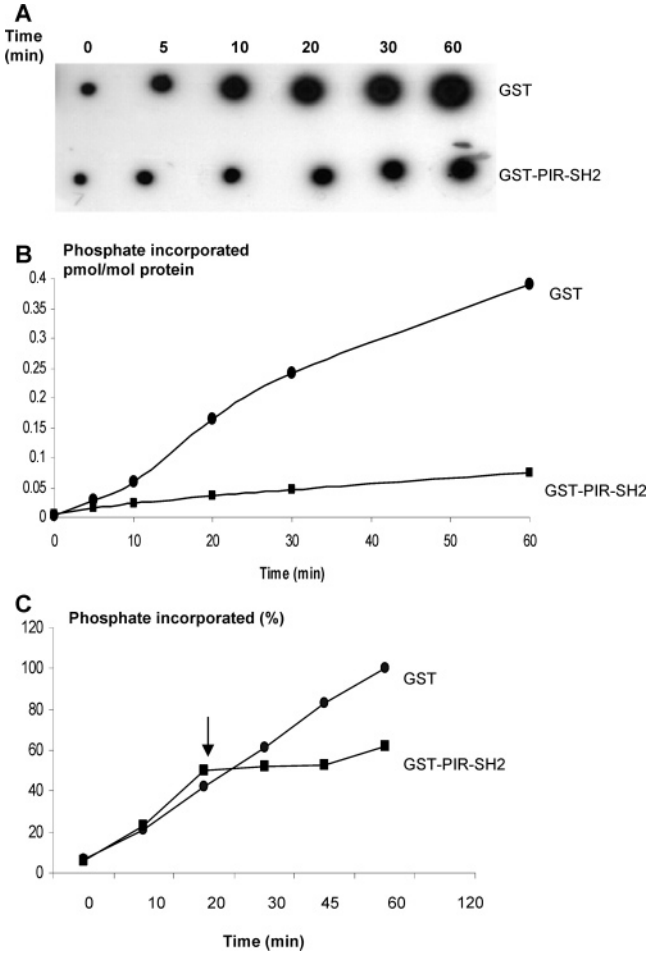

FIGURE 2: Time course of phosphorylation of IRS-1 peptide by insulin receptor in the presence and absence of Grb14 in vitro. IRS-1 peptide phosphorylation was carried out by IRβ in the presence of either GST or GST-PIR-SH2 domain of Grb14, and the reaction was initiated by the addition of [γ^{32}P] ATP. At various time intervals, indicated aliquots were removed and spotted on P81 phosphocellulose paper followed by analysis of phosphate incorporation. The P81 phosphocellulose papers were either subjected to autoradiography (A) or counted (B). (C) Here, the experiment was carried out similar to experiment B except that the fusion was (either GST or GST-PIR-SH2) added 20 min after the reaction was initiated. The reaction was followed at the times indicated.

69−96% (*23*). Our analysis indicated that of the 18 tyrosine residues in the Grb14, 12 are unlikely phosphorylation sites, with scores less than 0.336 (Table 1). The most likely tyrosine phosphorylation sites are 143, 186, 202, 234, 264, and 289 (Table 1).

Identification of NPXY Motif in Grb14. Out of six phosphorylation sites predicted by the phosphorylation prediction program, one of the tyrosine residues is present in a conserved sequence motif, NPXY. This motif is present between amino acids 199−202 in Grb14. Alignment of the NPXY motif found in the cytoplasmic tail of growth factor receptors and LDL receptor gene family (*24*, *25*) are given in Figure 3. It is interesting to note that at position −2, a phenylalanine is highly conserved in Grb14, SHIP, LDLR, LR8B, VLDLR, MEG-1, and MEG-3, where a tyrosine residue is conserved at −2 position in APP, APLP1, and APLP2. LRP1 and TrKA contain isoleucine at the −2 position, whereas serine is found at this position in IR and valine in EGFR.

