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Light-dependent Association of Src with Photoreceptor Rod Outer Segment Membrane Proteins *in Vivo**

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***In vivo* light exposure results in tyrosine phosphorylation of several rod outer segment (ROS) proteins (Ghalayini, A. J., Guo, X. X., Koutz, C. A, and Anderson, R. E. (1998) *Exp. Eye Res.* 66, 817–821). We now report the presence of Src in ROS and its increased association with bleached ROS membranes. Immunoprecipitation with anti-phosphotyrosine revealed that tyrosine kinase activity recovered from light-adapted ROS membranes was twice that recovered from dark-adapted ROS. Other experiments revealed the presence of both bleached rhodopsin and arrestin in immunoprecipitates of LROS, suggesting the formation of a multimeric complex containing Src, arrestin, and bleached rhodopsin. Additionally, when immobilized Src homology domains 2 and 3 (SH2 and SH3, respectively) were used to study the association of Src with ROS membranes, only bleached opsin and arrestin were found to associate with the SH2 domain of Src. These data strongly suggest that Src through its SH2 domain interacts with bleached rhodopsin and arrestin either directly or indirectly. Similar results were also obtained when dark-adapted and light-adapted retinas were used instead of ROS membranes. Our data strongly suggest that light exposure *in vivo* activates Src and promotes its association through its SH2 domain with a complex containing bleached rhodopsin and arrestin. A hypothesis for the functional significance of this phenomenon is presented.**

Tyrosine phosphorylation plays an important role in many cellular functions including growth, development, proliferation, and survival. Several extracellular signals including insulin, growth factors, mitogens, and others produce their intracellular effects through the activation of the appropriate receptor tyrosine kinases or nonreceptor protein-tyrosine kinases (1). This activation results in tyrosine phosphorylation of key intracellular proteins that are either enzymes or play the role of adaptor proteins between other intracellular signaling proteins (2). In the case of receptor tyrosine kinases, receptor activation causes a rapid autophosphorylation on tyrosine residues, which leads to association of these receptors with key intracellular proteins, resulting in tyrosine phosphorylation of these target proteins. Among the enzymes that are tyrosine-phosphorylated upon activation are Ras-GTPase-activating protein, phospholipase Cγ (PLCγ),[1] phosphatidylinositol 3-kinase, protein-tyrosine phosphatases including the ubiquitous SHP-2, and a growing list of other enzymes (2–5).

Although most of the current information regarding the biological significance of tyrosine phosphorylation involves receptor tyrosine kinases and nonreceptor protein-tyrosine kinases that are stimulated by growth factors and mitogens, there is recent evidence indicating the involvement of G-protein-coupled receptors (GPCRs) in the generation of tyrosine phosphorylation-based signals (for a recent review, see Ref. 6). One such example is the angiotensin II receptor (AT_1), a seven-transmembrane receptor with no intrinsic kinase activity, which has been shown to induce rapid tyrosine phosphorylation of several intracellular proteins including PLCγ_1 (7, 8), Shc, mitogen-activated protein kinase, and focal adhesion kinase (9–11). Other studies (12) have demonstrated the involvement of Src in GPCR activation of mitogen-activated protein kinase. More recently, the formation of a signaling complex between Src, β-arrestin, and β_2-adrenergic receptor has been reported (13). In earlier studies, we have shown that light adaptation *in vivo* stimulates tyrosine phosphorylation in rod outer segments (ROS) of rat retina (14, 15). The most likely mediator of such an effect of light in ROS is rhodopsin. This would imply that rhodopsin bleaching, similar to the activation of other GPCRs, stimulates an intracellular tyrosine kinase, leading to tyrosine phosphorylation of key intracellular ROS enzymes and proteins. More recently, we have reported the association of Src with ROS membranes following light exposure *in vivo* (16). In the current study, we have investigated the identity of the tyrosine kinase that may be activated by light. Our studies show that Src binds specifically to ROS membranes obtained from retinas exposed to light *in vivo*. We also demonstrate that this membrane-associated form of Src is the activated form of this enzyme. We present additional evidence that Src associates specifically with bleached rhodopsin and arrestin in immune complexes obtained with anti-Src and that this association is specifically mediated by the SH2 domain of Src.

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[1] The abbreviations used are: PLCγ, phospholipase Cγ; ROS, rod outer segment(s); SH2, Src homology 2; SH3, Src homology 3; PTB, phosphotyrosine binding domain; GPCR, G-protein-coupled receptor; GST, glutathione S-transferase; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; LROS, light-adapted ROS; DROS, dark-adapted ROS.

EXPERIMENTAL PROCEDURES

ROS Preparation—Albino Sprague-Dawley rats were dark-adapted overnight and sacrificed either under dim red light, or following 30 min of light exposure (~300 lux). ROS were prepared on a discontinuous sucrose gradient from either dark- or light-adapted retinas as previously described (17) with some modification. Retinas were homogenized in 1.5 ml of 47% sucrose solution containing 100 mM NaCl, 1 mM EDTA, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 mM Tris-HCl, pH 7.4 (buffer A). Retinal homogenates were transferred to 5-ml centrifuge tubes and sequentially overlaid with 1.0 ml of 42%, 1.0 ml of 37%, and 1.5 ml of 32% sucrose solutions dissolved in buffer A. The gradients were spun at 82,000 × *g* for 1 h at 4 °C. The 32/37% interfacial sucrose band containing ROS membranes was harvested; diluted with 10 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 1 mM EDTA, 1 mM orthovanadate; and centrifuged at 27,000 × *g* for 30 min. The ROS pellets obtained were resuspended in 10 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 1 mM EDTA, and 1 mM orthovanadate and stored at −20 °C. The non-ROS band designated band II (37/42% interface) and the remainder of the retina were also saved for comparison with ROS. All procedures were carried out under dim red light for dark-adapted retinas and in room light for light-adapted retinas. Protein was determined by the BCA reagent from Pierce.

SDS-PAGE and Immunoblot Analysis—SDS-PAGE was performed using 7.5 or 10% gels according to Laemmli (18). Resolved proteins were transferred to plastic backed nitrocellulose sheets (0.2 μm) or Immobilon P membranes using a Genie electroblotter (Idea Scientific Company, Minneapolis, MN) for 2–3 h. Membranes were blocked overnight with Tris-buffered saline (20 mM Tris-HCl, 300 mM NaCl), pH 7.5, containing 0.1% Tween 20 and 5% crystalline grade bovine serum albumin. Incubations with primary antisera were performed for 2–3 h at room temperature. Immunoreactions were detected either with alkaline phosphatase or horseradish peroxidase conjugated to goat anti-rabbit or goat anti-mouse IgG, followed by either alkaline phosphatase substrate nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate, *p*-toluidine salt or enhanced chemiluminescence substrates (ECL; Amersham Biosciences, Inc.). In some experiments, immunoblots were stripped by incubation in 100 mM Tris-HCl buffer, pH 6.7, containing 100 mM β-mercaptoethanol, 2% SDS for 1 h at 50 °C with gentle agitation. Blots were reblocked with 5% bovine serum albumin in Tris-buffered saline and probed as described above. Densitometric scan of immunoblots was performed using ONE-Dscan software (Scanalytics, Billerica, MA).

