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Association of the Tyrosine Phosphatase SHP-2 with Transducin-α and a 97-kDa Tyrosine-Phosphorylated Protein in Photoreceptor Rod Outer Segments

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Abstract: Increasing evidence indicates that tyrosine phosphorylation, controlled by the concerted action of tyrosine kinases and protein tyrosine phosphatases (PTPs), plays important roles in retinal photoreceptor rod outer segments (ROS). We characterized PTP activity in isolated bovine ROS that is significantly inhibited by orthovanadate. Incubating ROS in the presence of exogenous Mg^{2+}, ATP, and orthovanadate dramatically enhanced the tyrosine phosphorylation of several endogenous proteins. SHP-2, a PTP with two SH2 domains, was identified in ROS by immunoblot analysis and was found to associate with ROS membranes. Immunocytochemistry showed localization of SHP-2 in photoreceptor outer segments and possibly in the outer plexiform, inner nuclear, and inner plexiform cell layers of the retina as well. SHP-2 associated with transducin-α and a 97-kDa tyrosine-phosphorylated protein in ROS, suggesting the formation of a multimeric signaling complex. Based on its association with transducin-α and a 97-kDa protein, SHP-2 may regulate the tyrosine phosphorylation of endogenous proteins, including transducin-α, and may play a significant role in a novel signaling pathway in photoreceptors. **Key Words:** Protein tyrosine phosphatases—SHP-2—Retina—Rod outer segments—Transducin—Vanadate.
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Tyrosine phosphorylation of intracellular proteins is a key mechanism for regulating the proliferation, differentiation, and survival of cells in nonocular tissues. The level of tyrosine phosphorylation within cells is controlled by the concerted actions of tyrosine kinases and protein tyrosine phosphatases (PTPs) (reviewed by Sun and Tonks, 1994). The classical PTPs are a large and structurally diverse family of receptor-like and intracellular enzymes with a highly conserved catalytic domain (Van Vactor et al., 1998). Although PTPs are typically thought to inactivate tyrosine phosphorylation signaling cascades, PTPs have also been shown to play positive signaling roles (Byon et al., 1997).

SHP-2 [previously called SH-PTP2, PTP2C, PTP1D, and Syp (Adachi et al., 1996)] is a ubiquitously expressed intracellular PTP that contains two Src homology 2 (SH2) domains at its amino terminus, a catalytic phosphatase domain, and a carboxyl-terminal tail that can undergo tyrosine phosphorylation (reviewed by Feng and Pawson, 1994). The SH2 domains allow SHP-2 to bind to phosphotyrosine residues on activated growth factor receptors and other signaling proteins. SHP-2 becomes phosphorylated on tyrosine in response to stimulation of cells by various ligands, including epidermal growth factor, platelet-derived growth factor (Feng et al., 1993; Lechleider et al., 1993; Vogel et al., 1993), insulin-like growth factor-I (Ali et al., 1997), interleukin-3 (Welham et al., 1994), and growth hormones (Kim et al., 1998; Stofega et al., 1998). Activation of several G protein-coupled receptors, including α-thrombin (Rivard et al., 1995), AT_1 (Ali et al., 1997), and proteinase-activated receptors (Yu et al., 1997), has also been shown to stimulate the tyrosine phosphorylation of SHP-2. SHP-2 plays both negative and positive roles in various signaling pathways, not only through its phosphatase activity, but also by functioning as an adaptor protein linking activated receptors to signaling proteins (Bennett et al., 1994; Li et al., 1994). For example, SHP-2 is a positive mediator of mitogenic signals induced by platelet-derived growth factor and α-thrombin (Rivard et al.,

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Abbreviations used: ECL, enhanced chemiluminescence; *p*-NPP, *p*-nitrophenyl phosphate; PAGE, polyacrylamide gel electrophoresis; PB, 0.1 *M* phosphate buffer (pH 7.4); PTP, protein tyrosine phosphatase; PY, tyrosine-phosphorylated; PY-39 and PY-97, 37- and 97-kDa tyrosine-phosphorylated proteins, respectively; ROS, rod outer segments; SDS, sodium dodecyl sulfate; SH2, Src homology 2; Tα, α subunit of transducin; TNGT, 20 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, 10% glycerol, and 0.1% Triton X-100; TTBS, 0.1% (wt/vol) Tween 20 in 20 m*M* Tris-HCl (pH 7.4) and 410 m*M* NaCl.

1995) but acts as a negative regulator of the Ras pathway in T cell receptor signaling (Marengere et al., 1996). Although SHP-2 is widely expressed, it is most abundant in the brain, indicating that SHP-2 may have important roles in neural signal transduction (Suzuki et al., 1995). A case in point is that the activation of SHP-2 in neuronal cells stimulated by nerve growth factor, brain-derived neurotrophic factor, or neurotrophin-3 suggests that SHP-2 is involved in neurotrophin signaling (Goldsmith and Koizumi, 1997; Ohnishi et al., 1999).