Table 1: Prediction of Tyrosine Phosphorylation on Tyrosine Residues in Retinal Grb14

position of Tyr	sequence[a]	score[b]	prediction
113	VIKVYSEDE	0.008	
143	LKNH**Y**IDDH	0.577	"Y"
186	ENKL**Y**FRKN	0.695	"Y"
191	FRKN**Y**AKYE	0.066	
194	NYAKYEFFK	0.286	
202	KNPM**Y**FFPE	0.571	"Y"
234	SSST**Y**PEIH	0.806	"Y"
255	WKK**I**YFLLR	0.049	
264	RSGL**Y**FSTK	0.592	"Y"
289	NSDI**Y**VSLA	0.987	"Y"
304	APTN**Y**GFCF	0.021	
342	RLLKYGMQL	0.028	
347	GMQLYQNYM	0.336	
350	LYQNYMHPY	0.020	
354	YMHPYQGRS	0.121	
360	GRSGYSSQS	0.122	
520	LVEFYQLNK	0.039	
534	KLKHYCARI	0.008	

[a] The amino acid sequence surrounding the Tyr (Y). [b] Phosphorylation scores were calculated based on the phosphorylation site prediction program (*23*). Scores above 0.5 are deemed to be possible phosphorylation sites, and the higher the score, the more likely a particular site will be phosphorylated.

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GRB14  :  -FFKNPMYFFPEHMVS  :  15
IR     :  -ASSNPEYLSASDVFP  :  15
EGFR   :  -AVGNPEYLNTAQPTC  :  15
SHIP   :  -MFENPLYGSVSSFPK  :  15
LDLR   :  -NFDNPVYQKTTEDEL  :  15
LRP    :  -NFTNPVYATLYMGGH  :  15
APP    :  -GYENPTYKFFEQMQN  :  15
APLP1  :  HGYENPTYRFLEERP-  :  15
APLP2  :  -GYENPTYKYLEQMQI  :  15
LR8B   :  -NFDNPVYRKTTE---  :  12
VLDLR  :  -NFDNPVYLKTTE---  :  12
LRP1   :  -EIGNPTYKMYEG---  :  12
TrKA   :  -IIENPQYFSDACVHH  :  15
MEG-1  :  -IFENPMYAAKDN---  :  12
MEG-3  :  -NFENPIYAEMDS---  :  12
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FIGURE 3: Alignment of the NPXY motif found in the cytoplasmic tails of various receptors and cytoplasmic proteins. The multiple sequence alignment was generated using the clustalw algorithm and displayed using GeneDoc (*38*).

PTB Domain of IRS-1 Binds to the NPXY Motif of Grb14. To study the interaction between the PTB domain of IRS-1 and the NPXY motif of Grb14, we have cloned, expressed, and purified the retinal IRS-1 PTB domain as a His-tagged protein employing Ni-NTA resin (data not shown). The NPXY motif of Grb14 was expressed as a GST fusion protein as described in Experimental Procedures. To investigate the role of phosphorylation on the binding between the NPXY motif of Grb14 and the PTB domain of IRS-1, we made use of a system in which mammalian polypeptides are expressed and inducibly phosphorylated in bacteria (*20*). Following induction of elk expression, the GST-NPXY fusion protein became phosphorylated on tyrosine, as detected by Western blotting of isolated GST-fusion proteins with anti-PY antibodies (Figure 4B). The parental GST protein was not phosphorylated when coexpressed along with elk tyrosine kinase (data not shown). The GST fusion proteins in their nonphosphorylated (NPXY) or tyrosine-phosphorylated (NPX-pY) forms were incubated with the purified His-tag PTB domain of IRS-1, followed by GST pull-down assays. The