Immunoprecipitation—ROS membranes (100 μg) were solubilized in 0.2 ml of buffer B, pH 7.5, containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM orthovanadate, 1% Triton X-100 with or without 0.1% SDS; mixed thoroughly; and centrifuged at 27,000 × *g* for 30 min at 4 °C to remove any particulate material. The cleared supernatants were transferred to fresh microcentrifuge tubes and incubated with 50 μl of protein A-agarose/protein G-agarose for 30 min at 4 °C with gentle mixing. The suspension was centrifuged at 27,000 × *g* for 30 min, and the precleared supernatant was incubated with anti-Src (5 μg) and 50 μl of protein A-agarose (4 mg/ml) or protein G-agarose for 3–4 h at 4 °C with gentle mixing. The immunoprecipitates were collected by centrifugation at 2000 × *g* for 5 min, washed (four times) with 0.4 ml of buffer B. The immunoprecipitates were solubilized in sample buffer for SDS-PAGE and subjected to immunoblot analysis with anti-opsin (1:2000) or anti-arrestin (1:2000).

Immune Complex Tyrosine Kinase Assay—Typically, ROS membranes (100 μg) or "whole retinal" homogenates were solubilized in buffer B without SDS as described above. The cleared supernatants were transferred to fresh microcentrifuge tubes and incubated with 50 μl of protein A-agarose/protein G-agarose or GST-agarose for 60 min at 4 °C with gentle mixing. The suspension was centrifuged at 27,000 × *g* for 30 min, and the precleared supernatants were incubated with PY99-agarose (40 μg) or normal mouse IgG-agarose (40 μg) for 2 h at 4 °C with gentle mixing. The immunoprecipitates were collected by centrifugation at 2000 × *g* for 5 min, washed (three times) with 0.4 ml of buffer B (without SDS) and two times with 50 μl of kinase assay buffer. Tyrosine kinase activity in the immune complex was assayed as previously described (19) using exogenously added Raytide EL as substrate in kinase assay buffer containing 50 mM HEPES (pH 7.5), 0.1 mM EDTA, 0.02% Brij 35, and 5 μg of Raytide EL. The reaction was initiated by the addition of 10 μl of ATP mix (0.2 mM ATP and 30 mM $MgCl_2$) and 10 μCi of [γ-^{32}P]ATP. Incubations were carried out for 30 min at 37 °C in a final volume of 35 μl. The reaction was terminated by the addition of 120 μl of 10% phosphoric acid, and the reaction mixture was applied to a 2.5-cm diameter Whatman P81 filter paper and washed 5 × 10 min in 5 ml of 0.5% phosphoric acid. Radioactivity was quantitated by scintillation counting. Enzyme activity is expressed as difference between counts incorporated into Raytide using either PY99-agarose or normal mouse IgG-agarose.

Src SH2 Domain Binding—ROS membranes or retinal homogenates from dark- and light-adapted retinas were solubilized in buffer B without SDS as previously described, and equal aliquots based on total protein were precleared with 20 μg of GST-agarose for 1 h at 4 °C and incubated with either 20 μg of GST-agarose (control) or 20 μg of SH2-agarose overnight at 4 °C with gentle mixing. The agarose beads were centrifuged briefly at 2000 × *g* and washed four times with buffer B, and the associated proteins were solubilized in SDS-PAGE sample buffer and subjected to immunoblot analysis with either anti-opsin or anti-arrestin.

Preparation of Cytoskeletal-enriched Fraction—ROS membranes (100 μg) were solubilized in 0.2 ml of buffer B, pH 7.5, containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM orthovanadate, 1% Triton X-100; mixed thoroughly; and centrifuged at 27,000 × *g* for 30 min at 4 °C. The supernatant (solubilized membranes) was removed, and the detergent-insoluble pellet was washed a second time with the same buffer, centrifuged at 27,000 × *g* for 30 min, and the final pellet was resuspended in SDS-PAGE sample buffer followed by immunoblot analysis.

Immunocytochemistry—Eyes were fixed in perfix (20% isopopropyl alcohol, 2% trichloroacetic acid, 4% paraformaldehyde, and 2% zinc chloride) for 48 h; dehydrated in 70, 80, 95, and 100% ethanol for 45 min each; and embedded in paraffin. For immunocytochemistry, sections were dewaxed and rinsed sequentially with water and phosphate-buffered saline, and endogenous peroxidase was inactivated by incubation at room temperature for 20 min with H_2O_2 (0.05%). Sections were blocked for 1 h with phosphate-buffered saline, pH 7.4, containing 0.25% Triton X-100 and 0.3% bovine serum albumin. Incubations with anti-Src (N terminus; 2.5 μg/ml), or anti-arrestin (1:4000) were carried out for 2 h at room temperature in a humidified chamber. After rinsing the sections (three times) with phosphate-buffered saline, sections were incubated with biotinylated goat anti-rabbit secondary antibodies, followed by incubation with avidin-biotin complex (Vectastain kit; Vector Laboratories). After rinsing with phosphate-buffered saline (three times), immunoreactions were developed in 50 mM Tris-HCl (pH 7.5) containing 0.06% diaminobenzidine and 0.01% H_2O_2 for 7–20 min at room temperature. Slides were rinsed in tap water, and the reaction was visualized on a Zeiss Axiovert photomicroscope.

Antisera—Monoclonal anti-phosphotyrosine (PY99), polyclonal anti-Src (C terminus amino acids 509–533), polyclonal anti-Src (N terminus amino acids 3–18), SH2, SH3, and SH2-SH3 fusion proteins coupled to GST-agarose corresponding to Fyn were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). SH2-GST-agarose corresponding to the SH2 domain of Src was obtained from Calbiochem. Clone 28, a monoclonal antibody to activated Src (20) was a gift from Dr. Hisaaki Kawakatsu (University of California, San Francisco). Polyclonal anti-bovine arrestin was a gift from Dr. Igal Gery at the National Eye Institute (Bethesda, MD). Monoclonal anti-opsin antibody (Rho 4D2) was a gift from Dr. Robert Molday (University of British Columbia).

