Interaction of the Insulin Receptor β-Subunit with Phosphatidylinositol 3-Kinase in Bovine ROS

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PURPOSE. To identify the tyrosine-phosphorylated protein(s) in bovine rod outer segments (ROS) that are associated with phosphatidylinositol 3-kinase (PI3K).

METHODS. Glutathione-S-transferase (GST) fusion proteins containing two SH2 domains of the p85 regulatory subunit of PI3K—GST-p85 (N-SH2), GST-p85 (C-SH2), and respective SH2 mutants (N-SH2, R358A, and C-SH2, R649A)—were prepared and used to pull down tyrosine-phosphorylated proteins in bovine ROS. Protein identity was established by Western blot analysis. PI3K activity was determined in the pull-down mixtures and in immunoprecipitates by incubation with phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2) and [γ-32P]adenosine triphosphate (ATP).

RESULTS. The GST pull-down assays indicated the binding of a 97-kDa protein by GST-p85 (N-SH2) in tyrosine-phosphorylated (PY)-ROS that was not present in nonphosphorylated (N)-ROS. Binding was completely abolished when the Arg 358 in the N-SH2 domain was mutated to Ala. Increased binding of the p110α catalytic subunit to GST-p85 (N-SH2) fusion protein was also observed in the presence of the 97-kDa phosphorylated protein. Biochemical evidence indicated that the 97-kDa protein was the β-subunit of the insulin receptor β-subunit (IRβ). Immunoprecipitates of PY-ROS and N-ROS with anti-PY antibodies, probed with anti-IRβ, indicated the presence of IRβ only in PY-ROS. Immunoprecipitates of PY-ROS and N-ROS with anti-IRβ antibodies, probed with anti-p85 and anti-p110α antibodies, indicated increased amounts of both p85 and p110α in PY-ROS compared to N-ROS. Treatment of ROS with insulin, followed by immunoprecipitation with either anti-IRβ or anti-PY, resulted in increased PI3K activity. Expression and phosphorylation of the cytoplasmic tail of retina insulin receptor showed direct involvement with the p85 subunit of PI3K in vitro.

CONCLUSIONS. Tyrosine phosphorylation of the β-subunit of the insulin receptor is involved in the regulation of PI3K activity in ROS. (Invest Ophthalmol Vis Sci. 2001;42:3110–3117)

Phosphatidylinositol 3-kinase (PI3K) consists of an ~85-kDa regulatory subunit (p85) and a ~110-kDa catalytic subunit (p110), the latter of which is responsible for the phosphorylation of phosphatidylinositol lipids at the D3 position and serine phosphorylation of proteins.1-3 The p85 subunit contains a Src homology 3 (SH3) domain capable of binding to proline-rich sequences, a region of homology to the breakpoint cluster region (BCR) gene product, a p110 binding domain, and two SH2 domains (N- and C-terminal). PI3K activity increases in response to receptor activation by the direct binding of the p85 SH2 domain to tyrosine-phosphorylated sites on the receptor.4,5 PI3K activity can also be regulated by activated receptor tyrosine kinase substrates, such as insulin receptor substrate (IRS)-1.6 Ruderman et al.7 first demonstrated the activation of PI3K by insulin, either by stimulating Chinese hamster ovary (CHO) cells with insulin or by transfecting the CHO cells with human insulin receptor. Van Horn et al.8 showed a two-fold activation of PI3K by insulin receptor and also demonstrated the activation of PI3K by tyrosyl phosphopeptide derived from the insulin receptor C terminus in vitro. Thus, there is ample evidence to suggest that the class I p85/p110 complex of PI3K is a common element of numerous signaling pathways involving a large number of tyrosine kinases.9-11

Light stimulates tyrosine phosphorylation of several proteins in rat rod outer segments (ROS) in vivo.12 and bovine ROS contain an endogenous tyrosine kinase(s) that can phosphorylate at least 10 proteins in vitro.13,14 The activity of cyclic nucleotide-gated channels from salamander15 and bovine16 ROS could be substantially altered after tyrosine phosphorylation of the α-subunits of the channel protein. Phospholipase Cγ1, the enzyme responsible for hydrolysis of dioleoylphosphatidylinositol-4,5-bisphosphate (PI(4,5)-P2) and known to be stimulated by tyrosine phosphorylation,17,18 has been localized in bovine ROS.19 We have reported that bovine retinal ROS contain a class I p85/p110 enzyme complex20 that can be activated in vitro by light and tyrosine phosphorylation of proteins in these membranes.21 In the present study, we identified the mechanism of regulation of PI3K activity in bovine photoreceptor cells through tyrosine phosphorylation of the insulin receptor β-subunit (IRβ).