Currently, the role of tyrosine phosphorylation in retinal photoreceptors is unclear. Our laboratory has recently shown that light stimulates tyrosine phosphorylation in photoreceptor rod outer segments (ROS), indicating that tyrosine kinases and phosphatases are playing active roles in photoreceptors (Ghalayini et al., 1998*b*). Activation of epidermal growth factor, fibroblast growth factor (Fontaine et al., 1998), and insulin-like growth factor-1 (Zick et al., 1987; Waldbillig et al., 1988) receptor tyrosine kinases, which are expressed in photoreceptors, has also been shown to stimulate tyrosine phosphorylation in photoreceptors. The non–receptor tyrosine kinases Src (Ghalayini and Anderson, 1998) and Lck (Omri et al., 1998) are also expressed in photoreceptors, and our laboratory has identified several putative tyrosine kinase substrates in ROS, including phospholipase Cγ_1 (Ghalayini et al., 1998*a*) and the p85 subunit of phosphatidylinositol 3-kinase (Guo et al., 1997). Although several PTPs, such as RPTP-σ (Yan et al., 1993), PTP1B (Shock et al., 1995), and hVH-5 (Martell et al., 1995), have been shown to be expressed in the retina, PTP activity and the localization of PTPs within the retina have yet to be investigated. Modulation of the rod photoreceptor cyclic GMP-gated channel by tyrosine phosphorylation is inhibited by the PTP inhibitor pervanadate, suggesting that dephosphorylation of tyrosine residues on the channel or an associated regulatory protein increases the channel's sensitivity to cyclic GMP (Molokanova et al., 1997).

Given the significant role that PTPs have been shown to play in neuronal cells and tissues and their possible functions in retinal cells, we investigated PTPs in photoreceptor ROS. We characterized PTP activity in isolated bovine ROS and demonstrated that the PTP inhibitor orthovanadate significantly enhances the tyrosine phosphorylation of endogenous ROS proteins. We also immunolocalized the tyrosine phosphatase SHP-2 to photoreceptor cells and demonstrated that SHP-2 binds to the α subunit of rod transducin (rod Tα) and a 97-kDa tyrosine-phosphorylated (PY) protein (PY-97) in isolated ROS.

MATERIALS AND METHODS

Materials

BCA protein assay reagent kit and GelCode Coomassie stain were from Pierce (Rockford, IL, U.S.A.). Raytide tyrosine kinase substrate and c-Src tyrosine kinase were from Oncogene Research Products (Cambridge, MA, U.S.A.). Nonfat dry milk, nitrocellulose sheets, and molecular weight markers were from Bio-Rad (Hercules, CA, U.S.A.). Protogel (30% acrylamide) was from National Diagnostics (Atlanta, GA, U.S.A.). Monoclonal anti-phosphotyrosine PY20 and monoclonal anti-SHP-2 (PTP1D), which was generated against a 20-kDa protein fragment corresponding to the amino terminus, were from Transduction Laboratories (Lexington, KY, U.S.A.). Polyclonal anti-SHP-2 [SH-PTP2 (C-18)], which was raised against a peptide corresponding to amino acids 576–593 of the carboxy terminus, monoclonal anti-phosphotyrosine PY69, monoclonal anti-phosphotyrosine PY99, polyclonal anti-Tα (K-20), normal mouse IgG, and normal rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated donkey anti-rabbit IgG and sheep anti-mouse IgG, enhanced chemiluminescence (ECL) reagents, and Hyperfilm ECL were from Amersham Life Science (Arlington Heights, IL, U.S.A.). Avidin–biotin complex Vectastain kit, diaminobenzidine-peroxidase substrate kit, and biotinylated goat anti-rabbit IgG were from Vector Labs (Burlingame, CA, U.S.A.). Protein A-Sepharose CL-4B was from Pharmacia Biotech (Piscataway, NJ, U.S.A.). [γ-^{32}P]ATP was from NEN Life Science (Boston, MA, U.S.A.). Bovine serum albumin, ATP, *p*-nitrophenyl phosphate (*p*-NPP), Na_3VO_4, and all other reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All gels and immunoblots were digitized using a Hewlett Packard ScanJet 3C, and images for publication were processed with Adobe Photoshop version 4.0.

Preparation of ROS

ROS were prepared as previously described by Zimmerman and Godchaux (1982) with slight modifications. Fresh bovine eyes were obtained from a local abattoir, and the retinas were dissected within 2 h and placed in centrifuge tubes (maximum of eight retinas per tube) containing 0.5 ml of buffer A [20 m*M* Tris-HCl (pH 7.4), 100 m*M* NaCl, 2 m*M* $MgCl_2$, and 20% sucrose] per retina. The retinal suspensions were vortex-mixed for 30 s at maximal speed and centrifuged for 4 min at 400 *g* at 4°C. The supernatants were transferred to fresh tubes and centrifuged for 10 min at 8,000 *g* at 4°C to yield a crude ROS pellet. Pellets were resuspended in 3 ml of 65% sucrose containing 20 m*M* Tris-HCl (pH 7.4) and 100 m*M* NaCl and loaded at the bottom of a 35-ml continuous gradient of 25–50% sucrose containing 10 m*M* Tris-HCl (pH 7.4), 100 m*M* NaCl, and 2 m*M* $MgCl_2$. The gradients were centrifuged at 80,000 *g* for 2 h at 4°C. Broken and resealed ROS were collected from the gradients, pooled, diluted with an equal volume of buffer B [20 m*M* Tris-HCl (pH 7.4), 100 m*M* NaCl, and 10% sucrose], and centrifuged for 20 min at 17,000 *g* at 4°C. The final ROS pellet was resuspended in buffer B (~2.5 mg/ml) and frozen at −80°C. For fractionation of ROS from a continuous sucrose gradient, the final ROS pellet was resuspended in 3 ml of the 65% sucrose solution, loaded onto a second 35-ml continuous gradient of 25–50% sucrose, and centrifuged at 80,000 *g* for 2 h at 4°C. A 16-gauge needle connected to tubing was inserted into the side of the centrifuge tube near the bottom, and 1-ml fractions were slowly pumped out and collected. The total protein concentration of ROS and sucrose gradient fractions was determined using the BCA protein assay according to the manufacturer's protocol.