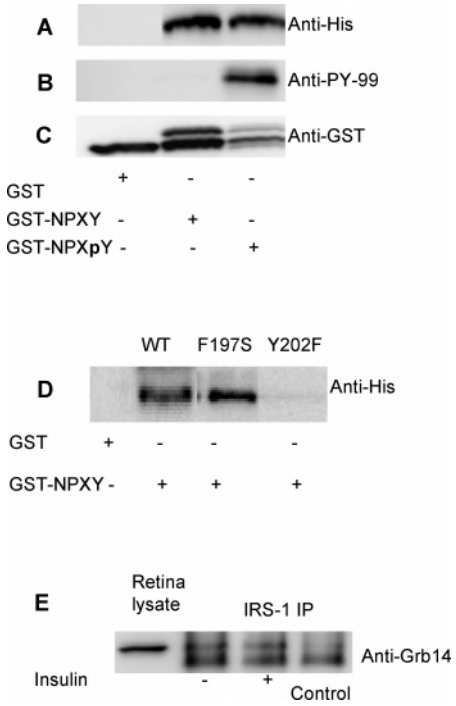

FIGURE 4: Interaction between the PTB domain of IRS-1 and Grb14 NPXY motif. GST, GST-Grb14 NPXY (nonphosphorylated), and GST-Grb14 NPXpY (elk phosphorylated) fusion proteins were incubated with His-tagged PTB domain of IRS-1 followed by GST pull-down assay. The bound protein was washed and subjected to Western blot analysis with either anti-His (A) or anti-PY99 (B) antibodies. The blot (A) was stripped and reprobed with anti-GST antibody (C). GST and nonphosphorylated forms of GST-NPXY (wild-type), GST-NPXY (F197S), and GST-NPXY (Y202F) mutant proteins were incubated with His-tagged PTB domain of IRS-1 followed by GST pull-down assay. The bound protein was washed and subjected to Western blot analysis with anti-His antibody (D). Insulin- and noninsulin-stimulated retina lysates were immunoprecipitated with anti-IRS-1 antibody followed by Western blot analysis with anti-Grb14 antibody (E). The immune complexes were not boiled (to avoid IgG heavy chain interference) but subjected to incubation at 37 °C for 10 min before being subjected to SDS−PAGE. Control immunoprecipitation was carried out omitting retina lysate.

bound proteins were subjected to SDS−PAGE followed by Western blotting analysis with anti-His antibody. The PTB domain of IRS-1 was pulled down by both nonphosphorylated and phosphorylated NPXY motifs but not by GST (Figure 4A). The blot was stripped and reprobed with anti-GST antibody to ensure the presence of fusion protein (Figure 4C). These experiments indicate that the PTB domain of IRS-1 interacts with the NPXY motif of Grb14 in vitro in a phosphorylation-independent manner.

Our results indicate a phosphorylation-independent binding between the NPXY motif of Grb14 and the PTB domain of IRS-1. However, substitution of Y202F in the NPXY motif of Grb14 abolished PTB domain binding (Figure 4D), suggesting the absolute requirement of tyrosine residue in this interaction. Substitution of F197S did not affect the interaction of Grb14 NPXY with the PTB domain of IRS-1 (Figure 4D).

Insulin- and noninsulin-stimulated retina lysates were immunoprecipitated with anti-IRS-1 antibody followed by Western blot analysis with anti-Grb14 antibody. The results indicate the recovery of Grb14-IRS-1 complexes independent of insulin stimulation (Figure 4E).

DISCUSSION

Using yeast two- and three-hybrid systems, we have shown that IRS-1 binds to retinal insulin receptor at Y960, IRS-2 at Y1146, Y1150, and Y1151, and p85 subunit of PI3K at Y1322 (*7*). In this study, GST-IRS-1 (PTB domain), GST-IRS-2 (novel domain), and GST-p85 (N-SH2 domain) fusion proteins were capable of bringing down the autophosphorylated insulin receptor. These results suggest that these domains could recognize the specific phosphorylation sites on the insulin receptor. IRS-1 binds to phosphorylated insulin receptor at Y960 (*7*, *22*, *26*, *27*). The absence of IRS-1 phosphorylation in response to insulin treatment in the retina (*7*, *8*) is not due to the lack of Y960 phosphorylation in the insulin receptor as GST-IRS-1 brings down the insulin receptor in a phosphorylation-dependent manner. These results suggest the expression of tissue-specific regulators of IRS proteins in the retina.