EXPERIMENTAL PROCEDURES

Materials

Polyclonal antisera to the p85 regulatory subunit of PI3K, p110α catalytic subunit of PI3K, mouse monoclonal phosphotyrosine (clone 4G10) antibody, and polyclonal IRβ were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY); polyclonal anti-PY99, polyclonal IRβ and IRS-1 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); [γ-32P]adenosine triphosphate (ATP) from New England Nuclear (Boston, MA); and anti-glutathione-S-transferase (GST) antibody from Amersham Pharmacia Biotechnology, Inc. (Piscataway, NJ). Echelon Research Laboratories Inc. (Salt Lake City, UT) provided PI(4,5)-P2. NIH3T3 cells transfected with the insulin receptor were obtained from Upstate Biotechnology, Inc. Cells harboring elk tyrosine kinase (Epicurian Coll TKX1 competent cells) were obtained from Stratagene (La Jolla, CA). All other reagents were of analytical grade and were from Sigma (St. Louis, MO).

Preparation of ROS

Fresh bovine eyes were obtained from a local abattoir and dissected on ice, and retinas were obtained within 2 hours. ROS were prepared

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from fresh retinas on a continuous sucrose gradient (25%-50%), as previously described.19 Protein determination was performed with bichinchoninic acid (BCA) reagents (Pierce, Rockford, IL), according to the manufacturer’s instructions.

Preparation of Tyrosine-Phosphorylated ROS

ROS prepared as described have an endogenous tyrosine kinase activity.13,14 Tyrosine-phosphorylated (PY)-ROS were prepared by incubating ROS for 15 minutes at 37°C in a phosphorylation buffer (50 mM Tris- HCl [pH 7.4], 100 mM NaCl, 2 mM MgCl₂, 1.5 mM ATP, and 0.2 mM Na₃VO₃). Nonphosphorylated (N)-ROS were prepared by incubating ROS in a similar buffer without ATP, MgCl₂, and Na₃VO₃. After incubation, PY-ROS and N-ROS were solubilized at 4°C for 30 minutes in a solubilization buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, and 10% glycerol).

Immunoprecipitation

PY-ROS and N-ROS were solubilized in a lysis buffer containing 1% Triton X-100, 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM EGTA, 1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM Na₃VO₃, 10 μg/ml leupeptin, and 1 μg/ml aprotinin. Insoluble material was removed by centrifugation at 17,000g for 20 minutes, and the solubilized ROS were precleared by incubation with 40 μl protein A-Sepharose for 1 hour at 4°C with mixing. The supernatant was incubated with anti-p85 (1:300), anti-p110α (4 μg), anti-p110β (4 μg), or anti-IRβ (4 μg) antibodies overnight at 4°C and subsequently with 40 μl protein A-Sepharose for 2 hours at 4°C. Immune complexes were washed twice with modified solubilization buffer (the 1% Triton X-100 was reduced to 0.1% and glycerol was removed) and once with phosphorylation buffer without ATP. Immune complexes were washed twice with modified solubilization buffer (the 1% Triton X-100 was reduced to 0.1% and glycerol was removed) and once with phosphorylation buffer without ATP. Precipitates were assayed for PI3K activity or subjected to immunoblot analysis.

SDS-PAGE and Western Blot Analysis

Protein were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes, and the blots were washed two times for 10 minutes with TTBS (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, and 0.1% Tween-20) and blocked with 10% bovine serum albumin in TTBS overnight at 4°C. Blots were then incubated with anti-p58 (1:4000), anti-p110α (1 μg/ml), anti-IRβ (1:1000), anti-PY (1 μg/ml), or anti-GST (1:5000) antibodies for 2 hours at room temperature. After primary antibody incubations, immunoblots were incubated with horseradish peroxidase (HRP)-linked secondary antibodies (anti-rabbit, anti-mouse, or anti-goat IgG) and developed by enhanced chemiluminescence (ECL), according to the manufacturer’s instructions. Quantitative analysis of bands of respective Western blot analysis was performed using NIH Image software, ver. 1.62 (provided in the public domain by the National Institutes of Health, Bethesda, MD, and available at http://rsb.info.nih.gov/nih-image/download.html).