RESULTS

Association of Src with Light-adapted ROS—Immunoblot analyses of retinal homogenates, non-ROS membranes, and ROS membranes from dark- (*D*) and light-adapted (*L*) retinas with anti-Src are shown in Fig. 1*A*. LROS membranes were significantly enriched in the amount of S*rc* over DROS. Densitometric scanning of the immunoblot showed a 6.6-fold enrichment of LROS over DROS. No detectable light-dark differences were observed between retinal homogenates or non-ROS membranes (band II), indicating that Src association with ROS membranes is light-dependent and that it occurs specifically in ROS. Fig. 1*B* shows the enrichment of arrestin (an independent indicator of light adaptation) in LROS over DROS. In addition, anti-arrestin recognized a band (*) of ~85 kDa, which was also recognized by anti-opsin (not shown), thus indicating that this may represent an opsin-arrestin aggregate. The enrichment of opsin in both DROS and LROS (Fig. 1*C*) over the other membrane fractions further validates the specificity of the membrane association of Src with LROS. Coomassie Blue-stained gel of all fractions used is shown in Fig. 1*D*.

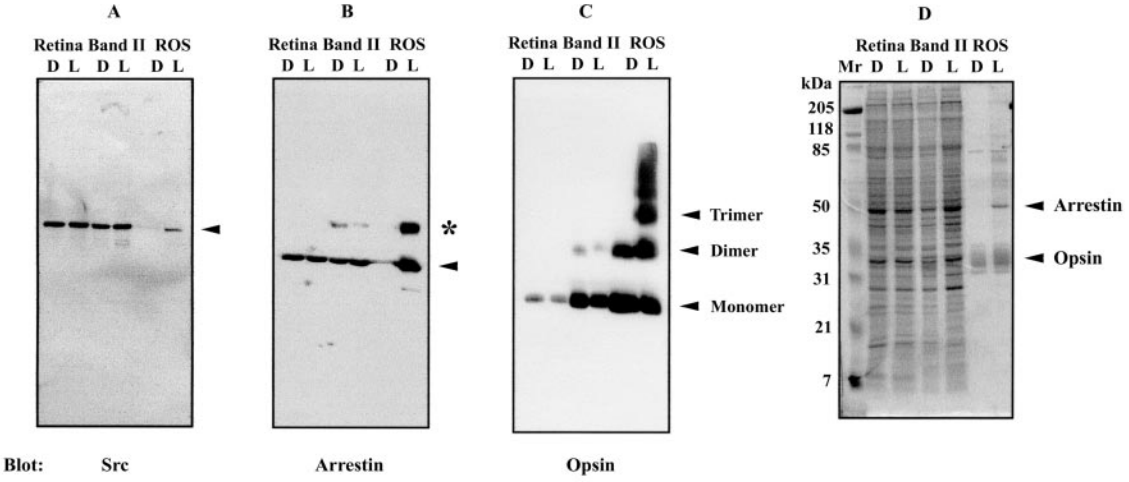

FIG. 1. **Light-dependent binding of Src to ROS.** *A*, immunoblot of "whole retinal" homogenates (*Retina*), non-ROS (*Band II*, 37/42% interface), and ROS membranes from dark- (*D*) and light-adapted (*L*) retinas *in vivo* with anti-*Src* at 1 μg/ml. Each *lane* contains 20 μg of protein. *B*, reprobe of the same immunoblot with anti-arrestin (1:2000). *C*, reprobe of the same immunoblot with anti-opsin (1:500). *D*, Coomassie Blue-stained gel of samples.

Light-induced Tyrosine Phosphorylation of ROS—In other experiments, we compared both the extent of tyrosine phosphorylation and amount of Src associated with DROS and LROS membranes (Fig. 2). Immunoblots of DROS and LROS with PY99 show that tyrosine phosphorylation of several proteins was increased in LROS over DROS in response to *in vivo* light exposure (Fig. 2*A*). The most pronounced increases were observed with proteins of apparent molecular mass of ~60, 70, 80, 110, 120, and 160 kDa. The 60-kDa protein showed the highest extent of tyrosine phosphorylation. Densitometric scanning of the blot showed that this represents a 1.9-fold increase in tyrosine phosphorylation of LROS over DROS. A reprobe of the same immunoblot with anti-Src antibody identified the 60-kDa protein as Src (Fig. 2*B*). Densitometric scans of anti-Src immunoblot showed a 2.3-fold increase in the amount of Src associated with LROS, indicating that the increase in phosphotyrosine immunoreaction represents the increase in the amount of Src associated with LROS and not an increase in tyrosine phosphorylation of Src. Other membranes (non-ROS) obtained from the same gradient (*DII* and *LII*) showed no apparent difference in either phosphotyrosine or Src content (Fig. 2, *A* and *B*, respectively), indicating the specificity of the effect of light on photoreceptor ROS membranes. The extent of enrichment of Src in LROS over DROS varied from 1.6- to 3.8-fold (Fig. 2*C*).

Light-dependent Activation of Tyrosine Kinase in ROS—In other experiments, PY99 immunoprecipitates from DROS and LROS were subjected to immunoblot analysis with anti-Src and assayed for tyrosine kinase activity *in vitro* (Fig. 3). As shown in Fig. 3*A*, Src was recovered in PY99 immunoprecipitates from both DROS and LROS (*lanes 3* and *4*, respectively), while it was absent in control immunoprecipitates with normal mouse IgG (*lanes 1* and *2*). When PY99 immunoprecipitates were assayed for tyrosine kinase activity using exogenously added Raytide as substrate, LROS immunoprecipitates showed twice the activity of DROS immunoprecipitates (Fig. 3*B*).

Light-adapted ROS Membranes Are Enriched in Activated Src—In other experiments, we compared the amount of Src associated with DROS and LROS using two different antibodies: a peptide-specific anti-Src and a monoclonal antibody, designated clone 28, that recognizes only the activated form of Src (20). Immunoblot analysis of D and LROS is shown in Fig. 4. Clone 28 recognized Src in LROS membranes, whereas immunoreaction was barely detectable in DROS, indicating that the activated form of Src preferentially associates with LROS and not DROS. Densitometric scans of the immunoblot showed a 4.6-fold enrichment of activated Src in LROS over DROS (Fig. 4*A*). When the same immunoblot was stripped and reprobed with anti-Src, a similar increase in the amount of Src was observed in LROS over DROS (Fig. 4*B*). Densitometric scans showed a 3.3-fold increase in LROS over DROS. DROS and LROS were immunoprecipitated with clone 28 to identify any associated proteins in the immunoprecipitate. When the immunoblot was sequentially probed for arrestin and Src, they were both detected in the immunoprecipitates from DROS and LROS (Fig. 4*C*); however, more of both Src and arrestin were recovered from LROS than DROS.

Association of Src with Bleached Rhodopsin and Arrestin—In an attempt to determine how Src associates with ROS membranes, solubilized DROS and LROS were immunoprecipitated with anti-Src, and the immunoprecipitates were subjected to immunoblot analysis with anti-opsin. As shown in Fig. 5*A*, opsin was detected only in immunoprecipitates of LROS, with the monomer and dimer of opsin being the predominant form in the immunoprecipitates. This indicates that Src associates specifically with bleached rhodopsin in ROS membranes. Arrestin was also detected in immunoprecipitates of LROS but was absent from immunoprecipitates with normal rabbit IgG (Fig. 5*B*). These results indicate the presence of a multimeric complex containing Src, bleached rhodopsin, and arrestin. When whole retinal homogenates from dark- and light-adapted retinas were immunoprecipitated with anti-opsin, Src was only detected in light-adapted immunoprecipitates (Fig. 5*C*).

