Insulin Growth Factor 1 Receptor/PI3K/AKT Survival Pathway in Outer Segment Membranes of Rod Photoreceptors

Ashok K. Dilly[1,2] *and Raju V. S. Rajala*[1,2,3]

Purpose. The authors previously reported that physiological light induces the tyrosine phosphorylation of insulin receptors (IRs), which leads to the activation of the phosphoinositide 3-kinase (PI3K) and Akt (serine/threonine protein kinase B) survival pathway in rod photoreceptor cells. Tissue-specific deletion of IRs from photoreceptors resulted in stress-induced photoreceptor degeneration. Insulin growth factor 1 receptor (IGF-1R) is highly related in sequence and structure to the IR and shares 70% sequence identity overall and 84% identity within the tyrosine kinase domain. The role of IGF-1R in photoreceptor function is unknown. In this study the authors examined IGF-1R signaling in rod outer segment (ROS) membranes.

Methods. IGF-1R localization was examined in the plasma and disc membranes of ROS. Activation of the IGF-1R/PI3K/Akt pathway was analyzed using specific antibodies against phospho-tyrosine, IGF-1R, and phospho-Akt. PI3K activity was determined in the anti–phospho-tyrosine and anti–IGF-1R immunoprecipitates. Glutathione-S-transferase fusion proteins containing two Src homology 2 (SH2) domains of the p85 subunit of PI3K and their mutants were used to study the molecular interaction between IGF-1R and p85. In vivo IGF-1R signaling was studied in rats exposed to physiological light or to constant light.

Results. IGF-1R is predominately localized to plasma membranes of ROS. These studies indicate that light stress results in an increase in tyrosine phosphorylation of IGF-1R and an increase in PI3K enzyme activity in anti–phosphotyrosine and anti–IGF-1R immunoprecipitates of ROS and retinal homogenates. The authors observed that light stress induces tyrosine phosphorylation of IGF-1R in ROS membranes, which leads to the binding of p85 through N-SH2 and C-SH2 domains. Finally, the authors observed a significant activation of Akt in light-stressed retinas, indicating activation of the Akt survival pathway downstream of IGF-1R activation.

Conclusions. Light stress induced the activation of PI3K through activation and binding of IGF-1R, which leads to activation of the Akt survival pathway in photoreceptors. (*Invest Ophthalmol Vis Sci.* 2008;49:4765–4773) DOI:10.1167/iovs.08-2286

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Insulin growth factor I receptor (IGF-1R) is involved in the proliferation of many retinal cell types: retinal pigment epithelial cells,[1,2] rod[3] and cone[4] photoreceptor cells, and Müller cells.[5] IGF-1R activation protects cultured retinal neurons from apoptosis through a phosphoinositide-3-kinase (PI3K) cascade.[6] Mice lacking insulin receptor substrate-2, the principal substrate of IGF-1R, experience photoreceptor degeneration.[7] IGF-1R activation has been shown to protect the retinal ganglion cells from death through PI3K-dependent Akt phosphorylation and inhibition of caspase-3 in vivo.[8] These studies suggest the importance of the IGF-1R/PI3K/Akt signaling pathway in the retina.

PI3K is an important component in the signal transduction cascade initiated by various receptor tyrosine kinase–specific growth factors. This kinase is a heterodimeric protein with a 110-kDa catalytic subunit (p110) and an 85-kDa regulatory adaptor subunit (p85) with two Src homology (SH2) domains[9] that interact with the cytosolic portion of the tyrosine kinase domain of the receptor. PI3K catalyzes the phosphorylation of the 3-OH position of inositol head groups of phosphoinositide lipids, namely phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PI-4-P), and phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2). This results in the formation of PI-3-P, PI-3,4-P_2 and PI-3,4,5-P_3, respectively. These are collectively termed 3′-phosphoinositide lipids.[9] Several studies have highlighted the importance of PI3K-generated phosphoinositides in the regulation of cell proliferation, cellular differentiation, cytoskeletal reorganization, membrane trafficking, glucose transport, survival and metabolism, and the prevention of apoptosis.[10]

Our previous study shows that bovine rod outer segments (ROS) contain the class 1 p85-p110 enzyme complex, which is significantly active in light-adapted retinas in vitro[11] and which can be activated in vivo through light-induced tyrosine phosphorylation of the insulin receptor (IR) in ROS.[12] We also demonstrated that photobleaching of rhodopsin regulates the phosphorylation state of IR.[13] IR, IGF-1R, and insulin-related receptor (IRR) belong to the same receptor-tyrosine kinase (RTK) family.[14,15] These receptors are composed of two extracellular α-subunits containing the ligand-binding domain and two transmembrane β-subunits containing tyrosine kinase activity. IGF-1R and IR share 70% sequence identity overall and 84% identity within the tyrosine kinase catalytic domains. The mechanism for receptor activation is thought to be similar for IR and IGF-1R.[16–19] We recently reported that IR activation is neuroprotective because its deletion in rods resulted in stress-induced photoreceptor degeneration.[20] This functional phenotype in IR knockout mice is attributed to the reduced PI3K/Akt survival signal in rods.[20] Because of the structural similarity between IR and IGF-1R, we hypothesized that IGF-1R activation might also result in the activation of PI3K/Akt survival pathway in rod photoreceptors. To test this hypothesis, we studied the IGF-1R/PI3K/Akt neuron survival pathway in physiological light and light stressed retinas in vivo.

Materials and Methods

Materials

Polyclonal anti-IGF-1Rβ, anti-IRβ, monoclonal anti-PY-99, and anti-Glut-1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-Akt and anti-pAkt antibodies were obtained from Cell Signaling (Danvers, MA). Polyclonal anti-insulin/IGF-1R-like growth factor receptor (Y1158, Y1162, Y1163) phosphospecific antibody was obtained from Biosource (Camarillo, CA). The actin antibody was obtained from Affinity BioReagents (Golden, CO). Monoclonal anti-opsin antibody (Rho 4D2) was a gift from Robert Molday (University of British Columbia). [γ-^{32}P] ATP was from PerkinElmer Life Sciences (Waltham, MA). Echelon Research Laboratories Inc. (Salt Lake City, UT) provided D-*myo*phosphatidylinositol- 4,5-bisphosphate (PI-4,5-P_2). IGF-1 and all other reagents were of analytical grade from Sigma (St. Louis, MO).