PI3K Assay

Enzyme assays were performed essentially as previously described.22 Briefly, assays were performed directly on immunoprecipitates in 50 μl of the reaction mixture containing 0.2 mg/ml PI-4,5-P₂, 50 μM ATP, 0.2 μCi [γ³²P]ATP, 5 mM MgCl₂, and 10 mM HEPES buffer (pH 7.5). The reactions were performed for 15 minutes at room temperature and stopped by the addition of 100 μl of 1 N HCl followed by 200 μl chloroform-methanol (1:1, vol/vol). Lipids were extracted and resolved on oxalate-coated thin-layer chromatography (TLC) plates (silica gel 60) with a solvent system of 2-propanol/2 M acetic acid (65:35, vol/vol). The plates were coated in 1% (wt/vol) potassium oxalate in 50% methanol (vol/vol) and then baked in an oven at 100°C for 1 hour before use. TLC plates were exposed to x-ray film overnight at ~70°C, and radioactive lipids were scraped and quantified by liquid scintillation counting.

GST-p85 Proteins and Pull-Down Experiments

GST-p85 fusion proteins were generated by PCR amplification of the indicated p85 regions and cloned into a vector (pGEX2T; Amersham Pharmacia Biotech). The amino acids of bovine p85α present in each fusion protein are N-SH2 (514–546) and C-SH2 (614–724), based on the sequence published by Otsu et al.23 The sequence of each clone was verified by DNA sequencing. All inductions yielded proteins of the expected size, as judged by Coomassie blue staining. Pull-down experiments were performed as described6 using 5 μg GST-fusion proteins that had been adsorbed onto GST-Sepharose 4B matrix. PY-ROS and N-ROS were incubated with GST/GST-p85 fusion proteins with continuous mixing at 4°C for 1.5 hours. The Sepharose beads were washed three times in 500 μl HINTG buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol) and centrifuged at 5000 rpm for 30 to 60 seconds at 4°C. GST-p85 fusion proteins and bound proteins were eluted by boiling in 2× SDS sample buffer 5 minutes before 10% SDS-PAGE. After SDS-PAGE, the gels were not stained, but instead were used in a Western blot analysis to visualize the specific protein present. For PI3K activity, the HINTG−washed GST-Sepharose beads were used directly. Blots were stripped and reprobed with anti-GST antibodies to ensure that comparable amounts of GST-p85 fusion proteins were present in each experiment.

Site-Directed Mutagenesis

Site directed mutagenesis was performed by using a quick-change site-directed mutagenesis kit (Stratagene Inc.). The reaction mixture contained SDM buffer (200 mM Tris-HCl [pH 8.8], 100 mM KCl, 100 mM NH₄SO₄, 20 mM MgSO₄, 1% Triton X-100, 1 mg/ml nuclease-free bovine serum albumin), 1 mM deoxyoctonucleotide mix (dATP, dCTP, dGTP, and dTDP), 50 ng GST-pGEX vector containing either p85 (N-SH2) or p85 (C-SH2) fusion proteins, and 125 ng sense and antisense primers with mutations, in a total volume of 50 μl, followed by the addition of 2.5 U phi DNA polymerase with a programmable thermal controller (PTC 100; MJ Research, Inc., Watertown, MA). The mutant primers were: R58A (sense: ACC TTG GTG GTA GAC GAC GCT TCT ACT AAA; antisense: TTT AGT AGA TGC GTG TGC TAC CAA AAA GGT) and R649A (sense: ACT TTT GTT GTC GCG GAA AGT AAA CAC; antisense: CTG TTT ACT GCT TTC CGC GAC AAG AAG AGT). The extension parameters of SDM were as follows: after denaturation at 95°C for 30 seconds, 16 cycles at 95°C for 30 seconds, 55°C for 1 minute, and at 68°C for 12 minutes (2 min/kb of plasmid length). After temperature cycling, the reaction was placed on ice for 2 minutes, after which 10 U DpnI restriction enzyme was added, mixed, and incubated at 37°C for 60 minutes. Transformation was performed using 1 μl of the DpnI treated reaction to Epicurean XL-blue supercompetent cells, and the reaction was placed on plates coated with Luria-Bertani (LB) agar-ampicillin (100 μg/ml). The cDNAs of all mutants were sequenced after PCR. The only modifications observed were those intentionally introduced to create each desired mutation. The clones were induced with isopropyl β-D-thiogalactopyranoside (IPTG; 1 mM), and the expressed fusion proteins were purified through GST-Sepharose 4B matrix.