Activated Src Is Associated with Bleached Rhodopsin—In other experiments, tyrosine phosphorylated proteins from "whole retina" were affinity-eluted from PY99-agarose with phenylphosphate, and the eluates were either assayed for tyrosine kinase activity or subjected to immunoblot analysis with anti-Src or anti-opsin (Fig. 6). As shown, Src was recovered in phenylphosphate eluates from both dark- and light-adapted retinas; however, only the eluates from light-adapted retinas contained opsin indicating a light-dependent association between Src and opsin (Fig. 6*A*, *lanes 2* and *4*). Neither Src nor opsin was detectable in control eluates using GST-agarose instead of PY99-agarose (Fig. 6*A*, *lanes 1* and *3*). When eluates from PY99 immunoprecipitates were assayed for tyrosine kinase activity using exogenously added Raytide, light-adapted

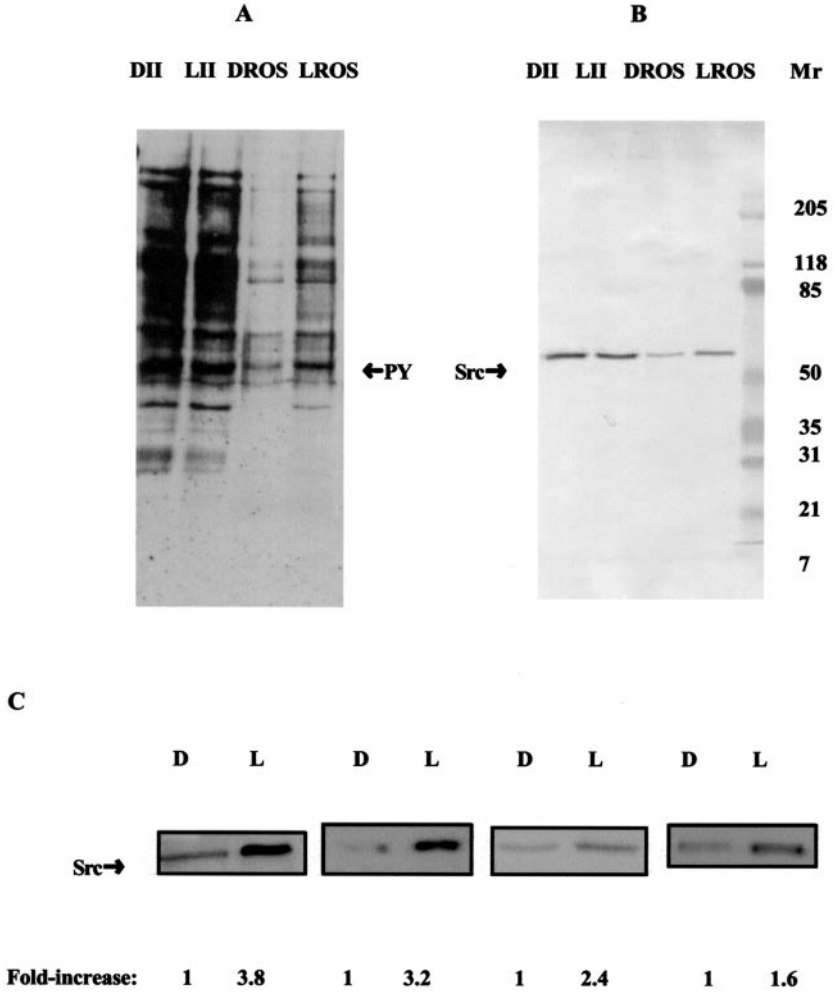

FIG. 2. **Light-mediated tyrosine phosphorylation of ROS proteins.** *A*, immunoblot of DROS and LROS membranes (17 μg/lane) with anti-phosphotyrosine (PY99) at a concentration of 1 μg/ml. *D II* and *L II* contained 40 and 39 μg/lane, respectively. *B*, a reprobe of the same immunoblot with anti-Src at 1 μg/ml. *C*, immunoblot of four pairs of DROS and LROS preparations with anti-Src. Each pair contained an equal amount of total ROS protein. The -fold increases based on densitometric scans of immunoblots are shown *below*.

retinas had 1.5-fold the activity of dark-adapted retinas (Fig. 6*B*), indicating that activated Src is specifically associated with bleached rhodopsin either directly or indirectly.

Src SH2 Domain Mediates Association with Bleached Opsin—In other experiments, we tested the interaction of Src fusion proteins with DROS and LROS as well as dark- and light-adapted "whole retinal" homogenates (Fig. 7). As shown in Fig. 7*A*, both bleached opsin and arrestin were found to associate with Src SH2 domain (*lane 4*), whereas neither opsin nor arrestin was detected in DROS (*lane 2*). Control incubations of DROS and LROS with GST-agarose (*lanes 1* and *3*) also showed the absence of opsin and arrestin, indicating the specificity of interaction of Src SH2 domain with bleached opsin. Similar results were obtained when dark- and light-adapted "whole retinal" homogenates were used to test the interaction with the Src SH2 domain (Fig. 7*B*). Comparison of the interaction of different Src domains with dark- and light-adapted "whole retinal" homogenates showed that bleached opsin associates predominantly with the SH2 domain of Src and to a much lower extent with the SH3 domain (Fig. 7*C*). Moreover, the amount of opsin recovered from SH2-SH3 fusion protein was similar to that recovered with SH2 fusion protein, suggesting that bleached rhodopsin interaction with Src occurs predominantly through the SH2 domain of Src.

Colocalization of Arrestin and Src to ROS Cytoskeleton—A cytoskeletal enriched fraction obtained from DROS and LROS was subjected to immunoblot analysis with anti-Src and anti-arrestin (Fig. 8). As shown, the light-adapted cytoskeletal pellet was significantly enriched with Src and arrestin over the dark-adapted pellet, indicating that light exposure specifically promotes the association of Src and arrestin with the ROS cytoskeleton.