To identify new binding partners of the retinal insulin receptor, we chose to screen a library constructed from bovine retina, a nontarget tissue for insulin. From this library, we have isolated several clones, and most of the clones represent the same protein, Grb14. This protein was initially thought to be expressed only in insulin-responsive tissues (*17*), and this is the first study to demonstrate the presence of Grb14 in retinal tissues.

The phosphorylation prediction program on Grb14 indicated six potential tyrosine residues with scores more than 0.5 (*23*). We have recently used this program to identify phosphorylation sites in retinal IR, and the validity of the prediction was verified by site-directed mutagenesis (*7*). Substitution of tyrosine residues with phenylalanine individually in IR predicted by the phosphorylation prediction program failed to interact with downstream targets in a yeast two-hybrid system (*7*). Out of six potential phosphorylation sites in Grb14, one of the tyrosine residues is present in a conserved sequence motif, NPXY, between amino acids 199 and 202 in Grb14. The NPXY motif was absent in Grb7 and Grb10 (*12*, *13*). It is possible that other tyrosine residues in Grb14 may also be involved in intracellular signaling.

The NPXY motif was first recognized in low-density lipoprotein receptor-related protein (LRP) and subsequently recognized in numerous other cell surface receptors (*24*). In addition to cell surface receptors, the NPXY motif is also present in the cytoplasmic protein, SHIP (*28*). NPXY signals were initially thought to be involved in endocytosis, but recent studies have shown that NPXY motifs are recognized by proteins containing PTB or PID motifs (*25*). The biological function of the PTB domain is to drive recruitment of signaling adapters such as IRS-1 or Shc to NPXpY on activated receptor tyrosine kinases. Some PTB domain-containing proteins actually display a substantial higher selectivity for NPXY than for NPXpY (*25*), and others bind sequences unrelated to this consensuses motif. The Grb14 NPXY motif was found to interact with the PTB domain of IRS-1 in vitro, and this interaction does not require phosphorylation. Furthermore, phosphorylated Grb14 NPXpY did not increase the binding of the PTB domain of IRS-1.

In several proteins, PTB domains have been found to participate in phosphotyrosine-independent interaction. The X11 family of proteins contains a PTB domain that binds

peptides in a phosphotyrosine-independent fashion (*29*). The homologue of X11 in *C. elegans* in the *lin-10* gene crucial for receptor targeting to the basolateral surface of body wall epithelia (*29*). The X11/Lin-10 proteins are found in a complex with two other proteins, Lin-2 and Lin-7, which have also been implicated in basolateral targeting in worm epithelia (*29*). This protein complex is important in the targeting of cell surface proteins in mammalian neurons and epithelia. The PTB domain proteins disabled-1 (Dab1) and disabled-2 (Dab2) bind to cell-surface receptors of the low-density lipoprotein receptor (LDLR) family, which contain FXNPXY internalization signals in their cytoplasmic domains in a phosphorylation-independent manner (*25*, *30*).

The tyrosine residue in the NPXY motif is critical for binding to PTB domains as substitution of tyrosine with phenylalanine (Y202F) in the Grb14 NPXY motif completely eliminated the binding to PTB domain of IRS-1. In proteins such as Shc and IRS-1, the PTB domains bind to the NPXY motif in a phosphotyrosine-dependent fashion (*19*). The NPXY motif in the insulin receptor is essential for IRS-1 interaction (*19*). Substitution of Y960F in IR eliminates IRS-1 interaction, and also the kinase inactive receptor fails to interact with IRS-1 suggesting the phosphorylation-dependent binding (*7*, *19*).