Tyrosine Kinase Assay

A synthetic peptide corresponding in sequence to residues 6-20 of p58α, cdc2 (KVEKIGEGTYGVVKK), was used as a substrate for Src tyrosine kinase.21 The phosphorylation reaction was performed essentially as described by Cheng et al,24 in a total volume of 25 μl of 50 mM Tris-HCl buffer (pH 7.0), 50 mM MgCl₂, 5 mM MnCl₂, 50 mM Na₃VO₃, 7 μg/ml p-nitrophenyl phosphate, and protein kinase Src. The reaction was initiated by adding 2.5 μl [γ³²P]ATP (2186 counts per minute per picomole) to reach a final concentration of 146 cpm/μl, and the reaction was terminated by adding 10 μl 50% (vol/vol) acetic acid. Twenty-five microliters of assay mixture was spotted onto phosphocellulose filter paper discs (1.5 × 1.5 cm), which were immersed in a solution containing 0.75% phosphoric acid (vol/vol), as described.24 The filter paper discs containing the bound phosphorylated peptide were washed three times with phosphoric acid and rinsed in acetone.
Radioactivity was quantified in 7.5 ml of a liquid scintillation cocktail and counted (Ready Safe Liquid Scintillation Cocktail and Liquid Scintillation Counter; Beckman, Fullerton, CA).

Cloning of CTIR and Generation of Phosphorylated CTIR
The carboxyl terminal tail of the cytoplasmic tail of insulin receptor (CTIR) was amplified from rat retina cDNA that was reverse transcribed from rat retina total RNA, using rat liver insulin receptor–specific primers (sense 5′-GGA TCC TCT CAC TGT CAG AGA GAG GCT; antisense 3′-GAA TTC TTA GGA AGG GTT CGA CCT CGG CGA). All PCR products were sequenced, and the insert was subcloned into the bacterial expression plasmid pGEX-2TK. The PGEX-2TK plasmid was transformed to TKX1-competent cells (Stratagene) that harbor a plasmid-encoded, inducible tyrosine kinase, elk tyrosine kinase, under the control of the trp promoter. The bacterial cells were grown at 37°C to an optical density at 600 nm (OD 600) of approximately 1 and were incubated for 2 hours with 0.1 mM IPTG to induce expression of the GST fusion proteins. The cells were then centrifuged at 2000g, and the pellet was resuspended in TK-induction medium (M9 medium containing indoleacrylic acid) for 2 hours, according to the manufacturer’s instructions. The cells were collected and frozen at −80°C until used. Frozen cell pellets were sonicated in ice-cold PASE lysis buffer (50 mM HEPES, [pH 7.5], 50 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF) and clarified by centrifugation. The supernatants were incubated with GST-Sepharose 4B matrix for 30 minutes at 4°C. The beads were then washed several times with ice-cold PBS, and the pellets were resuspended in PASE lysis buffer and used for in vitro binding after examining the phosphorylation on the insulin receptor kinase tail, using phosphotyrosine antibodies. In vitro binding assays were performed by using the nonphosphorylated and phosphorylated CTIR domain by incubating the beads with solubilized N-ROS for 90 minutes followed by GST pull-down assays. The GST beads were used for Western blot analysis with the anti-p85 regulatory subunit of PI3K and measured the PI3K activity, using PI-4,5-P2 as substrate.

RESULTS
Effect of Tyrosine Phosphorylation on PI3K Activity in ROS
The effect of tyrosine phosphorylation on PI3K activity was determined in immunoprecipitates of PY-ROS and N-ROS, using PI-4,5-P2 as a substrate (Figs. 1A, 1D). PI3K activity was approximately two times higher in anti-p85 IPs from PY-ROS than in N-ROS (Figs. 1A, 1D). p110α IPs from PY-ROS also had higher PI3K activity (~1.5-fold) than those from N-ROS (Figs. 1B, 1D). The strongest effect was found in anti-PY IPs, where PY-ROS had approximately 10 times the PI3K activity as N-ROS (Figs. 1C, 1D). The relative amount of p85 in the IPs was determined by densitometric scans of Western blot probed with anti-p85 antibody, and the density values are shown below the top panels. Anti-p85 and anti-p110 IPs from PY-ROS and N-ROS contained a similar amount of p85, whereas the amount of p85 in anti-PY IPs was approximately 10 times higher in PY-ROS.

p85 Protein Associated with Anti-PY IPs
Anti-PY IPs from PY-ROS and N-ROS were subjected to Western blot analysis with an anti-p85 antibody to determine whether