Immunolocalization of Src in Rat Retina—Immunocytochemistry of sections from dark- and light-adapted albino rat retinas are shown in Fig. 9. In the outer retina, immunoreaction was detected throughout the inner and outer segments of photoreceptor cells in both dark- and light-adapted retinas. However, immunoreaction with light-adapted sections was much more pronounced that of dark-adapted sections (Fig. 9*A*), possibly indicating exposure of a specific Src epitope in response to light. No apparent difference in immunoreaction of this antibody with dark- and light-adapted retinal homogenates was observed (Fig. 9*C*), suggesting that this is unlikely to represent an increase in the amount of Src protein in the light. However, we cannot rule out that the observed difference in immunocytochemistry represents a light-dependent modification of Src. Sections incubated in the absence of primary antibody showed no reaction (not shown). As a positive control, immunolocalization of arrestin in dark- and light-adapted sections is shown in Fig. 9*B*. Arrestin was present predominantly in the inner segments of photoreceptors of dark-adapted retinas but was present only in the outer segment of light-adapted retinas, in agreement with previous reports (22). The translocation of arrestin between the inner and outer segments is quite evident; however, Src shows only differences in the intensity of reaction and not localization in response to light. These results suggest that Src might either translocate from the cytosol to the disc membranes/cytoskeleton or undergo a conformational change exposing a specific epitope in response to light.

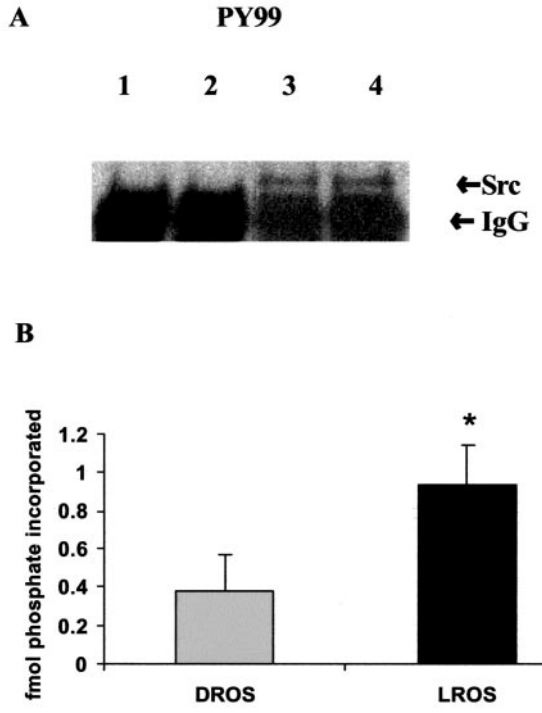

FIG. 3. **Src and tyrosine kinase activity in PY99 of DROS and LROS.** *A*, immunoprecipitates from DROS and LROS (100 μg each) with either normal mouse serum (*lanes 1* (*DROS*) and *2* (*LROS*), respectively) or PY99 (*lanes 3* (*DROS*) and *4* (*LROS*), respectively). *B*, tyrosine kinase activity in PY99 immunoprecipitates from DROS and LROS (50 μg each) using Raytide as substrate. Activity represents the difference in counts incorporated between PY99 immunoprecipitates and control immunoprecipitate from normal mouse IgG. *, significantly different; $p < 0.05$, two-tailed, equal variance t test, $n = 3$.

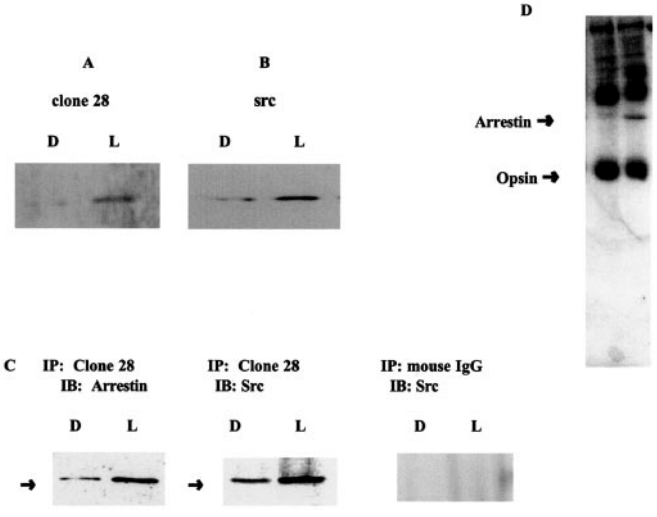

FIG. 4. **Enrichment of LROS with activated Src and its association with arrestin.** *A*, immunoblot of DROS and LROS (10 μg/lane) with clone 28 at 1 μg/ml. *B*, reprobe of immunoblot with anti-*Src* at 1 μg/ml. *C*, immunoprecipitates (*IP*) of DROS and LROS membranes (100 μg) with clone 28 (2 μg) followed by immunoblot (*IB*) analysis with anti-Src and anti-arrestin. *D*, Coomassie Blue-stained gel of ROS (20 μg/lane) used in these experiments.

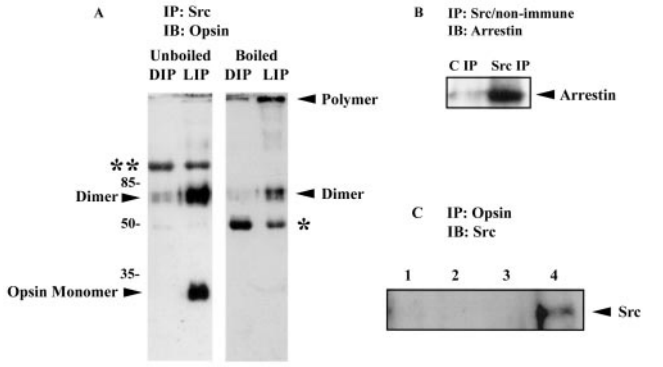

FIG. 5. **Association of Src with bleached rhodopsin and arrestin.** *A*, immunoprecipitates (*IP*) of DROS and LROS (100 μg) with anti-Src (5 μg) were separated on 10% gels, transferred to nitrocellulose, and probed with anti-opsin (1:1000). Equal aliquots from the immunoprecipitates were either boiled for 5 min or not boiled in denaturing buffer prior to SDS-PAGE. The *arrows* show the mobility of opsin monomer, dimer, and polymers. **, IgG heavy chain aggregate; *, IgG heavy chain. *B*, immunoprecipitates of LROS (80 μg) with either normal rabbit serum (*CIP*; 5 μg) or anti-Src (5 μg) were separated on 7.5% gels, transferred to nitrocellulose, and probed with anti-arrestin (1:2000). *C*, immunoprecipitation of "whole retinal" homogenates (150 μg each) with anti-opsin (*lanes 2* (*D*) and *4* (*L*)) or with normal mouse IgG (*lanes 1* (*D*) and *3* (*L*)), followed by immunoblot (*IB*) with anti-Src. Data are representative of three independent experiments.