PTP assays

PTP activity in bovine ROS was assayed using either *p*-NPP or ^{32}P-labeled Raytide as substrate. For *p*-NPP, ROS (20 μg) were incubated in sodium acetate (pH 5.0) buffer containing 1 m*M* dithiothreitol and 16 m*M* *p*-NPP for 30 min

at 37°C in a final volume of 100 μl. The reaction was quenched by addition of 900 μl of 1 *M* NaOH, and the amount of *p*-nitrophenol released was determined by measuring the absorbance at 410 nm.

Raytide was phosphorylated on a single tyrosine residue with c-Src and [γ-^{32}P]ATP overnight at 37°C according to the manufacturer's protocol and purified as described by Krueger et al. (1990). The PTP assay was carried out essentially as described by Vambutas et al. (1995). In brief, 40 μg of ROS or an equal volume of ROS isotonic wash or washed membranes was incubated in 25 m*M* HEPES (pH 7.4) buffer containing 5 m*M* EDTA, 10 m*M* dithiothreitol, and ^{32}P-labeled Raytide (1×10^5 cpm) for 1 h at 37°C in a final volume of 100 μl. The reaction was terminated by adding 750 μl of acidic charcoal mix (4% Norit A, 0.9 *M* HCl, 90 m*M* $Na_4P_2O_7$, and 2 m*M* NaH_2PO_4) and centrifuged at 13,000 *g* for 2 min. Equal volumes of the supernatants were added to 10 ml of scintillation cocktail, and radioactivity was quantitated by scintillation counting.

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis of tyrosine-phosphorylated ROS proteins

ROS (1 mg/ml) were incubated in 50 m*M* Tris-HCl (pH 7.4), 1 m*M* ATP, 1 m*M* EGTA, 5 m*M* $MgCl_2$, and 1 m*M* Na_3VO_4 at 37°C for 30 min. Incubations were terminated by addition of SDS-PAGE sample buffer, and the samples were resolved by 8 or 10% SDS-PAGE and stained with GelCode Coomassie stain or transferred to nitrocellulose. After transferring, blots were washed for three times each for 5 min in TTBS [0.1% (wt/vol) Tween 20 in 20 m*M* Tris-HCl (pH 7.4) and 410 m*M* NaCl] and blocked with 1% nonfat dry milk and 1% bovine serum albumin in TTBS for 1 h at room temperature or overnight at 4°C. Blots were incubated with anti-PY69 (0.25 μg/ml), anti-PY99 (0.25 μg/ml), monoclonal anti-SHP-2 (1:500), polyclonal anti-SHP-2 (0.5 μg/ml), or anti-Tα (K-20; 0.1 μg/ml) for 2 h at room temperature. Following primary antibody incubations, blots were washed three times each for 5 min with TTBS, incubated for 1 h with horseradish peroxidase-linked secondary antibodies (1:3,000), washed four times each for 10 min with TTBS, and developed by ECL. Membranes were stripped by incubation in stripping buffer [62.5 m*M* Tris-HCl (pH 6.8), 2% SDS, and 100 m*M* 2-mercaptoethanol] for 30 min at 50°C.

Washing of ROS

ROS (1 mg/ml) were suspended in 100 m*M* Tris-HCl (pH 7.4), 1 m*M* ATP, 10 m*M* $MgCl_2$, and 0.1 m*M* EGTA, with or without 1 m*M* Na_3VO_4, and homogenized by repeated extrusion through a 26-gauge needle. The ROS were incubated at 37°C for 1 h, an aliquot was removed for determining total ROS, and the remaining ROS were centrifuged at 45,000 *g* for 30 min. The supernatant (isotonic wash 1) was removed, and the ROS were resuspended in an equal volume of the following buffers for sequential washes: once in isotonic wash buffer [100 m*M* Tris-HCl (pH 7.4), 3 m*M* $MgCl_2$, and 1 m*M* dithiothreitol], twice in hypotonic wash buffer [10 m*M* Tris-HCl (pH 7.4), 1 m*M* $MgCl_2$, and 1 m*M* dithiothreitol], once in GTP buffer [100 μ*M* GTP or 10 μ*M* guanosine 5′-*O*-(3-thiotriphosphate) in hypotonic buffer], and once in hypertonic buffer [10 m*M* Tris-HCl (pH 7.4), 1 m*M* $MgCl_2$, 1 m*M* dithiothreitol, and 1 *M* NH_4Cl]. The final washed ROS were resuspended in isotonic wash buffer, and all washes were centrifuged again to remove contaminating membranes.

Immunocytochemistry

Adult bovine eyes obtained from a local abattoir were opened by an encircling cut, the anterior segment and vitreous humor were removed, and the light-adapted retina in the eyecup was fixed by immersion in 4% paraformaldehyde in 0.1 *M* phosphate buffer (PB; pH 7.4) for 3 days at 4°C. The eyecups were washed with PB, dissected into retinal-scleral pieces, cryoprotected in 30% sucrose in PB overnight, embedded in OCT medium, and frozen. Retinal pieces were sectioned at 14 μm on a Leica Cryocut 1800 and collected on gelatin-coated slides. Sections were incubated in 10% normal goat serum and 0.5% Triton X-100 in PB for 1 h before incubation with polyclonal anti-SHP-2 (1 μg/ml), neutralized anti-SHP-2 (1 μg/ml), anti-Tα (1 μg/ml), or nonimmune rabbit IgG (1 μg/ml) at room temperature overnight in a humidity chamber. The primary antibodies were diluted in 3% normal goat serum, 0.5% Triton X-100, and 0.02% sodium azide in PB. Polyclonal anti-SHP-2 was neutralized by incubating with a 10-fold excess of blocking peptide overnight at 4°C. The sections were rinsed three times with PB, incubated for 1 h with biotinylated goat anti-rabbit IgG (1:1,000), rinsed again with PB, and incubated for 1 h with Vectastain avidin–biotin complex. After rinsing with PB, the immunoreaction was developed using a diaminobenzidine–peroxidase substrate kit according to the manufacturer's protocol. Slides were rinsed in distilled water, coverslipped, visualized on a Nikon Eclipse E800 digital microscope, and captured using Adobe Photoshop version 4.0.