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932901/)
the higher PI3K activity observed in anti-PY IPs from PY-ROS is due to increased amounts of p85. The p85-immunoreactive band was seen only in anti-PY IPs from PY-ROS (Fig. 1C, top). However, tyrosine phosphorylation of p85 was not observed on Western blot analysis of either anti-p85 or anti-p110α IPs from PY-ROS and N-ROS probed with anti-PY (data not shown). These results indicate that a tyrosine-phosphorylated protein in bovine ROS could be binding to the SH2 domain(s) of the p85 subunit of PI3K. The apparent molecular weight of the phosphorylated protein was similar to that of the p85 subunit of PI3K. The higher PI3K activity observed in anti-PY IPs from PY-ROS is due to increased amounts of p85. The p85-immunoreactive band was seen only in anti-PY IPs from PY-ROS (Fig. 1C, top). However, tyrosine phosphorylation of p85 was not observed on Western blot analysis of either anti-p85 or anti-p110α IPs from PY-ROS and N-ROS probed with anti-PY (data not shown). These results indicate that a tyrosine-phosphorylated protein in bovine ROS could be binding to the SH2 domain(s) of the p85 regulatory subunit, and this protein may be involved in the activation of PI3K activity. This speculation is consistent with our results showing that the anti-PY IP pulled down the p85 in response to ROS phosphorylation.

Identification of the 97-kDa Protein from GST-p85 Pull-Down Experiments

PY-ROS and N-ROS were incubated with GST and respective p85 wild-type and mutated fusion proteins in a GST pull-down assay. After incubation, the fusion proteins were resolved by SDS-PAGE and transferred to nitrocellulose, and the resultant Western blots were probed with anti-PY antibody. We identified a 97-kDa immunoreactive band only in the GST-p85 (N-SH2) domain on Western blot analysis (Fig. 2A). No binding was observed in GST-p85 (N-SH2, R358A) mutant fusion protein (lane 4), demonstrating the specificity of the phosphorylation-dependent binding. When the blot was stripped and reprobed with anti-p110α antibody (Fig. 2B), an increased amount of p110α immunoreactivity was observed in the GST-p85 (N-SH2) domain. PI3K activity was also higher in the GST-p85 (N-SH2) fusion protein samples (data not shown). These results suggest that the increased PI3K activity in anti-PY IPs is due to the increased amount of p110α bound to the 97-kDa tyrosine-phosphorylated protein.

Characterization of the 97-kDa Tyrosine-Phosphorylated Protein

To test the possibility that the identified tyrosine-phosphorylated protein could be a member of the Src tyrosine kinase family or a higher molecular weight isoform, we eluted the bound tyrosine-phosphorylated protein with phenyl phosphate and performed a tyrosine kinase assay using the 97-kDa protein as substrate, using c-Src as the protein kinase. To determine whether the 97-kDa protein could be a member of the Src tyrosine kinase family, we used the 97-kDa protein as substrate, using c-Src as the protein kinase. The results show that the 97-kDa protein does not phosphorylate the peptide substrate, under conditions in which both are phosphorylated by c-Src (Table 1). This experiment ruled out the possibility that the 97-kDa protein is a nonreceptor tyrosine kinase family member.

Identification of the 97-kDa Protein as the IRβ

The apparent molecular weight of the phosphorylated protein bound by the GST-p85 (N-SH2) fusion protein was similar to that of the β-subunit of the insulin receptor. To test this possibility, ROS was probed with an antibody to IRβ and indicated an abundant presence of IRβ in these membranes (Fig. 3A). NIH3T3 cells transfected with the insulin receptor were used as a positive control. The molecular weight of NIH3T3 cell IRβ subunit is slightly higher than IRβ from ROS, possibly because of the extent of glycosylation that could alter the mobility of the protein. To determine whether the 97-kDa protein identified as IRβ is involved in PI3K binding, we incubated the GST and GST-p85N-SH2 fusion proteins were eluted with 100 mM phenyl phosphate and dialyzed overnight against phosphorylation buffer without ATP. The dialyzed protein was subjected to phosphorylation with c-Src tyrosine kinase.
p85 (N-SH2) fusion proteins with PY-ROS or N-ROS and subjected each to GST pull-down assays. After incubation, the fusion proteins were resolved by SDS-PAGE and the resultant Western blots were probed with anti-IRβ antibody. An immunoreactive band of IRβ was observed only in PY-ROS that were pulled down with the GST-p85 (N-SH2) domain (Fig. 3B). The blot was then stripped and reprobed with an anti-PI3K antibody to demonstrate that equal amounts of GST were present in each sample (Fig. 3C).

The other approach we used to identify IRβ was to elute the material from the GST-binding experiment described earlier with phenyl phosphate and electrophorese it on a 10% SDS-PAGE 0.7-mm-thick gel and stain with Coomassie blue. Once the band was visualized on the gel, it was cut with a clean razor blade and placed onto another 10% SDS-PAGE 1.0-mm-thick gel, to facilitate the placement of the 0.75-mm-thick slice of the first gel. The gel was run at 100 V and transferred to nitrocellulose, and the resultant Western blot was probed with the anti-IRβ antibody. An immunoreactive band of IRβ (Fig. 4), further confirming the identity of the 97-kDa protein as IRβ.