DISCUSSION

We have previously reported that light stimulates tyrosine phosphorylation of photoreceptor ROS in rat retina *in vivo* (15). In the current report, we have observed that light exposure of albino rats promotes a specific association of Src with LROS membranes purified on a discontinuous sucrose gradient. LROS membranes were enriched 1.6–6.6-fold with Src over DROS membranes. Arrestin, which has been documented to bind to bleached and phosphorylated rhodopsin (21) was similarly enriched in these same membranes over DROS. LROS membranes also showed a significant increase in tyrosine phosphorylation of several proteins ranging in size from 60 to 140 kDa. The 60-kDa protein was identified as Src by sequential probing first with PY99 followed by anti-Src. Densitometric scanning of both blots revealed a similar increase in tyrosine phosphorylation and amount of Src (1.9- and 2.3-fold, respectively) associated with LROS, indicating that the apparent increase in the extent of tyrosine phosphorylation of Src simply represents the increase in the amount of Src associated with LROS. The identity of the 60-kDa protein was further verified by immunoprecipitation with PY99. These immunoprecipitates recovered Src from both DROS and LROS. Moreover, tyrosine kinase activity in PY99 immunoprecipitates from LROS was twice that of DROS, indicating that Src recovered from LROS is the activated form of the enzyme.

In other experiments, we investigated if the membrane-associated Src is an activated form of Src. There are two known phosphorylation sites on Src, which are Tyr^{419} and Tyr^{530} (for a review, see Ref. 23). It has been shown that the Tyr^{530} is an autoregulatory (inhibitory) phosphorylation site and that the unphosphorylated Tyr^{530} is the activated (open configuration) form of Src (20, 23). We have used monoclonal antibodies (clone 28), which specifically recognize the activated form of Src (13, 20), to probe DROS and LROS. Our results showed a significant immunoreaction of clone 28 with LROS but not DROS, whereas polyclonal anti-Src recognized Src in DROS and LROS. This indicates that the increase in membrane association of Src with LROS represents an increase in the activated form of Src. This was further verified by immunoprecipitation of both DROS and LROS with clone 28. Activated Src was present only in LROS immunoprecipitates, whereas it was absent from DROS immunoprecipitates. These findings indicate that light promotes the association of activated Src (unphosphorylated Tyr^{530}) with ROS membranes. However, we cannot rule out that this activation may also involve tyrosine phosphorylation of Tyr^{419}.

To investigate the mechanism of light-dependent membrane association of Src with ROS membranes, DROS and LROS

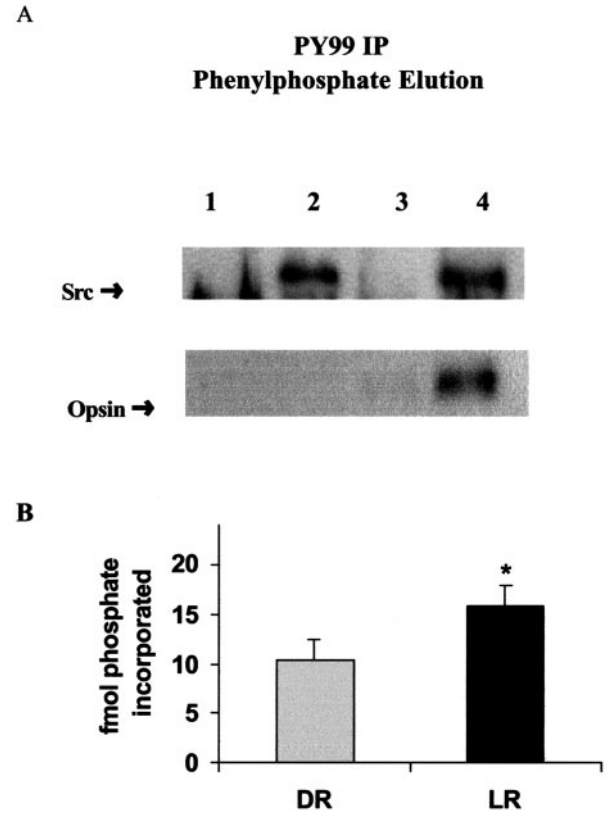

FIG. 6. **Phenylphosphate elution of Src and opsin from PY99-agarose from dark- and light-adapted retinas.** *A*, "whole retinal" homogenates (from dark- and light-adapted (30 min) retinas (*DR* and *LR*, respectively) were solubilized as described under "Experimental Procedures," and equal amounts of protein (100 μg each) were immunoprecipitated (*IP*) using either PY99-agarose (*lanes 2* (*D*) and *4* (*L*)), or GST-agarose (*lanes 1* (*D*) and *3* (*L*)). Immunoprecipitated proteins were eluted by incubation with 25 mM phenylphosphate for 1 h at 4 °C, and the eluate was probed on immunoblots with either anti-Src or anti-opsin. *B*, PY99 immunoprecipitations were performed as in *A*, except 150 μg of total protein/immunoprecipitate was used, and the immune complex was assayed for tyrosine kinase activity with Raytide as substrate. Activity represents the difference in counts incorporated between PY99-agarose immunoprecipitates and control immunoprecipitate from GST-agarose. *, significantly different; $p < 0.05$, two-tailed, equal variance *t* test, $n = 4$.

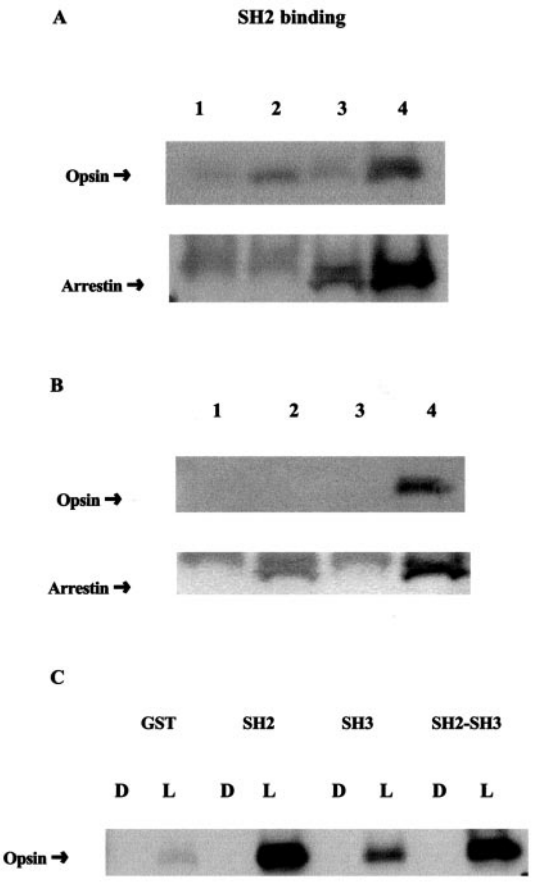

FIG. 7. **Association of bleached opsin and arrestin with Src SH2 domain.** *A*, DROS and LROS (63.5 μg each) were solubilized and incubated with either Src SH2-agarose (20 μg) or GST-agarose (control). Proteins recovered were subjected to SDS-PAGE and immunoblot analysis with either anti-opsin or anti-arrestin. *Lanes 1* and *3*, dark and light GST-agarose controls, respectively; *lanes 2* and *4*, dark and light proteins recovered from SH2 domain, respectively. *B*, experiments performed as in *A*, except solubilized "whole retinal" homogenates (from dark- and light-adapted (30 min)) retinas were used, and equal amounts of protein (100 μg each) were incubated with either Src SH2-agarose (20 μg) or GST-agarose (control). Proteins recovered were subjected to SDS-PAGE and immunoblot analysis with either anti-opsin or anti-arrestin. *Lanes 1* and *3*, dark and light GST-agarose controls, respectively; *lanes 2* and *4*, dark and light proteins recovered from SH2 domain, respectively. *C*, experiments performed as in *B*, except 150 μg of "whole retinal" lysate were used per incubation, and 40 μg of either SH2-agarose, SH3-agarose, or SH2-SH3-agarose were used. Proteins recovered were subjected to SDS-PAGE and immunoblot analysis with anti-opsin.