Immunoprecipitation

ROS (200 μg) were incubated for 1 h at 37°C in 50 m*M* Tris-HCl (pH 7.4), 1 m*M* EGTA, and 5 m*M* $MgCl_2$, with or without 1 m*M* ATP and/or 1 m*M* Na_3VO_4, in a final volume of 0.2 ml. Incubated ROS were diluted to 0.5 mg/ml with solubilization buffer [20 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, 10% glycerol, and 1% Triton X-100] and sonicated for 5 min. For immunoprecipitation using anti-Tα, 0.1% SDS was also included in the solubilization buffer. Insoluble material was removed by centrifugation at 17,000 *g* for 20 min, and the solubilized ROS were precleared by incubating with 25 μl of protein A-Sepharose for 1 h at 4°C on a rotator. PY proteins, SHP-2, and Tα were immunoprecipitated by incubating precleared ROS with 1 μg of anti-PY20, 2 μg of polyclonal anti-SHP-2, 2 μg of anti-Tα, or an equal amount of nonimmune IgG (control) overnight at 4°C on a rotator. Immune complexes were incubated with 25 μl of protein A-Sepharose for 1 h and then washed three times with TNGT [20 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, 10% glycerol, and 0.1% Triton X-100]. Anti-SHP-2 and anti-Tα immune complexes were heated for 5 min at 85°C in 30 μl of SDS-PAGE sample buffer, and the supernatants were resolved by SDS-PAGE. PY proteins were eluted from anti-PY20 immune complexes by incubating with 40 m*M* phenyl phosphate in TNGT for 2 h at 4°C with gentle shaking and resolved by SDS-PAGE.

RESULTS

Characterization of PTP activity in ROS

Bovine ROS were assayed for PTP activity in the absence or presence of several inhibitors and effectors using exogenous *p*-NPP as a substrate (Fig. 1A). In the absence of effectors (control), specific PTP activity in ROS was 56.7 ± 3.4 nmol of *p*-nitrophenol produced/min/mg of protein. Two PTP inhibitors, orthovanadate (Na_3VO_4) and molybdate (Na_2MoO_4), inhibited ~75%

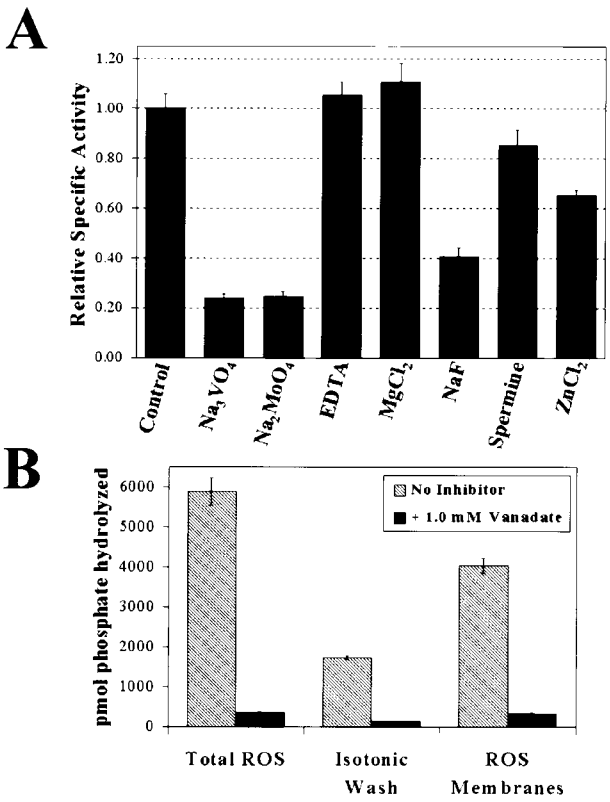

FIG. 1. PTP activity in bovine ROS. **A:** ROS were assayed for PTP activity using *p*-NPP as a substrate in the absence (Control) or presence of the following inhibitors and effectors—0.1 m*M* Na_3VO_4, 1.0 m*M* Na_2MoO_4, 1.0 m*M* EDTA, 2.0 m*M* $MgCl_2$, 20 m*M* NaF, 1.0 m*M* spermine, and 1.0 m*M* $ZnCl_2$. All activities are relative to control. **B:** Isolated ROS were washed under isotonic conditions, and the total (prewashed) ROS, isotonic wash, and washed ROS membranes were assayed for PTP activity in the presence or absence of 1.0 m*M* orthovanadate (Na_3VO_4) using ^{32}P-labeled raytide as a substrate.

of the PTP activity in ROS. The remaining noninhibited activity may be due to alkaline phosphatases in ROS that can also hydrolyze *p*-NPP. Another known PTP inhibitor, Zn^{2+}, inhibited PTP activity in ROS by 35%, whereas the serine/threonine phosphatase inhibitor NaF produced a 59% decrease in PTP activity. EDTA, Mg^{2+}, and spermine were largely without effect on PTP activity. ATP at 1.0 m*M*, GTP at 1.0 m*M*, and guanosine 5′-*O*-(3-thiotriphosphate) at 100 μM, in the absence or presence of Mg^{2+}, also had no effect on PTP activity in ROS (data not shown). PTP activity in ROS isolated from dark-adapted bovine retinas was not affected by light (data not shown).