To examine the association between IRβ and the p85-p110 complex, we immunoprecipitated IRβ from PY-ROS and N-ROS and probed the ensuing Western blots with anti-IRβ, anti-p85, or anti-p110α. The presence of IRβ was also examined in PY-ROS and N-ROS immunoprecipitated with an anti-PY antibody. The results indicate no difference in the amount of IRβ in either PY-ROS or N-ROS (Fig. 5A), when immunoprecipitated with the anti-IRβ antibody, which was the expected result. IPs of PY-ROS and N-ROS, using the anti-PY antibody, probed with anti-IRβ antibody, indicated the presence of IRβ only in PY-ROS (Fig. 5B), suggesting that tyrosine-phosphorylated IRβ can be pulled down by the anti-PI3K antibody. When Western blots of anti-IRβ IPs were probed with anti-p85 or anti-p110α antibodies, there were increased amounts of each (Figs. 5C, 5D) in PY-ROS compared with N-ROS. IPs of PY-ROS and N-ROS with anti-IRβ probed with anti-PY antibody showed tyrosine phosphorylation of IRβ only in PY-ROS (Fig. 5E). The weaker signal with anti-PY compared with anti-p110 and -p85 antibodies in the PY-ROS could be due to differences in the relative strength of the antibodies. As can be seen from the blot, there was no apparent immunoreactivity with the anti-PY antibody in N-ROS, although we saw anti-p110 and -p85 immunoreactivity in N-ROS. It is known that basal phosphorylation is associated with the insulin receptor. Thus, the anti-PY antibodies may not detect the low signal, which could be detected by the anti-p110 and -p85 antibodies. These results suggest that tyrosine phosphorylation of IRβ is essential for interaction with the p85–110α complex of PI3K through the N-SH2 domain of p85.

**Figure 4.** Identification of the 97-kDa protein as IRβ. (A) NIH3T3 cells transfected with the insulin receptor served as a positive control. (B) Protein eluted with phenyl phosphate was subjected to 10% SDS-PAGE with 0.75-mm-thick gels stained with Coomassie blue. The band was visualized, excised, and placed on a second 10% SDS-PAGE 1.0-mm-thick gel. The gel was transferred to nitrocellulose and probed with anti-IRβ antibody.

**Figure 5.** Immunoblots of ROS immunoprecipitated with anti-IRβ or anti-PY antibodies. PY-ROS and N-ROS (500 μg each) were subjected to immunoprecipitation with anti-IRβ and probed with (A) anti-IRβ, (C) anti-p110α, (D) anti-p85, or (E) anti-PY antibodies. PY-ROS and N-ROS (500 μg) were subjected to immunoprecipitation with anti-PY antibody and probed with (B) anti-IRβ antibody.

**Effect of Insulin on PI3K Activity**

To test the effect of insulin on PI3K activity, N-ROS were either phosphorylated or treated with insulin and immunoprecipitated with anti-IRβ, and the IPs were assayed for PI3K activity with PI-4,5-P2 as a substrate. Increased PI3K activity was associated with the ROS treated either with ATP (Fig. 6, lane 1) or insulin (Fig. 6, lane 5) compared with untreated ROS (lanes 2, 4, 6). Furthermore, N-ROS incubated with insulin followed by immunoprecipitation with anti-PY antibody had significantly higher PI3K activity than nontreated N-ROS (Fig. 6, lane 5). Western blot analysis of the anti-PY IPs of ROS probed with the anti-IRβ antibody showed increased immunoreactivity in the insulin-treated samples (data not shown). These results suggest that phosphorylation and insulin treatment have the same effect—that is, the phosphorylation of IRβ.

**Direct Interaction of the p85 Subunit of PI3K with the Phosphorylated CTIR**

The rat CTIR was cloned by RT-PCR, using rat liver cDNA primers (Fig. 7A) and coexpressed in *Escherichia coli* with an inducible tyrosine kinase. After induction of *elk* expression, the GST-CTIR fusion protein became phosphorylated on tyrosine, as detected by Western blot analysis of isolated GST-fusion proteins with anti-PY antibodies (Fig. 7B). The parental GST protein was not phosphorylated when coexpressed with *elk* tyrosine kinase (Fig. 7B). The GST fusion proteins in their nonphosphorylated or tyrosine-phosphorylated forms were immobilized on glutathione-agarose beads and incubated with solubilized N-ROS, followed by GST pull-down assays. There was increased p85 binding (Fig. 7C) and PI3K activity (Fig. 7E) to phosphorylated GST-CTIR, compared with nonphosphorylated GST-CTIR. When the p85 blots were stripped and re-
probed with anti-IRS-1 antibody, no binding of IRS-1 was found, either to phosphorylated or nonphosphorylated CTIR (data not shown), showing the direct interaction of p85 with phosphorylated CTIR independent of IRS-1. The blot was then stripped and reprobed with an anti-GST antibody to demonstrate that equal amounts of GST were present in each sample (Fig. 7D).