Animals

All animal work was conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All protocols were approved by the IACUC at the University of Oklahoma Health Sciences Center and the Dean McGee Eye Institute. Rats were dark-adapted overnight and killed under dim red light or after 30 minutes of normal light exposure (300 lux). In all experiments, rats were killed by asphyxiation with carbon dioxide before the retinas were harvested.

Exposure of Animals to Light Stress

Sprague–Dawley albino rats were born and raised in 5 lux cyclic light. Rats were exposed to constant light for 3 hours at 5000 lux provided by white fluorescent light bulbs suspended 50 cm above the floor of the cage. During light exposure, rats were maintained in transparent polycarbonate cages with stainless steel wire bar covers. Drinking water was supplied by a bottle attached to the side of the cage so that there was no obstruction between the light and the animal, and food was placed on bedding in the bottom of the cage. At the end of the exposure, retinas were removed. Control experiments were performed on unexposed dark-adapted rats.

Retinal Organ Cultures

Retinas were removed from Sprague–Dawley albino rats that were born and raised in dim cyclic light (5 lux; 12 hours on/12 hours off) and incubated at 37°C in Dulbecco modified Eagle medium (Invitrogen) in the presence or absence of 1.3 nM IGF-1 for 10 minutes. At the indicated times, retinas were snap-frozen in liquid nitrogen and stored at −80°C until analyzed. The retinas were lysed in lysis buffer (1% Nonidet P-40, 20 mM HEPES [pH 7.4], and 2 mM EDTA) containing phosphatase inhibitors (100 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM $NaVO_3$, and 1 mM molybdate) and protease inhibitors (10 μM leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and kept on ice for 10 minutes followed by centrifugation at 4°C for 20 minutes.

Preparation of Rat Rod Outer Segments

Rats were either unexposed or exposed to constant light for 3 hours at 5000 lux. After exposure, the ROS were prepared using a discontinuous sucrose gradient centrifugation as previously described.[12] Retinas were homogenized in 4 mL ice-cold 47% (wt/vol) sucrose solution containing buffer A (100 mM NaCl, 1 mM EDTA, 1 mM $NaVO_3$, 1 mM PMSF, and 10 mM Tris-HCl [pH 7.4]). Retinal homogenates were transferred to 15 mL centrifuge tubes and sequentially overlaid with 3 mL of 42% (wt/vol), 3 mL of 37% (wt/vol), and 4 mL of 32% (wt/vol) sucrose dissolved in buffer A. Gradients were spun in a swinging bucket rotor at 82,000*g* for 1 hour at 4°C. The 32%/37% interfacial sucrose band containing the ROS membranes was harvested and diluted with 10 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and 1 mM EDTA. The band solution was then centrifuged at 27,000*g* for 30 minutes at 4°C. ROS pellets were resuspended in 10 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and 1 mM EDTA and were stored at −20°C. Protein concentrations were determined by BCA reagent (Pierce, Rockford, IL) according to the manufacturer's instructions.

Preparation of Osmotically Intact Rod Outer Segment Discs

Osmotically intact ROS discs were prepared by Ficoll flotation.[21] ROS was prepared from frozen bovine retinas according to the method described.[22] The ROS pellet was resuspended in 30 mL Ficoll in distilled water, and the suspension was kept at 4°C under nitrogen for at least 2 hours to allow the ROS plasma membrane to burst. This suspension of ROS was divided between two small Beckman SW-28 centrifuge tubes and layered with cold water. After 2 hours of centrifugation in a swinging bucket rotor (SW-20; Beckman, Fullerton, CA) at 25,000 rpm, the intact discs were collected from the 5% Ficoll water interface. The bottom pellet contained the plasma membrane–enriched fraction.

Immunoprecipitation

Retinal lysates were prepared as described.[23] Retinal lysates or ROS were solubilized for 30 minutes at 4°C 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 $MgCl_2$, 1 mM PMSF, 0.2 mM Na_3VO_4, 10 μg/mL leupeptin, and 1 μg/mL aprotinin. Insoluble material was removed by centrifugation at 17,000*g* for 20 minutes at 4°C, and the solubilized proteins were precleared by incubation with 40 μL protein A-Sepharose for 1 hour at 4°C with mixing. The supernatant was incubated with anti-IGF-1R or anti-PY99 antibodies overnight at 4°C and subsequently with 40 μL protein A-Sepharose for 2 hours at 4°C. After centrifugation at 17,000*g* for 1 minute at 4°C, immune complexes were washed three times with ice-cold wash buffer (50 mM HEPES [pH 7.4], 118 mM NaCl, 100 mM NaF, 2 mM $NaVO_3$, 0.1% [wt/vol] SDS, and 1% [vol/vol] Triton X-100). IGF-1R immunoprecipitates were immunoblot analyzed or assayed for PI3K activity, whereas PY-99 immunoprecipitates were assayed only for PI3K activity.

PI3K Assay

Enzyme assays were carried out as described previously.[24] Briefly, assays were performed directly on immunoprecipitates in 50 μL reaction mixture containing 0.2 mg/mL PI-4,5-P_2, 20 μM ATP, 0.2 μCi of [γ-^{32}P]ATP, 5 mM $MgCl_2$, and 10 mM HEPES buffer (pH 7.5). Reactions were carried out for 30 minutes at room temperature and stopped by the addition of 100 μL of 1 N HCl followed by 200 μL chloroform/methanol (1:1). Lipids were extracted and resolved on oxalate-coated TLC plates (silica gel 60) with a solvent system of 1-propanol/2 M acetic acid (65:35). Plates were coated in 1% (wt/vol) potassium oxalate in 50% (vol/vol) methanol 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 Pull-Down Experiments

Pull-down experiments were carried out as described[25] using 5 μg GST-fusion proteins that had been adsorbed onto GST-Sepharose 4B matrix. IGF-1 treated or untreated (in vitro) retina lysates or light-stressed and dark-adapted retina lysates or solubilized ROS were incubated with GST-p85 (N-SH2), GST-p85 (N-SH2) R358A, GST-p85 (C-SH2), and GST-p85 (C-SH2) R649A fusion proteins[26] at 4°C overnight with continuous mixing. The bound proteins to GST-Sepharose fusions were washed three times in 500 μL wash buffer and centrifuged at 5000 rpm for 30 to 60 seconds at 4°C. Bound proteins were eluted by boiling in 2× SDS sample buffer for 5 minutes before 10% sodium dodecyl sulfide-polyacrylamide gel electrophoresis (SDS-PAGE). After