Distribution of PTP activity in ROS was analyzed by isotonically washing isolated ROS and assaying the total unwashed ROS, isotonic wash (cytosolic proteins), and washed ROS membranes (peripheral and membrane proteins) for PTP activity using ^{32}P-labeled Raytide as a substrate (Fig. 1B). Unwashed ROS had a specific PTP activity of 4.9 nmol of $^{32}P_i$ released from Raytide/min/mg of ROS. Approximately 70% of the total PTP activity remained bound to the ROS membranes, whereas 30% fractionated with the cytosol. The activity in all of the fractions could be inhibited >95% by 1.0 m*M* orthovanadate. Additional isotonic and hypotonic washes of the ROS membranes had no detectable PTP activity (data not shown).

Effect of orthovanadate on tyrosine phosphorylation of ROS proteins

We investigated the effect of orthovanadate on the tyrosine phosphorylation of endogenous ROS proteins. ROS were incubated in a buffer containing 5 m*M* $MgCl_2$, 1 m*M* ATP, and increasing concentrations of orthovanadate (0–1.0 m*M*) and subjected to immunoblot analysis using a monoclonal antibody to phosphotyrosine, anti-PY69 (Fig. 2A). In the presence of $MgCl_2$ and ATP, orthovanadate significantly enhanced tyrosine phosphorylation in ROS at concentrations as low as 1 μM, with maximal stimulation occurring at 100 μM and 1.0 m*M*. We also examined the time course of tyrosine phosphorylation of ROS proteins in the absence or presence of orthovanadate. Tyrosine phosphorylation of ROS proteins was highest at 20 min in the absence of orthovanadate and decreased thereafter with increasing incubation time (Fig. 2B, top). In the presence of orthovanadate, tyrosine phosphorylation increased with incubation time and reached a maximum at 90–120 min (Fig. 2B, bottom).

Distribution of SHP-2 in ROS

We identified the tyrosine phosphatase SHP-2 in bovine ROS by immunoblot analysis using both polyclonal and monoclonal anti-SHP-2. Both antibodies immunoreacted with a 66-kDa protein, which is the apparent molecular mass of SHP-2 (Fig. 3A). The distribution of SHP-2 in ROS was investigated by subjecting isolated ROS to sequential washes in buffers of various ionic strengths. Before washing, the ROS were incubated in a buffer containing Mg^{2+} and ATP and either without or with orthovanadate. Equal volumes of the total prewashed ROS, supernatants from washes, and washed ROS membranes were subjected to immunoblot analysis using polyclonal anti-SHP-2. The first isotonic wash contained a significant amount of SHP-2, but very little or no detectable amount of SHP-2 was present in the following isotonic, hypotonic, hypotonic with GTP, or hypertonic washes. Most of the SHP-2 remained bound to the ROS membranes during these sequential washes. Tyrosine phosphorylation of ROS proteins by preincubating with orthovanadate before washing did not have an effect on SHP-2 distribution (Fig. 3B, bottom).

To ensure that the presence of SHP-2 and PTP activity in ROS was not due to cytosolic contamination from other cell types, ROS were purified on two successive continuous sucrose gradients, and the second gradient was fractionated into 1-ml aliquots. The gradient fractions were resolved by 8% SDS-PAGE and subjected to GelCode Coomassie staining or immunoblot analysis using anti-Tα or anti-SHP-2. Gra-

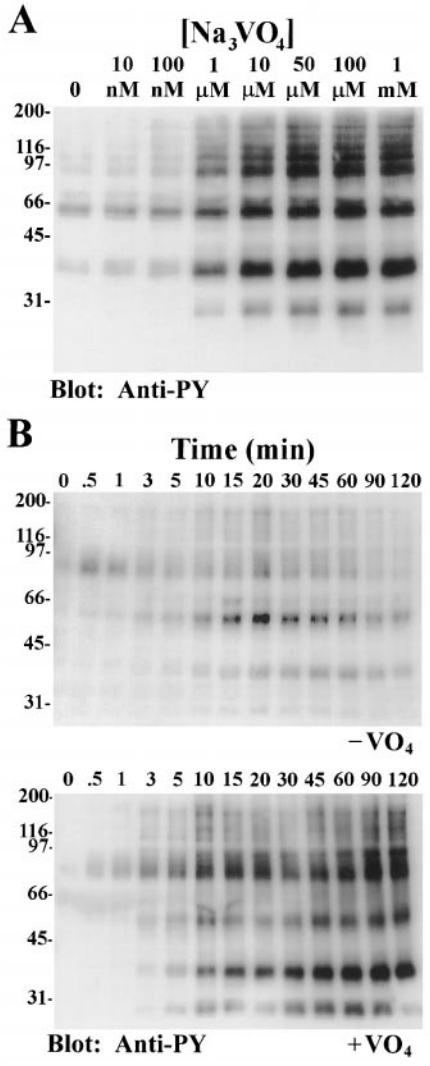

FIG. 2. Effect of orthovanadate (Na_3VO_4) on tyrosine phosphorylation of endogenous proteins in ROS. **A:** ROS (10 μg) were incubated for 30 min at 37°C in a buffer containing 5 m*M* $MgCl_2$, 1 m*M* ATP, and increasing concentrations (0–1.0 m*M*) of Na_3VO_4. The samples were resolved on a 10% gel and subjected to immunoblot analysis using anti-PY69. **B:** ROS (200 μg) were incubated at 37°C in buffers containing $MgCl_2$, ATP, and either without (**top**) or with (**bottom**) 1.0 m*M* orthovanadate (VO_4). Aliquots of ROS (10 μg) were removed at the indicated time points (0–120 min) and solubilized in SDS-PAGE sample buffer. The samples were resolved on an 8% gel and subjected to immunoblot analysis using anti-PY69.