The phosphorylation dependency and independency of the NPXY motif toward PTB domain binding is dictated by the flanking sequences around the NPXY motif (*25*). The insulin receptor NPXY motif has a serine residue at position −2, and the Grb14 NPXY motif has a phenylalanine in this position. To examine whether the serine residue confers the phosphorylation dependency, we have substituted phenylalanine with serine (F197S), but this mutant still binds to the PTB domain of IRS-1 similar to wild-type NPXY motif. These results suggest that other residues flanking the NPXY motif could be contributing to confer phosphorylation dependency.

There is widespread expression of IRS-1 throughout the retina, and IRS-1 is found in regions of the retina abundant in insulin receptors, including outer segments (*6*). However, we (*7*) and others (*8*) have shown that IRS-1 is not phosphorylated in response to insulin, under conditions that led to the activation of Akt (*7*, *8*). Further, insulin stimulated-tyrosine phosphorylation of IRS-1, Shc, and Dok is also decreased in CHO-IR/Grb14 cells compared to CHO-IR (*17*, *18*). A similar effect was reported in CHO-IR cells overexpressing Grb-IR, an isoform of Grb10 (*31*). It has been suggested that the decrease in IRS-1 activation could be due either to the sequestration of the protein when Grb14 is overexpressed or to an inhibitory effect of Grb14 on insulin signaling (*17*). These authors also observed phosphorylated IRS-1 in anti-Grb14 immunoprecipitates from insulin-stimulated rat liver (*17*).

Our studies also suggest that Grb14 does not contain any intrinsic phosphatase activity, and the decreased substrate phosphorylation of IRS-1 peptide is likely due to the inhibition of insulin receptor kinase activity. Grb14 has been shown to reduce insulin receptor kinase activity without loss of autophosphorylation, in part by competing with PTP1b binding (*32*, *33*). Tissue-specific differences were found in Grb14 knockout mice, such that liver had enhanced insulin receptor phosphorylation and increased IRS-1 tyrosine phosphorylation and Akt activation, whereas skeletal muscle had normal insulin receptor phosphorylation but increased IRS-1 and Akt activation (*34*). If Grb14 inhibits insulin receptor kinase activity in vivo, one would expect to see the absence of IRS-1 and IRS-2 phosphorylation. We (*7*) and others (*8*) have shown the absence of IRS-1 and the presence of IRS-2 phosphorylation in response to insulin treatment. These results suggest that the lack of IRS-1 phosphorylation in the retina is due, at least in part, to Grb14. It appears from our data that Grb14−IRS-1 complexes are more favorable than Grb14−insulin receptor complexes in the retina. Consistent with this, we failed to detect the in vivo interaction between Grb14 and insulin receptor in the retina (data not shown). This could be due to either absence of or very weak/transient interactions between insulin receptor and Grb14 which might dissociate during the co-immunoprecipitation experiments. Development of substrate-trapping mutants would identify such interactions, as demonstrated for the identification of physiological substrates of protein tyrosine phosphatases (*35*). On the basis of our data, we suggest that the Grb14 NPXY motif could be acting as a dominant negative for IRS-1 functions in retina, and this hypothesis is supported by a recent study showing that Grb14-deficient mice exhibit enhanced IRS-1 phosphorylation and activation of protein kinase B (*34*).

Emerging evidence which comes from various laboratories indicates that PTB domain-containing proteins play an important role in neuronal functions. Disabled (Dab) proteins have an important role in neuronal development in both flies and mammals (*36*, *37*). Both Dab-1 and Dab-2 proteins are expressed in the retina (Rajala, unpublished data). It is tempting to speculate that overexpression of Grb14 in retina may interfere with the proteins containing PTB domains and their intracellular signaling. Studies are underway in our laboratory to test this hypothesis.

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