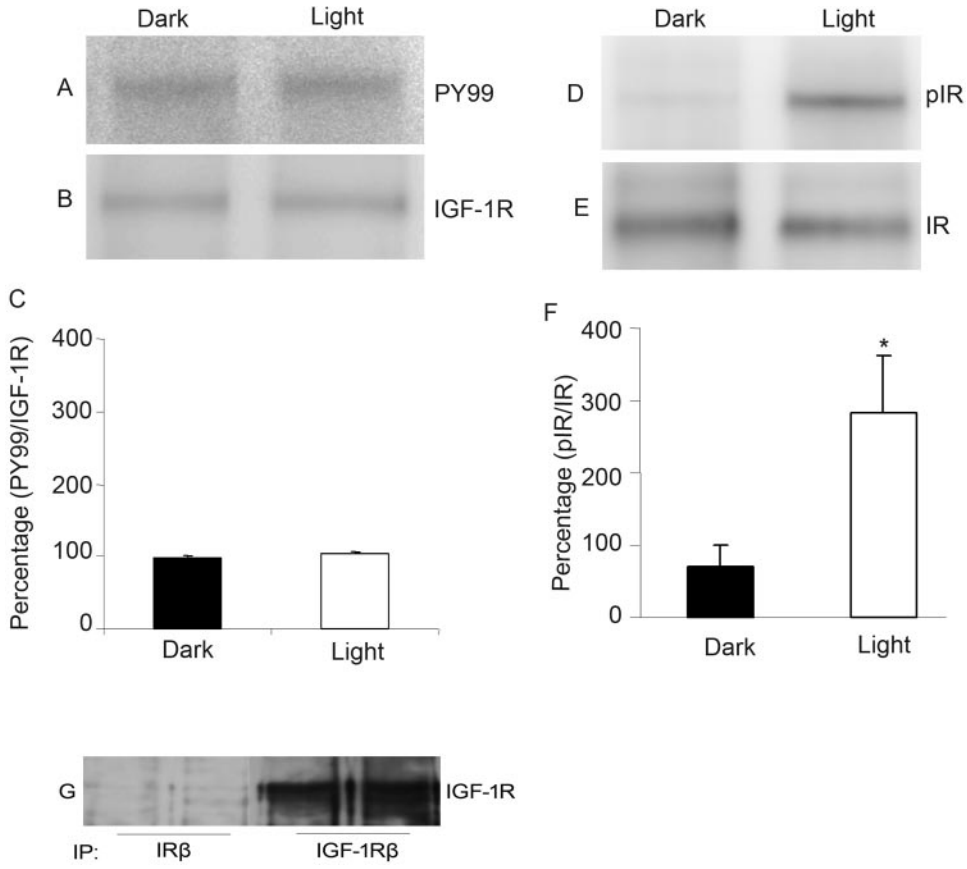

FIGURE 1. Effect of normal light on IGF-1R and IR phosphorylation in vivo. Rats were either dark- or light-adapted, as described. Retinas were lysed, and equal amounts of protein were immunoprecipitated with anti–IGF-1R (**B**) or anti–IRβ (**E**) antibodies and immunoblotted with anti-PY99 (**A**) or anti–phospho-IR (Y1158, Y1162, Y1163) antibody (**D**). The blots were stripped and reprobed with anti–IGF-1R (**B**) or anti–IRβ (**E**) antibodies. Quantitative analysis of bands of respective Western blot analyses was performed with imaging software. The PY99/IGF-IR or pIR/IR ratio was expressed as a percentage of control (**C**, **F**). Data are mean ± SD ($n = 4$; $^*P < 0.05$). Retinal proteins were immunoprecipitated with anti-IRβ or anti–IGF-1R antibody followed by Western blot analysis with anti–IGF-1R antibody (**G**). Two independent retinal lysates were used for immunoprecipitation.

SDS-PAGE, the gels were subjected to Western blot analysis with anti–IGF-1R antibody. To ensure equal amounts of fusion protein in each experiment, the blot was reprobed with anti–GST antibody.

SDS-PAGE and Western Blotting

Proteins were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The blots were washed twice for 10 minutes with TTBS (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, and 0.1% Tween-20) and were blocked with either 5% bovine serum albumin or nonfat dry milk powder (Bio-Rad, Hercules, CA) in TTBS for 1 hour at room temperature. Blots were then incubated with anti–PY99 (1:1000) and anti–IGF-1R (1:1000), anti–actin (1:1000), anti–Glut-1 (1:1000), pAkt (1:1000), or Akt (1:1000) antibodies overnight at 4°C. After washing with TTBS, the blots were incubated with horseradish peroxidase–coupled secondary (anti–rabbit or anti–mouse) antibodies and were developed by enhanced chemiluminescence according to the manufacturer's instructions (Eastman Kodak, Rochester, NY).

RESULTS

Effect of Normal Light on IGF-1R Phosphorylation In Vivo

We previously reported that physiological light induced the tyrosine phosphorylation of retinal IR in vivo.[12] IR and IGF-1R belong to the same family of receptor tyrosine kinases; therefore, we were interested in examining whether IGF-1R tyrosine phosphorylation is light dependent. To demonstrate the light effect, we dark adapted the rats overnight and subjected half to normal room light for 30 minutes before harvesting the retinas. The retinas were lysed and the lysates were immunoprecipitated with anti–IGF-1R; this was followed by Western blot analysis with anti–PY99 antibody. The results indicated that there was no significant difference in IGF-1R phosphorylation between dark- and light-adapted retinas (Fig. 1A–C). The results also suggested constitutive IGF-1R phosphorylation in the retina. Control experiments carried out on anti–IR immunoprecipitates showed the light-induced tyrosine phosphorylation of the retinal IR (Figs. 1D–F). These results suggested that even though IR and IGF-1R belong to the same family of receptor tyrosine kinases, their phosphorylation state was differentially regulated (light regulates IR phosphorylation but not IGF-1R). The existence of IR/IGF-1R hybrid receptors in the retina been reported previously.[26] To determine the specificity of IGF-1R antibody, we immunoprecipitated IR with an anti-IRβ and IGF-1R with an anti–IGF-1R antibody, followed by Western blot analysis with anti–IGF-1R antibody. The results indicated that IGF-IR antibody recognized IGF-IR immunoprecipitates but not IR immunoprecipitates (Fig. 1G), attesting to the specificity of IGF-1R antibody toward retinal IGF-1R.