dient fractions 11–19, which represent the ROS fractions normally collected from the first gradient, were enriched in both rhodopsin and transducin (Fig. 4A and B). Fraction 13, which has previously been shown to represent sealed ROS (Zimmerman and Godchaux, 1982), had the highest protein concentration (Fig. 4D) and content of rhodopsin and Tα. Immunoblot analysis using anti-SHP-2 showed that SHP-2 was enriched in fractions 11–17 and its level was highest in fraction 13 (Fig. 4C). Tyrosine phosphatase activity using *p*-NPP as a substrate was also enriched in fractions 11–19 and highest in fraction 13 (Fig. 4D).

Immunocytochemical localization of SHP-2 in bovine retina

Immunocytochemistry of frozen sections from bovine retina using anti-SHP-2 showed strong immunoreactivity with the photoreceptor outer segment layer and to a lesser extent with the outer plexiform, inner nuclear, and inner plexiform cell layers (Fig. 5). The SHP-2 immunoreaction in the outer segment layer was completely abolished by neutralizing the primary antibody before incubation with the C-terminus peptide it was raised against. The specificity of the immunoreactions in the outer plexiform, inner nuclear, and inner plexiform cell layers is unclear because it was not completely reversed on neutralization of the SHP-2 antibody. As a positive control, immunocytochemistry of similar sections with a polyclonal antibody to Tα, a rod photoreceptor-specific protein, showed labeling only in the photoreceptor outer segment layer. Control sections incubated with normal nonimmune rabbit IgG did not show any reaction.

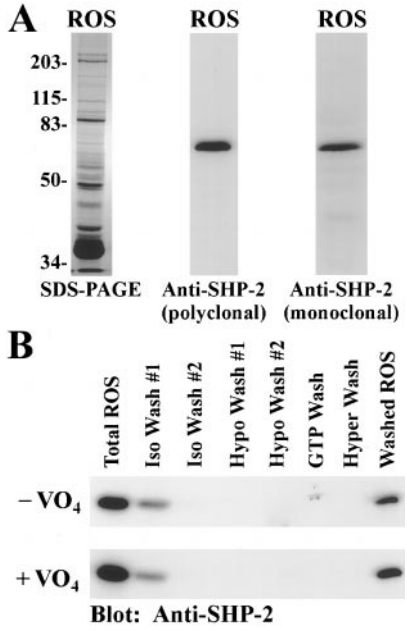

FIG. 3. Identification and association of SHP-2 tyrosine phosphatase with ROS membranes. **A:** ROS (25 μg) were resolved on a 7.5% gel and visualized by GelCode Coomassie staining (left lane) or subjected to immunoblot analysis using polyclonal anti-SHP-2 (center lane) or monoclonal anti-SHP-2 (right lane). The migration of SHP-2 (66 kDa) is indicated. **B:** ROS (1 mg/ml) were incubated for 30 min at 37°C in buffers containing $MgCl_2$ and ATP, either without (**top**) or with (**bottom**) 1.0 m*M* orthovanadate (VO_4). The samples were subjected to sequential washes, and equal volumes of the total (prewashed) ROS (50 μg), washes, and final washed ROS membranes were subjected to immunoblot analysis using polyclonal anti-SHP-2.

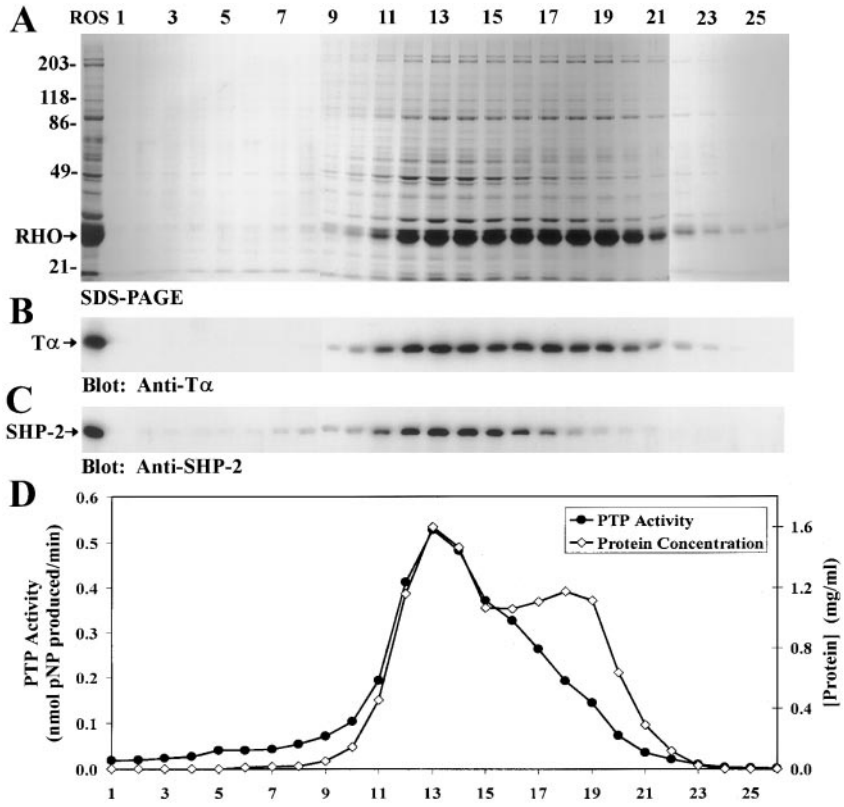

FIG. 4. Fractionation of SHP-2 and PTP activity in ROS. ROS purified on a second continuous sucrose gradient were fractionated into 1-ml aliquots. **A:** ROS (20 μg) and equal aliquots (15 μl) of gradient fractions 1–26 were resolved on an 8% gel and stained with GelCode. Migration of rhodopsin (RHO) is indicated. ROS (15 μg) and equal aliquots (11 μl) of gradient fractions 1–26 were subjected to immunoblot analysis using (**B**) anti-Tα and (**C**) polyclonal anti-SHP-2. Tα (39-kDa) and SHP-2 are indicated by arrows. **D:** Equal aliquots of gradient fractions 1–26 were assayed for protein concentration (5 μl) and PTP activity (40 μl) using *p*-NPP as a substrate.