**DISCUSSION**

The involvement of protein tyrosine phosphorylation in the regulation of PI3K activity is well documented. In response to various stimuli, such as platelet-derived growth factor (PDGF) and insulin, tyrosine phosphorylation of p85 has been shown to occur in several cell types. In most cases, however, PI3K is regulated through the receptor and non-receptor tyrosine kinases without apparent phosphorylation of the p85 subunit. To understand whether tyrosine phosphorylation is involved in the activation of PI3K in ROS, in vitro conditions that favor tyrosine phosphorylation of p85 were used. The increase of PI3K activity in anti-p85 and anti-p110 IPs from PY-ROS could be due to a greater amount of enzyme in the IP, activation of the enzyme by phosphorylation, or activation by some other mechanism. To investigate these possibilities, anti-p85 and anti-p110 IPs from PY-ROS and N-ROS were subjected to Western blot analysis, with anti-p85 or anti-p110 antibodies as probes. The amount of p85 and p110 in the IPs was not different between PY-ROS and N-ROS. Blots probed with anti-PY antibody showed that neither p85 nor p110 was phosphorylated, suggesting that the twofold increase in enzyme activity is probably due to the influence of some tyrosine-phosphorylated protein in the IPs.

Because the phosphorylation conditions used in this study are designed to promote tyrosine phosphorylation, anti-PY IPs from PY-ROS and N-ROS were obtained and assayed for PI3K activity and p85 protein expression. PI3K activity in anti-PY IPs from PY-ROS was 10 times that from N-ROS, and Western blot analysis probed with the anti-p85 antibody showed that the amount of p85 in IPs from PY-ROS was also approximately 10 times that in the IPs from N-ROS. These results indicate that the p85 regulatory subunit is most likely bound to and coimmunoprecipitates with a tyrosine-phosphorylated protein, in response to phosphorylation of ROS.

Src homology 2 (SH2) domains are found in a variety of cytoplasmic proteins involved in mediating signals from cell surface receptors to various intracellular pathways. They fold as molecular units and are capable of recognizing and binding to proteins and linear peptide sequences containing phosphorylated tyrosine residues. To identify and characterize the tyrosine-phosphorylated protein(s) in ROS, we constructed two GST-p85 (SH2) mutant fusion protein (N- and C-SH2 domains). GST pull-down assays on PY-ROS and N-ROS followed by Western blot analysis using an anti-PY antibody indicated the binding of a 97-kDa tyrosine-phosphorylated protein to GST-p85 (N-SH2) fusion protein. No binding was observed in
GST-p85 (N-SH2, R358A) mutant fusion protein, suggesting the specificity of phosphorylation-dependent binding. Van Horn et al.\(^a\) reported a similar finding, although they also saw a low level of binding to the C-terminus of their GST-p85 fusion protein. We found increased binding of p110\(^\alpha\) and an increase in PI3K activity in the presence of the 97-kDa protein bound to the GST-p85 (N-SH2) domain, which suggests that a p85-p110\(\alpha\)-97-kDa protein complex is sufficient to explain the increased PI3K activity in anti-PY IPs. However, the involvement of other proteins could not be ruled out by these experiments.

Several studies have shown that the \(\beta\)-subunit of the insulin receptor and the insulin-like growth factor (IGF)-1 receptor can be phosphorylated in whole retina and ROS in response to insulin\(^{27,38}\) and IGF-1.\(^{39,40}\) Using immunocytochemistry techniques, Rodrigues et al.\(^{41}\) found the insulin receptor to be localized in photoreceptor and neuronal cell bodies, with lower immunoreactivity in ROS. Bell et al.\(^{13,14}\) reported a 97-kDa protein in ROS that was actively phosphorylated in vitro under conditions that favor tyrosine phosphorylation, and Ghalavini et al.\(^{12}\) showed that a 97-kDa protein is phosphorylated in rat ROS in a light-dependent manner in vivo. Based on these studies, we suspected that the 97-kDa protein we found bound to the p85 regulatory subunit of PI3K could be the IR\(\beta\) subunit, and the experiments described herein show that this is indeed the case.