Light Stress–Induced Activation of PI3K In Vivo

To determine whether light stress induces the activation of PI3K, we exposed the rats to 5000 lux light for 3 hours, after which the retinas were removed and homogenized and the lysates used directly or to prepare ROS membranes. Retina lysates or solubilized ROS membranes were subjected to immunoprecipitation with anti–PY99 antibody followed by measurement of the PI3K activity. PI3K enzyme activity was significantly higher in anti–PY99 immunoprecipitates from retinal lysate (Figs. 2A, 2B) and ROS membranes (Figs. 2C, 2D) from light-stressed rats compared with dark-adapted rats. These results suggest that light stress induces the association of PI3K to tyrosine phosphorylated protein(s) and activates the PI3K enzyme in vivo.

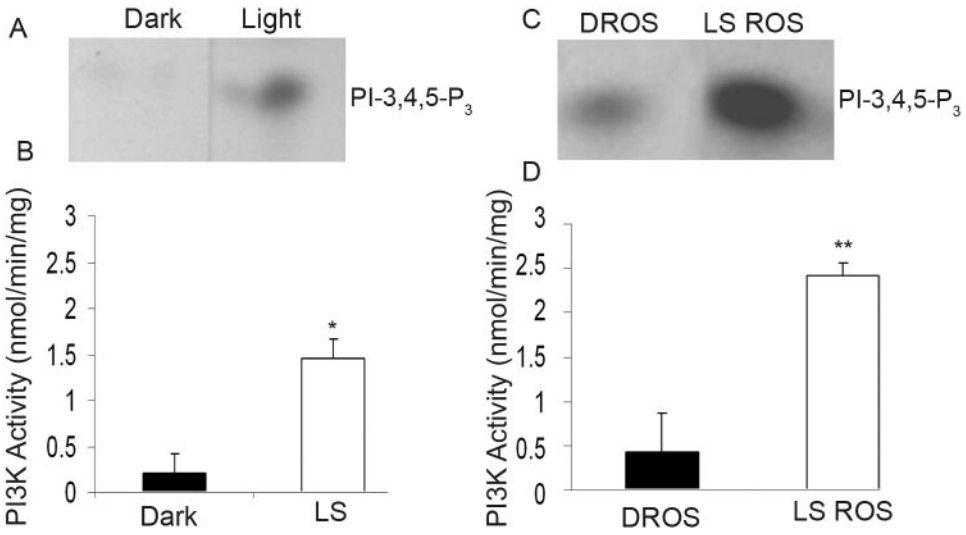

FIGURE 2. Light stress–induced activation of PI3K in vivo. Dark-adapted and light-stressed rat retinal lysates or solubilized ROS were subjected to immunoprecipitation with anti–PY99 antibody, and PI3K activity was measured using [γ^{32}P] ATP with PI-4,5-P_2 as a substrate. Reaction products were separated by TLC and visualized by autoradiography (**A**, **C**). The radioactive PI-3,4,5-P_3 spots were scraped and counted (**B**, **D**). Data are mean ± SD ($n = 3$; $^*P < 0.05$; $^{**}P < 0.01$).

Light Stress–Induced Tyrosine Phosphorylation of IGF-1R In Vivo

To determine whether light stress activates IGF-1R in vivo, we immunoprecipitated retinal lysates and solubilized ROS membranes from dark- and light-stressed rats with anti–IGF-1R antibody. The immune complexes were subjected to Western blot analysis with anti–PY99 antibody. The results indicated that light stress induces the tyrosine phosphorylation of IGF-1R in retinal lysates and in ROS membranes (Figs. 3A, 3C). The blots were stripped and reprobed with anti–IGF-1R antibody to ensure equal amounts of IGF-1R in each immunoprecipitate (Figs. 3B, 3D). These results suggested that light stress induces the activation of IGF-IR in vivo.

Localization of IGF-1Rs in ROS Plasma Membrane

ROS, plasma membranes, and disc proteins were subjected to Western blot analysis with anti–IGF-1R, anti-Glut1, and anti-opsin antibodies. The results indicated that IGF-1R, Glut1, and opsin immunoreactivity were present in the ROS (Fig. 4). IGF-1R and the plasma membrane marker Glut1 was enriched in the plasma membrane fraction of the ROS (Figs. 4A, 4B). Opsin blot showed the enrichment of opsin in the disc membranes (Fig. 4C). These results suggest that IGF-1R is localized to the ROS plasma membrane.

Light Stress–Induced IGF-1R–Associated PI3K Activity In Vivo

Figure 3 shows that light stress induced the tyrosine phosphorylation of IGF-1R. To determine whether the light stress–activated IGF-1R is able to associate with PI3K in vivo, we immunoprecipitated the IGF-1R from retina lysates and solubilized ROS membranes and then measured the PI3K activity associated with the anti–IGF-1R. PI3K activity was significantly higher in retina lysates (Figs. 5A, 5B) and ROS (Figs. 5C, 5D) prepared from light-stressed rats compared with dark-adapted rats. These results suggest that light stress induces the activation of IGF-1R, which leads to the association and activation of PI3K in vivo.