Association of SHP-2 with Tα and a PY-97

To determine if SHP-2 shows tyrosine phosphorylation in bovine ROS, anti-PY20 was used to immunoprecipitate PY proteins from ROS that were incubated for 1 h at 37°C in buffers containing 5 m*M* $MgCl_2$ only (Fig. 6, lane 1), $MgCl_2$ and 1 m*M* ATP (Fig. 6, lane 2), or $MgCl_2$, ATP, and 1.0 m*M* orthovanadate (Fig. 6, lane 3). Immunoblot analysis using anti-PY99 showed immunoreactivity with several PY proteins in the anti-PY20 immunoprecipitates from PY ROS incubated in the presence of $MgCl_2$ and ATP, with or without orthovanadate (Fig. 6A). The apparent molecular masses of the PY proteins were 33, 39, 60, 66, 83, 97, 120, 140, and 180 kDa. SHP-2 was identified by immunoblot analysis in the anti-PY20 immunoprecipitates from PY ROS (Fig. 6B) and corresponded in size to the 66-kDa PY protein observed on the anti-PY99 immunoblot. SHP-2 was not present in either anti-PY20 immunoprecipitates from ROS incubated without orthovanadate (Fig. 6, lanes 1 and 2) or control immunoprecipitates from PY ROS using normal (nonimmune) mouse IgG (Fig. 6, lane C).

SHP-2 was also immunoprecipitated from ROS incubated under the same conditions described in Fig. 6. SHP-2 was recovered in all of the polyclonal anti-SHP-2 immunoprecipitates from ROS (Fig. 7A). Immunoblot analysis of the anti-SHP-2 immunoprecipitates from PY ROS using anti-PY99 did not show immunoreactivity with a 66-kDa protein corresponding in size to SHP-2 but did show immunoreactivity with a 39-kDa PY protein (PY-39) and PY-97 coimmunoprecipitating with SHP-2 (Fig. 7B, lane 3). PY-39 was identified as rod Tα by immunoblot analysis with an antibody specific for rod Tα (Fig. 7C). Tα was present in SHP-2 immunoprecipitates from both PY ROS (incubated with orthovanadate) and nonphosphorylated ROS (incubated without orthovanadate). SHP-2, PY proteins, and Tα were not present in control immunoprecipitates from PY ROS using normal (nonimmune) rabbit IgG (Fig. 7C).

To examine further the association of Tα with SHP-2, we performed immunoblot analysis of anti-Tα immunoprecipitates using polyclonal anti-SHP-2. SHP-2 was present in anti-Tα immunoprecipitates from both nonphosphorylated ROS and PY ROS (Fig. 8A, lanes 2 and 4). The anti-Tα immunoprecipitates from PY ROS appear to contain higher amounts of SHP-2, suggesting that the association of SHP-2 with Tα may be enhanced by tyrosine phosphorylation. Neither SHP-2 nor Tα was present in control immunoprecipitates from nonphosphorylated ROS and PY ROS using normal (nonimmune) rabbit IgG (Fig. 8, lanes 1 and 3).

DISCUSSION

PTPs, acting in concert with tyrosine kinases, play a significant role in neuronal development and function (reviewed by Naegele and Lombroso, 1994). In the current study, we have characterized PTP activity in isolated bovine photoreceptor ROS using the exogenous sub-