Although it is known that PI3K can be activated in other tissues by insulin,\(^7\) there is no evidence that the insulin receptors in the retina undergo any physiological response after insulin stimulation. In our study, we observed a basal level of insulin receptor phosphorylation in N-ROS accompanied by low PI3K activity (Fig. 6, lanes 2, 4, 6). Under conditions favorable for phosphorylation, we observed an increase in the PI3K activity that could be due to the increased phosphorylation of insulin receptor (Fig. 6, lane 1). This phenomenon is also true when the ROS were treated in the presence of insulin, followed by immunoprecipitation with anti-IR\(\beta\), which resulted in increased PI3K activity (Fig. 6, lane 3). Increased PI3K activity was also observed from the IPs of PY from insulin-treated ROS (Fig. 6, lane 5), further confirming that insulin-induced, tyrosine phosphorylation-autophosphorylation of IR\(\beta\) leads to the increased PI3K activity.

The regulatory p85 subunit of PI3K binds to phosphotyrosine at a YXXM motif\(^{42}\) in IRS-1 and thereby activates the catalytic p110 subunit.\(^{15}\) It is surprising that inactivation of the IRS-1 gene in the mouse, by the homologous recombination approach, did not result in any dramatic pathologic phenotype, suggesting the possible existence of alternative signaling pathways.\(^{14,44}\) It has also been shown that wild-type insulin receptor binds tightly to the SH2 domains of p85, whereas the mutant insulin receptor truncated by \(\alpha\) amino acids at the C terminus binds poorly to the SH2 domains that do not have the Y\(^{112}\)-THM motif.\(^{44}\) To examine the binding properties of the noncatalytic region of the insulin receptor containing the YTHM motif, this sequence was expressed in bacteria and inducibly phosphorylated on tyrosine, thereby mimicking receptor autophosphorylation normally induced by ligand binding. The phosphorylated C-terminal tail of the insulin receptor bound to the p85 subunit of PI3K in N-ROS, and the resultant complex contained PI3K activity. These results are consistent with the notion that noncatalytic cytoplasmic regions of growth factor receptors provide phosphorylation-dependent binding sites for SH2-containing signaling proteins. Furthermore, because there was no binding of IRS-1 to the phosphorylated C-terminal tail of the insulin receptor, the evidence supports a direct interaction of p85 subunit of PI3K with the CTIR, independent of IRS-1.

Our experiments on PI3K activation through its interaction with the 97-kDa protein identified as IR\(\beta\) could not rule out the possibility of the involvement of the IGF-1 receptor \(\beta\)-subunit, which also has an apparent molecular weight of 97 kDa.\(^{38}\) Indeed, both insulin and IGF-1 can stimulate PI3K in bovine lens.\(^{46}\) Our phosphorylation and binding experiments could have included IGF-1\(\beta\), which would not have been detected by the anti-IR\(\beta\) antibody. Similarly, the experiments using immunoprecipitation with the anti-PY antibody could not differentiate between the \(\beta\)-subunits of insulin and IGF-1 receptors. However, the antibody we used against IR\(\beta\) does not cross-react with IGF-1\(\beta\) (catalog no. SC-711; Santa Cruz Biotechnol-ogy), and immunoprecipitation with anti-IR\(\beta\) followed by Western blot analysis with the anti-p85 antibody or measuring the PI3K activity clearly showed an association between IR\(\beta\) and PI3K. Also, the phosphorylated GST-CTIR fusion protein that bound the p85 subunit of PI3K contained the insulin sequence, which is different from the IGF-1 sequence. Therefore, we feel confident in concluding that phosphorylation of the \(\beta\)-subunit of the insulin receptor in ROS leads to its association with PI3K. Studies are currently under way to determine whether PI3K also associates with IGF-1 receptors in photoreceptor cells.

In vitro\(^{20,21}\) and in vivo\(^7\) studies in our laboratory have now shown that the activity of PI3K in the retina can be controlled by light and tyrosine phosphorylation. We speculate that the molecular mechanism of the activation is through binding of the p85 regulatory subunit of PI3K to the \(\beta\)-subunit of the insulin receptor. The role of PI3K in photoreceptor outer segments is not known. However, its function could be related to the activities driven by light, such as shedding of photoreceptor tips, biogenesis of new ROS membranes through addition of newly synthesized membranes at the base of the ROS, or light adaptation. Also, environmental and genetic (mutation) stresses lead to death of rod and cone photoreceptor cells. In some neuronal cell types, such as cerebellar granular neurons\(^{48}\) and PC-12 cells,\(^{39}\) receptor activation of PI3K has been shown to protect these cells from stress-induced neurodegeneration. Whether PI3K activation protects the retina from stress is currently under study in our laboratory.

**References**