Binding of Light Stress–Induced Tyrosine-Phosphorylated IGF-1R to N-SH2 and C-SH2 Domains of p85 Subunit of PI3K

We observed that light stress–induced tyrosine phosphorylation of IGF1-R binds to PI3K in vivo. To determine specifically which SH2 domain is involved in the interaction with the tyrosine-phosphorylated IGF-1R, we incubated dark- and light-stressed solubilized ROS membranes with wild-type (N-SH2 and C-SH2) and mutant (N-SH2-R358A and C-SH2-R649A) GST-p85 fusion proteins and subjected them to GST pull-down assays. The proteins bound to the fusions were subjected to Western blot analysis with anti–IGF-1R antibody. The results indicated that N-SH2 and C-SH2 domains of p85 were able to

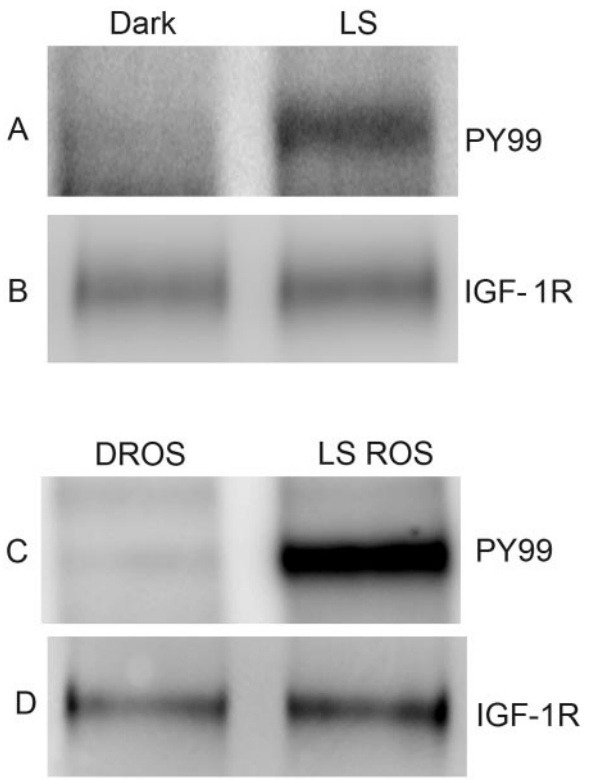

FIGURE 3. Light stress–induced tyrosine phosphorylation of IGF-1R in vivo. Equal amounts of protein from dark-adapted or light-stressed rat retinal lysates and dark-stressed or light-stressed (LS) ROS were immunoprecipitated with anti–IGF-1R antibody. Immunoprecipitates were subjected to Western blot analysis with anti–PY99 antibody (**A**, **C**). The blot was stripped and reprobed with anti–IGF-1R antibody to ensure equal amounts of receptor in each lane (**B**, **D**).

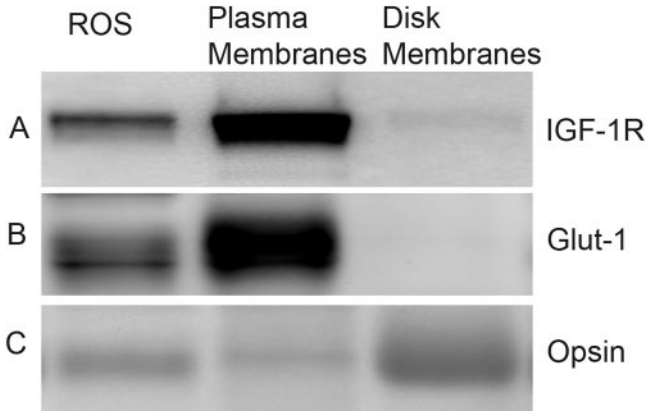

FIGURE 4. IGF-1Rs are localized to ROS plasma membrane. Bovine ROS, plasma membrane, and disc membrane proteins were subjected to Western blot analysis with anti–IGF-1R (**A**), anti–Glut1 (**B**), and anti–opsin (**C**) antibodies.

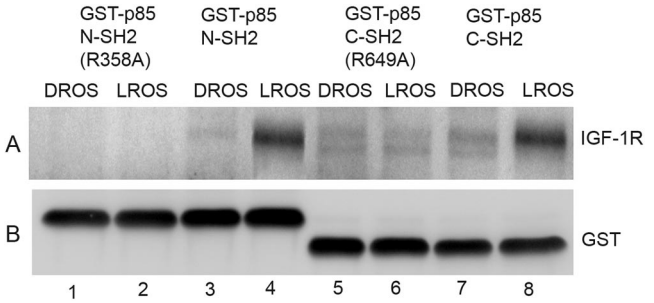

FIGURE 6. Binding interaction between IGF-1R and SH2 domains of p85. ROS from dark- and light-stressed rats were incubated with GST-NSH2, GST-NSH2 (R358A), GST-CSH2, or GST-CSH2 (R649A) fusion proteins and subjected to GST pull-down assay. Bound proteins were subjected to Western blot analysis with anti–IGF-1R antibody (**A**). Blots were reprobed with anti–GST antibody to ensure equal amounts of fusion in each pull-down (**B**). *Lanes 1, 3, 5, 7*: dark adapted. *Lanes 2, 4, 6, 8*: light stressed.

bring down IGF-IR from light-stressed ROS but not from DROS (Fig. 6A). The mutant p85 fusion proteins failed to bring down IGF-1R from dark- and light-stressed ROS (Fig. 6A). The blot was reprobed with anti–GST antibody to ensure equal amounts of fusion in each pull-down (Fig. 6B). These experiments indicate that the interaction is phosphorylation dependent and that both SH2 subunits of p85 are involved in the interaction with IGF-1R.

Light Stress-Induced Activation of Retinal Akt In Vivo

Our experiments on IGF-1 showed the activation of PI3K and Akt ex vivo. Furthermore, our light stress experiments indicated the activation of IGF-1R–associated PI3K activity. To determine whether light stress induced the activation of Akt, we subjected the retinal proteins from the dark- and light-stressed rats to Western blot analysis with anti–pAkt antibody. The results indicated the increased phosphorylation of Akt in light-stressed retinas compared with dark-adapted retinas (Fig. 7A). The blot was stripped and reprobed with anti–Akt and anti–actin antibodies to ensure equal amounts of proteins in each lane (Figs. 7B, 7C). This result suggested that light stress induced the activation of Akt in vivo.