were immunoprecipitated with anti-Src. Immunoprecipitates from LROS were significantly enriched with rhodopsin over DROS, indicating that light specifically increases the association of Src with bleached rhodopsin. In other experiments, PY99 immunoprecipitates from dark- and light-adapted retinas showed the presence of both Src and bleached opsin in light-adapted retinas, whereas only Src was recovered from dark-adapted retinas. Furthermore, tyrosine kinase activity in PY99 immunoprecipitates from light-adapted retinas was 1.5-fold higher than dark-adapted retinas. These observations taken together suggest that light exposure promotes the formation of a complex that contains Src, arrestin, and bleached rhodopsin in photoreceptor outer segments and that activated Src is associated with bleached opsin. Studies with SH2 and SH3 binding domains also demonstrated the association of bleached opsin with Src SH2 domain. These observations would indicate that opsin may interact with Src SH2 domain either directly or indirectly through an adaptor protein. The presence of both rhodopsin and arrestin in immunoprecipitates of LROS with activated Src suggests a light-dependent formation of a multimeric complex containing rhodopsin, arrestin, and activated Src. The specific intermolecular mechanism of association of these proteins remains to be elucidated. Src can putatively interact with other signaling molecules (receptors, adaptor proteins, or enzymes) through its SH2 and SH3 domains, catalytic domain (24), or Tyr(P) residues binding to the Tyr(P) binding domain (PTB; for a review, see Ref. 22). The association of Src with bleached opsin or arrestin could occur through any of these mechanisms. Our observation using ROS membranes and "whole retinal" homogenates strongly suggest that this association involves the SH2 domain binding to either bleached opsin, arrestin, or a yet unidentified protein that forms a complex with opsin and arrestin.

To date, there is no evidence of tyrosine phosphorylation of either rhodopsin or arrestin; however, such a modification has not been addressed rigorously. Recent studies (13), have shown the presence of a trimeric complex consisting of Src, β-arrestin, and the β_2-adrenergic receptor, whose formation is stimulated by isoproterenol. This interaction was shown to be mediated by the SH3 region of Src binding to a proline-rich region in the N terminus of β-arrestin. Visual arrestin does not contain the

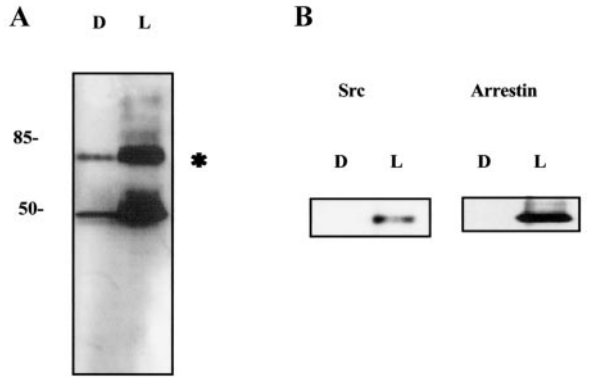

FIG. 8. **Light-dependent association of Src and arrestin with ROS cytoskeleton.** ROS membranes (100 μg) were solubilized in buffer B (without SDS) and centrifuged at 27,000 $\times$ *g* for 30 min. The Triton X-100-insoluble pellet was washed again with 0.4 ml of buffer B, centrifuged at 27,000 $\times$ *g* for 30 min, and solubilized in SDS-PAGE sample buffer. *A*, immunoblot of solubilized DROS and LROS membranes (15.5 μg/lane) used for preparation of cytoskeletal pellet with anti-arrestin; *, immunoreaction with arrestin-opsin aggregate. *B*, the detergent insoluble pellet was subjected to immunoblot analysis with anti-Src or anti-arrestin.

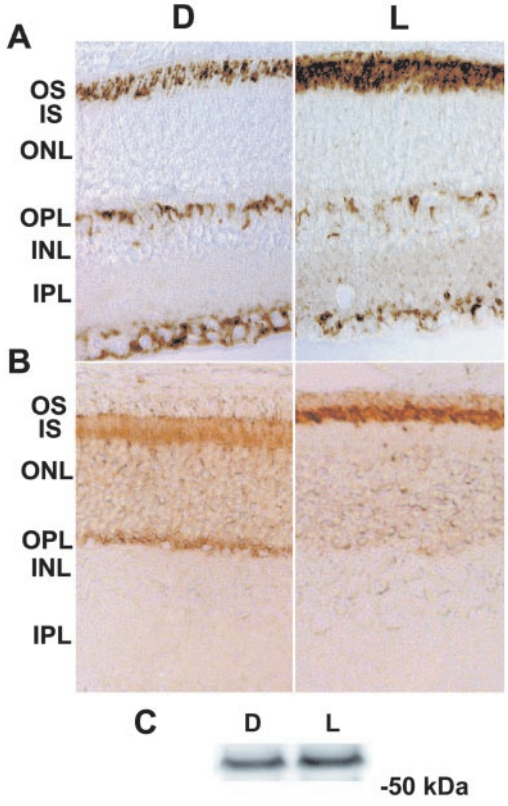

FIG. 9. **Immunolocalization of Src and arrestin in photoreceptor cells.** Immunocytochemistry of dark- (*D*) or light-adapted (*L*) (30 min) paraffin-embedded rat retinal sections with anti-Src (N terminus) at 2.5 μg/ml (*A*) or anti-arrestin at 1:4000 (*B*). *OS*, outer segment; *IS*, inner segment; *OPL* and *IPL*, outer and inner plexiform layers, respectively; *ONL* and *INL*, outer and inner nuclear layers, respectively. Data are representative of six independent eyecups (three dark and three light). *C*, immunoblot of dark and light (30 min *in vivo*) retinal homogenates (10 μg each), solubilized in buffer containing 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 60 mM octyl glucoside, 100 mM NaCl, and 1 mM EDTA with anti-Src (N terminus) at 1 μg/ml. Densitometric scans of three independent dark- and light-adapted samples showed no significant difference in reactivity with this antibody (dark-adapted integrated density = 2.03 $\pm$ 1.10; light-adapted integrated density = 2.40 $\pm$ 1.08; $p = 0.7$, two-tailed *t* test, $n = 3$).

proline rich sequence present in β-arrestin, indicating that its interaction with Src may involve a different module of protein-protein interaction or an interaction mediated by rhodopsin. Other reports have shown that β-arrestin as well as visual arrestin interact with the catalytic domain of Src (24). Additionally, Src has been recently shown to interact directly with the β_3-adrenergic receptor through its SH3 domain (25). These observations along with our current observations clearly suggest several mechanisms for agonist-mediated interaction between Src, arrestins, and GPCRs. The specific module of interaction may depend on cell type, receptor type, and stimulus.