IGF-1–Induced Activation of the IGF-1R/PI3K/Akt Signaling in Ex Vivo Retinal Organ Cultures

Rat retinas in culture were stimulated or unstimulated with IGF-1 for 10 minutes, and the retinas were lysed and subjected to immunoprecipitation with anti–IGF-1R antibody followed by Western blot analysis with anti–PY99 antibody (Fig. 8A) or directly assayed for PI3K activity (Figs. 8C, 8D). Increased tyrosine phosphorylation was observed on IGF-1R in response to IGF-1 treatment (Fig. 8A). To ensure equal amounts of IGF-1R, PY-99 blots were stripped and reprobed with anti–IGF-1R antibody (Fig. 8B). A significant increase in IGF-1R–associated PI3K activity was observed in the anti–IGF-1R immunoprecipitates of IGF-1–stimulated retinas when compared with nonstimulated retinas (Figs. 8C, 8D).

Binding of IGF-1–Induced Tyrosine-Phosphorylated IGF-1R to N-SH2 and C-SH2 Domains of p85

This experiment was conducted to examine differences in the binding of IGF-IR to the p85 subunit of PI3K under ligand and light-stressed conditions. Under light stress, IGF-1R binds to the N- and C-SH2 domains of p85 with equal binding. To determine whether the ligand-mediated activation of IGF-1R displays the

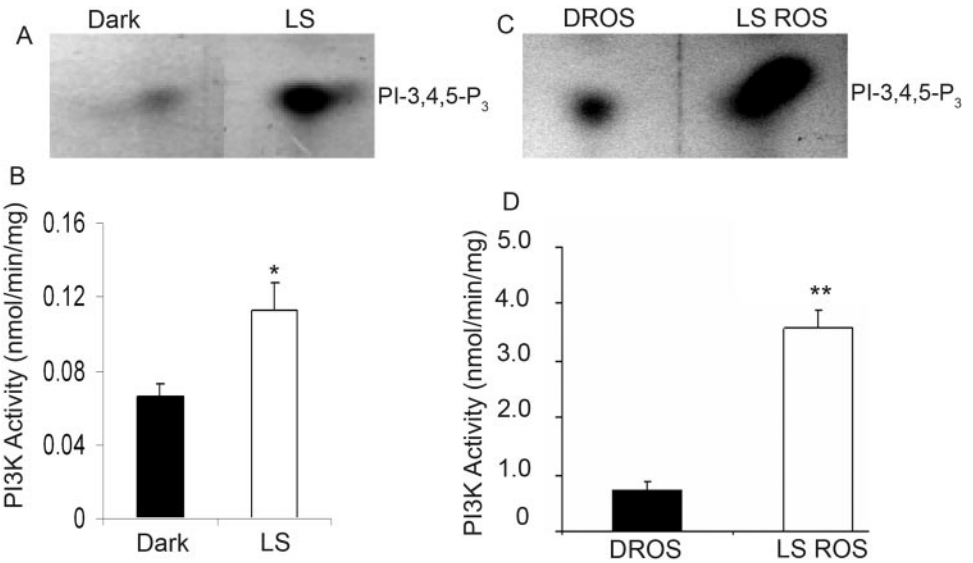

FIGURE 5. Light stress–induced IGF-1R–associated PI3K activity in vivo. Dark- and light-stressed rat retinal lysates or solubilized ROS were subjected to immunoprecipitation with anti–IGF-1R antibody, and PI3K activity was measured using [γ^{32}P] ATP with PI-4,5-P_2 as a substrate. Reaction products were separated by TLC and visualized by autoradiography (**A**, **C**). Radioactive PI-3,4,5-P_3 spots were scraped and counted (**B**, **D**). Data are mean ± SD ($n = 3$; $^*P < 0.05$; $^{**}P < 0.001$).

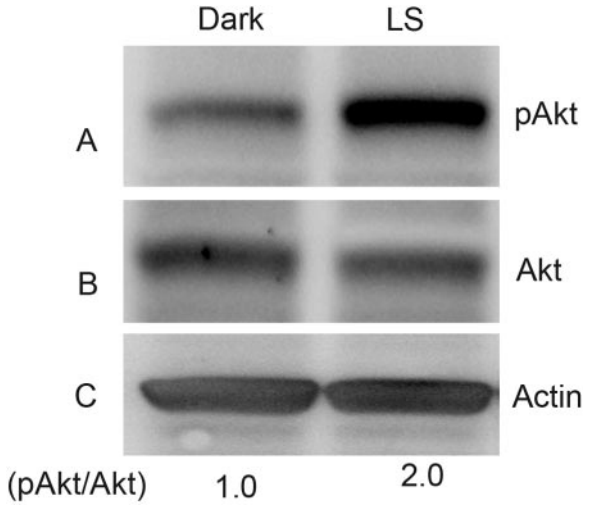

FIGURE 7. Activation of Akt in vivo and ex vivo. Dark- and light-stressed retinas were homogenized, and the lysates were subjected to Western blot analysis with anti–pAkt antibody (**A**). The blot was stripped and reprobed with anti–Akt (**B**) and anti–actin (**C**) antibodies to ensure equal amounts of protein in each lane.

same binding interaction with p85 that we observed in light stress, we performed GST pull-down assays with wild-type and mutant SH2 domains of p85. Proteins from IGF-1–treated or untreated retinas were incubated with wild-type (N-SH2 and C-SH2) and mutant (N-SH2-R358A and C-SH2-R649A) GST-p85 fusion proteins and were subjected to GST pull-down assays. The bound proteins were subjected to Western blot analysis with anti–IGF-1R antibody. Results indicated that increased binding of IGF-1 stimulated IGF-1R to the C-SH2 domain of p85 compared with the N-SH2 domain (Fig. 8E). The blot was reprobed with anti–GST antibody to ensure an equal amount of fusion in each pull-down (Fig. 8F). These results suggested that the differential binding affinities observed in ligand compared with light stress conditions could have resulted from differential phosphorylation states on the IGF-IR.

IGF-1–Induced Activation of Akt Ex Vivo

A significant increase in Akt phosphorylation was also observed in IGF-1–stimulated retinas compared with nonstimulated retinas (Fig. 8G). The blots were stripped and reprobed with anti–Akt and anti–actin antibodies to ensure equal amounts of protein in each lane (Figs. 8H, 8I). These results suggested that IGF-1Rs are functional and are responsive to growth factor IGF-1. Collectively, these results suggested that IGF-1R activation leads to the activation of PI3K and Akt survival signaling ex vivo.

DISCUSSION

We previously reported that physiological light induces the tyrosine phosphorylation of retinal IR, which leads to the activation of the anti-apoptotic enzyme PI3K[12] and the subsequent activation of Akt (data not shown). IR and IGF-1R belong to the same family of receptor tyrosine kinases. Interestingly, we found that IGF-1R activation is not light dependent. These observations suggest that even though these receptors belong to the same family of receptor tyrosine kinases, their activation may be differentially regulated. In this study, we also found an increased activation of IGF-1R in response to light stress. The activated IGF-1R associates with PI3K in the retina and in ROS. We also observed that the activation of Akt, the downstream effector molecule of PI3K, is also activated in response to light stress. These observations suggest that IGF-1R activation may be neuroprotective. However, further studies are required to understand the role of IGF-1R in photoreceptor functions.

The involvement of protein tyrosine phosphorylation in the regulation of PI3K activity is well reported.[24,27–30] In most cases, however, PI3K is regulated through the receptor and nonreceptor tyrosine kinases without apparent phosphorylation of the p85 subunit.[31,32] As previously shown,[31,32] we observed increased association of PI3K activity with anti–phosphotyrosine immunoprecipitates in light stress retina and in ROS. We previously observed the light stress–induced activation of retinal IR.[20] In the present study, our results suggested that IGF-IR activation is also light stress dependent. We cannot rule out the possibility of the involvement of other receptor tyrosine kinase(s) in the activation of PI3K in light stress.

Previous studies demonstrated that high-affinity association between p85 and the IGF-1R is dependent on the phosphorylation state of the receptor and occurs through the SH2 domains of p85.[33] Because of the well-known ability of SH2 domains to tightly bind to phosphotyrosine-containing peptides,[34–40] it is likely that one or both of these SH2 domains are responsible for this interaction. We previously reported that retinal IR binds to the N-SH2 but not the C-SH2 domain of p85.[41] The results from our study indicated that the N- and C-SH2 domains of p85 are involved in the binding interaction with IGF-IR under light stress conditions compared with IGF-IR activation in response to IGF-1. The differential binding could have been attributed to the differential phosphorylation states on IGF-1R in response to ligand compared with light stress conditions.

This study demonstrates that light stress activates tyrosine phosphorylation of ROS proteins, including IGF-1R, which promotes the binding of PI3K to ROS membranes. The mechanism behind the light stress–induced activation of IGF-1R is unknown. The possibility that light stress might induce the ligands for the activation of IGF-IR cannot be ruled out. In addition, we observed a differential binding pattern of IGF-IR to isolated N- and C-SH2 domains of p85. Light stress–activated IGF-1R binds to N- and C-SH2 domains of p85, whereas IGF-1–stimulated IGF-1R preferentially binds to the C-SH2 rather than the N-SH2 domain of p85. These observations suggest that other regulatory mechanisms may be involved in the light stress–induced activation of IGF-IR. Another mechanism may involve the activation of a non–receptor tyrosine kinase(s) in response to light. It has been shown that the non–receptor tyrosine kinase Src phosphorylates insulin and insulinlike growth factor receptors on autophosphorylation sites.[42,43] Thus, the Src kinase has been shown to substitute for the ligand-dependent receptor activation.[42,43] It has previously been shown that c-Src associates with light-activated opsin.[44] The IGF-1 receptors in ROS are localized to plasma membrane, and the insertion of the IR into the plasma membrane is necessary for hyperosmotic stress-induced receptor activation, as reported previously.[45] It is possible that light stress–induced conformational changes in the plasma membrane may result in the activation of the IGF-1R. In recent years it has become apparent that receptor tyrosine kinases (RTKs) and the signaling pathways they activate are part of a large signaling network that can be regulated by multiple extracellular cues, such as cell adhesion, agonists of G protein–coupled receptors, lymphokines, and stress signals.[46] RTKs have also been shown to be activated by membrane depolarization by various stress responses, including hyperosmotic conditions, ultraviolet radiation, white light, and G protein–coupled receptors.[47] Consistent with these studies, we recently reported that the state of IR phosphorylation is regulated through the photobleaching of G protein–coupled receptor rhodopsin.[13] Photoreceptor cell membranes are more susceptible to light-induced depolariza-