Immunocytochemistry of Src in photoreceptor cells shows a significant increase in intensity of reaction in light-adapted sections over dark-adapted sections, suggesting a light-dependent conformational change resulting in the exposure of the N terminus of Src. On the other hand, arrestin is immunolocalized in a more dramatic fashion to the outer segments of light-adapted sections. In this regard, our observations of 1) both Src and arrestin being enriched in a Triton X-100-insoluble, cytoskeleton-enriched fraction of LROS (Fig. 8) and 2) the light-dependent formation of a complex containing Src, arrestin, and bleached opsin (Figs. 4, 5, and 7) support the hypothesis that the interaction of bleached opsin, Src, and arrestin may be indicative of a light-mediated translocation of photoreceptor proteins between the inner and outer segment and/or a light-mediated membrane/cytoskeleton association within the outer segment. A recent report demonstrates a light- and GTPγS-dependent association of transducin α subunit and the rod cGMP-phosphodiesterase with detergent-resistant "lipid rafts" isolated from bovine ROS (26). We have recently reported the association of transducin α subunit with caveolin, a scaffolding protein enriched in detergent-resistant microdomains obtained from bovine ROS (27). These results along with our current observations regarding Src and arrestin may suggest a role for detergent-resistant lipid rafts in the recruitment and formation of signaling complexes within photoreceptor outer segments in a light-dependent manner.

The consequence of light activation of tyrosine phosphorylation in photoreceptor outer segments remains unknown. Our data suggest that Src is a light-activated tyrosine kinase in photoreceptor cells and may, in part, be responsible for the increase in tyrosine phosphorylation in ROS membranes. Several putative Tyr(P) substrates have been identified in ROS, including phosphatidylinositol 3-kinase (28), SHP-2 (29), transducin α (19), and PLCγ_1, which binds to bleached ROS membranes *in vitro* and translocates from the inner and outer segments of photoreceptors in response to light adaptation *in vitro* (30). The possible regulation of the former enzymes by light *in vivo* is currently under investigation. A recent report has demonstrated tyrosine phosphorylation of the epidermal growth factor and fibroblast growth factor receptors in response to epidermal growth factor and fibroblast growth factor treatment of photoreceptor cells in culture (31). In nonocular tissue, the epidermal growth factor receptor has been shown to associate with Src and to be regulated by it (32). Other reports have shown that the photoreceptor cGMP-gated channel is modulated by tyrosine phosphorylation (33, 34).

Functionally, *in vivo* light-activated tyrosine phosphorylation could serve to establish an intracellular communication network between the outer and inner segments of photoreceptors. This intracellular communication is likely to be important in the maintenance of the functional/structural polarization of photoreceptor cells. The hypothesis we propose can be summarized as follows: light $\rightarrow$ rhodopsin $\rightarrow$ activation of tyrosine kinase (Src) in the inner segment $\rightarrow$ tyrosine phosphorylation of other cytosolic photoreceptor proteins in the inner segment $\rightarrow$ direct recruitment/transport of cytosolic signaling molecules

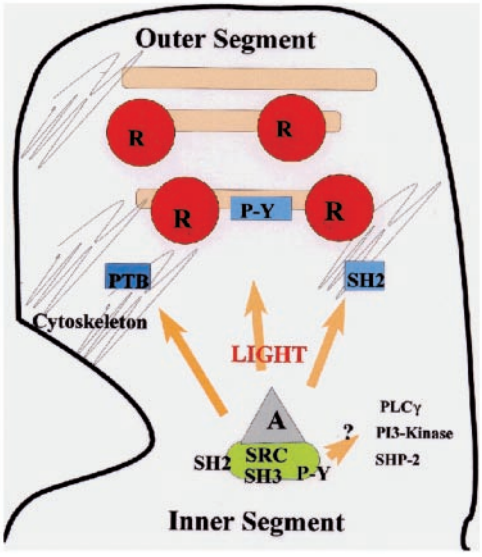

FIG. 10. **Model for the role of light activation of Src in photoreceptor cells.** Light activation of Src initiated by bleaching of rhodopsin (*R*), results in tyrosine phosphorylation of several ROS proteins. Phosphotyrosine residues (*P-Y*) generated on disc membranes, plasma, or cytoskeleton provide high affinity docking sites for signaling molecules with either an SH2 or PTB domain, resulting in the recruitment of these signaling components to the outer segment in a light-dependent manner. Alternatively, Tyr(P) residues generated on cytosolic photoreceptor proteins (PLCγ1, SHP-2) allow their association with SH2 and PTB domains in integral ROS components, resulting in their recruitment to the outer segment. The recruitment of arrestin (*A*) to the outer segment in response to light could involve association with an adaptor protein such as Src. The outcome of Src activation by light may contribute to the maintenance of functional or structural polarization of photoreceptor cells.

to ROS (*e.g.* PLCγ_1) or recruitment/transport of cytosolic proteins (*e.g.* arrestin) through their association with an adaptor molecule that is tyrosine-phosphorylated (Fig. 10). Tyr(P) residues on these cytosolic proteins bind to SH2 or PTB binding domains present in an integral ROS protein at the base of the outer segment or cytoskeletal element present throughout the photoreceptor, which ultimately leads to their transport from the inner to the outer segment. Alternatively, light → rhodopsin → activation of tyrosine kinase (Src) → translocation/membrane association of Src with ROS → tyrosine phosphorylation of an integral ROS membrane protein/cytoskeletal component providing high affinity binding sites (Tyr(P)) for signaling proteins with either an SH2 domain (*e.g.* PLCγ_1, phosphatidylinositol 3-kinase, or SHP-2) or a PTB. In either model, Tyr(P) residues generated in a light-dependent manner provide a module for protein-protein interaction between cytosolic photoreceptor proteins and integral membrane/cytoskeletal components of ROS. This module provides docking sites for transport of cytosolic proteins between the inner and outer segment either through association with the cytoskeleton or an integral ROS protein. Depending on the identity of the signaling protein recruited, the outcome will either modulate the photoresponse (light adaptation/turn-off; *e.g.* PLCγ_1 or arrestin translocation) or contribute to other light-regulated (entrained) events that are important for the maintenance of structural integrity of photoreceptor cells (disc shedding/morphogenesis).

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