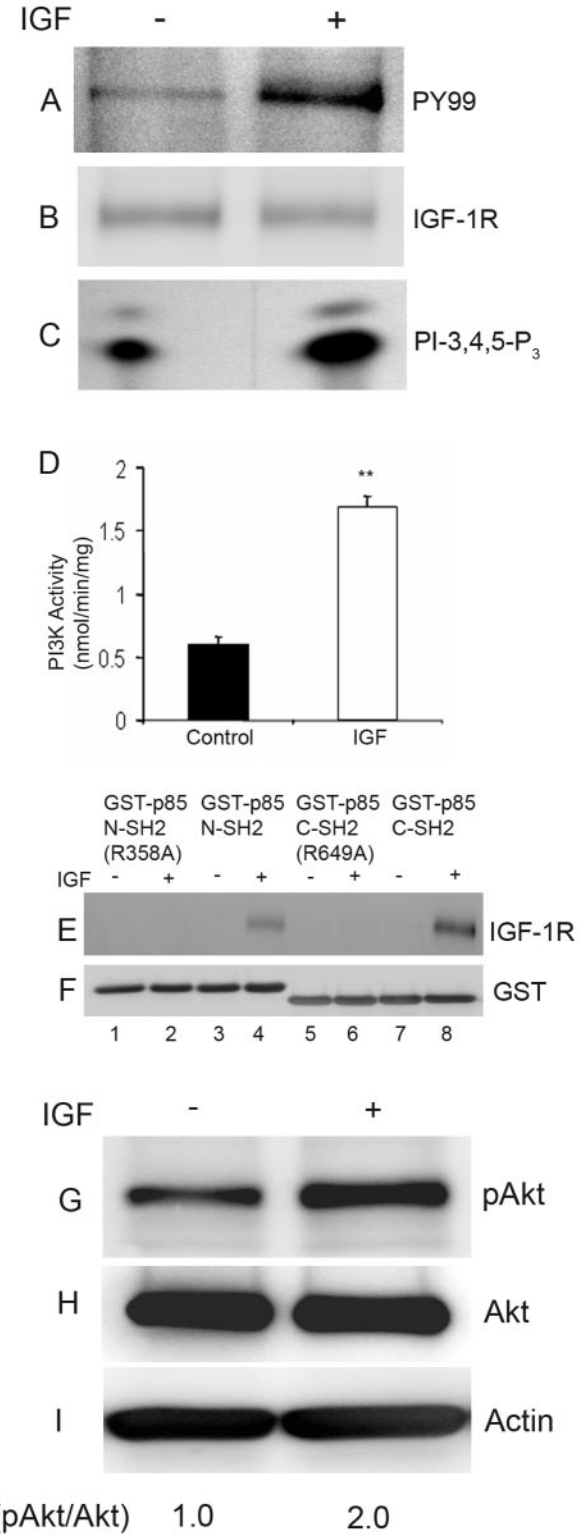

FIGURE 8. IGF-1–induced activation of IGF-1R, PI3K, and Akt ex vivo. Rat retinas were stimulated with or without 1.3 nM IGF-1 in organ cultures, lysed, and subjected to immunoprecipitation with anti–IGF-1R antibody and then to Western blot analysis with anti–PY-99 antibody (**A**). The blot was stripped and reprobed with anti–IGF-1R antibody to ensure equal amounts of protein in each immunoprecipitation (**B**). PI3K activity was measured in anti–IGF-1R immunoprecipitates of IGF-stimulated and unstimulated retinas using PI-4,5-P_2 and [γ^{32}P] ATP as substrates (**C**). Radioactive spots of PI-3,4,5-P_3 were scraped from TLC plates and counted (**D**). Data are mean ± SD ($n = 3$; $^{**}P < 0.01$). IGF-1–treated or untreated retinal lysates were incubated with GST-NSH2, GST-NSH2 (R358A), GST-CSH2, or GST-CSH2 (R649A) fusion proteins and subjected to the GST pull-down assay. Bound proteins were subjected to Western blot analysis with anti–IGF-1R antibody (**E**). Blots were reprobed with anti–GST-antibody to ensure equal amounts of fusions in each pull down (**F**). *Lanes 1, 3, 5, 7*: without IGF-1. *Lanes 2, 4, 6, 8*: with IGF-1. Activation of Akt ex vivo. IGF-1– treated or untreated retinas were homogenized, and the lysates were subjected to Western blot analysis with anti–pAkt antibody (**G**). The blot was stripped and reprobed with anti–Akt (**H**) and anti–actin (**I**) antibodies to ensure equal amounts of protein in each lane.

tion.[47] These possibilities cannot be ruled out in the light stress–induced activation of IGF-1 receptors.

Previous studies have shown that the IGF-1R is present in rat retina and can be phosphorylated in response to IGF-1 ex vivo.[26] Consistent with this observation, we also observed the activation of IGF-1R in response to IGF-1 ex vivo. IGF-1 is also known to activate PI3K in nerve growth cones.[48] Collective evidence shows that IGF-1 and its receptor system plays a critical role in maintaining the neuronal function and survival of many retinal cell types in vitro and in vivo.[6,49–52] Schlueter et al.[53] showed that targeted knockdown or specific inhibition of IGF-1R signaling resulted in reduced body size, slowed developmental rate, and increased embryonic lethality in zebrafish. IGF-1R–deficient embryos had significant defects in the retina and other neural tissues caused by elevated apoptosis.[53] The same group also demonstrated that overexpression of bcl2 in IGF-1R–deficient embryos led to a recovery of these tissues, strongly suggesting that IGF-1R signaling regulates the development of the retina and possibly other neuronal tissues by promoting cell survival. Collectively, previous studies[53] and our data showed that IGF1-R is involved in a protective role in retinal degeneration, indicating the activation of IGF-1R, PI3K, and Akt survival pathway in rod photoreceptors.

The functional consequence of light stress–induced activation of the IGF-1R–associated PI3K activity in photoreceptor cells is unknown. Activation of the IGF-1R signaling pathway has been shown to have complex physiological roles in different organelles.[54] It is known that focal injury to rat retina protects photoreceptors from degeneration.[55] In the Royal College of Surgeons rat with inherited photoreceptor degeneration, mechanical injury produced by the injection of saline into the subretinal space or into the vitreous, or even a needle insertion without injection, protects photoreceptors near the wound.[55,56] In light-induced photoreceptor degeneration, similar injury-dependent protection is also observed.[57] These findings imply that the retina has a self-protective mechanism whose activation protects photoreceptors from damage and death. The exact mechanism for the retinal self-protective role is unknown. Activation of the IGF1-R/PI3K/AKt survival pathway may serve a protective role in the retina. In this study, we demonstrated that increased IGF-1R–associated PI3K activity in photoreceptor cells in response to light stress could be the result of an innate self-protection mechanism. In some neuronal cell types, such as cerebellar granular neurons[58] and PC-12 cells,[59] receptor activation of PI3K has been shown to protect these cells from stress-induced neurodegeneration. Our studies suggest that IGF-1R activation may be neuroprotective.

IR and IGF-1R are structurally similar. Both are found at the cell surface as $\alpha_2\beta_2$ heterotetramers with transmembrane ligand–binding α-subunits and intracellular, tyrosine kinase–containing β-subunits. Furthermore, both receptors, once activated, can phosphorylate or interact with the same intracellular protein substrates, including members of the insulin receptor substrate (IRS) family, and Src homology and collagen domain protein (Shc). IR and IGF-1R also activate

many of the same downstream signaling molecules, such as PI3K and mitogen-activated protein kinase.[60,61] In retinal endothelial cells, insulin and IGF-1 receptors respond differentially to oxygen.[62] The insulin and IGF-1 signaling in endothelium play a role in retinal neovascularization through the expression of vascular mediators. The effect is that insulin is more important in this process than IGF-1, as demonstrated by the advent of endothelial cell–specific insulin and IGF-1 receptor knockout mice.[62] In the photoreceptors, IR[20] and IGF-1 receptor (present study) activate neuroprotective PI3K and Akt. These studies suggest the redundant and nonredundant roles of IR and IGF-1 receptors in various biological functions in a tissue-specific manner. Further studies are required to understand the functional role of IGF-1 receptors in photoreceptor functions.

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