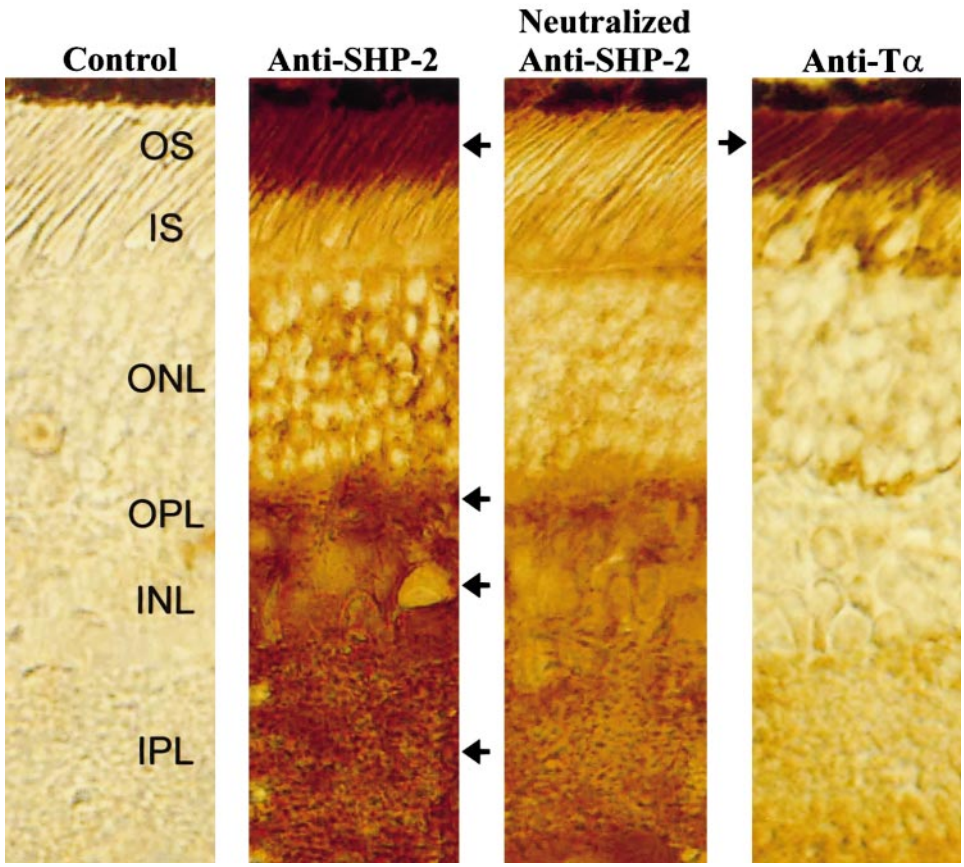

FIG. 5. Immunolocalization of SHP-2 in bovine retina. Frozen sections of bovine retina were subjected to immunocytochemistry using (from left to right) nonimmune rabbit IgG (Control), polyclonal anti-SHP-2, anti-SHP-2 neutralized with blocking peptide, and anti-Tα. Arrowheads point to areas of immunoreaction. OS and IS, photoreceptor outer and inner segment layers, respectively; ONL and OPL, outer nuclear and plexiform layers, respectively; INL and IPL, inner nuclear and plexiform layers, respectively.

strates *p*-NPP and ^{32}P-labeled Raytide. PTP activity in ROS was especially sensitive to the well-established PTP inhibitor orthovanadate, a phosphate analogue that inhibits PTPs by presumably binding as a transition state analogue (Swarup et al., 1982; Huyer et al., 1997). Approximately 70% of the PTP activity was associated with ROS membranes, suggesting the presence of a receptor-like PTP(s) and/or a non–receptor PTP(s) that is tightly associated with ROS membranes. The tyrosine phosphorylation of endogenous proteins in isolated ROS was significantly enhanced by orthovanadate. In the absence of orthovanadate, the tyrosine phosphorylation of endogenous proteins was highest at different times and decreased thereafter; however, the level of tyrosine phosphorylation in ROS incubated with orthovanadate continued to increase until it reached a maximum at 90–120 min, indicating that the dephosphorylation of ROS proteins by PTPs is inhibited by orthovanadate.

SHP-2 is ubiquitously expressed in the developing rat brain but is compartmentalized to postmitotic neurons in the adult brain, suggesting that it may play important roles in neural signal transduction (Reeves et al., 1996; Servidei et al., 1998). In this report, we demonstrate that SHP-2 is also present in isolated bovine photoreceptor ROS. The majority of the SHP-2 protein in isolated ROS is tightly associated with the membranes, and its distribution in ROS is not affected by tyrosine phosphorylation. Suzuki et al. (1995) have shown that SHP-2 also binds tightly to synaptic membranes in rat brain by an unknown mechanism. Although the mechanism of SHP-2 association with ROS membranes is also unknown, our results indicate that it may involve interactions with Tα or a PY-97.

We verified that PTP activity and SHP-2 in isolated ROS were not cytosolic contaminants from other retinal cells by purifying osmotically intact ROS a second time on a continuous sucrose gradient and analyzing gradient fractions. The content of rhodopsin and rod Tα, which are expressed only in rod photoreceptors and are significantly enriched in isolated ROS, was highest in the fractions that have been shown to contain sealed ROS (Zimmerman and Godchaux, 1982). PTP activity and SHP-2 content were also enriched in the same sealed ROS fractions. The presence of SHP-2 in ROS was further verified by immunocytochemistry, which showed strong localization of SHP-2 in photoreceptor outer segments.

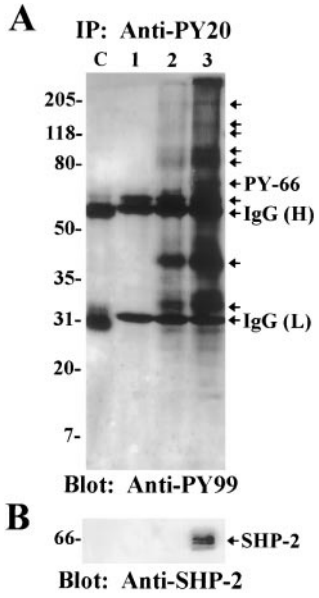

FIG. 6. Identification of SHP-2 in anti-PY20 immunoprecipitates from PY ROS. **A:** ROS (500 μg) were incubated in buffers containing (lane 1) $MgCl_2$ only, (lane 2) $MgCl_2$ and ATP, or (lanes C and 3) $MgCl_2$, ATP, and 1.0 m*M* orthovanadate. The samples were solubilized and subjected to immunoprecipitation using either (lane C) nonimmune mouse IgG (control) or (1–3) anti-PY20. Immunoprecipitates (IP) were resolved on a 10% gel and subjected to immunoblot analysis using anti-PY99. Arrows designate the major PY proteins of apparent molecular masses 33, 39, 60, 66, 83, 97, 120, 140, and 180 kDa. The migration of the 66-kDa PY protein (PY-66) and the heavy (H) and light (L) IgG chains are indicated. **B:** The anti-PY20 immunoprecipitates were also probed with polyclonal anti-SHP-2.

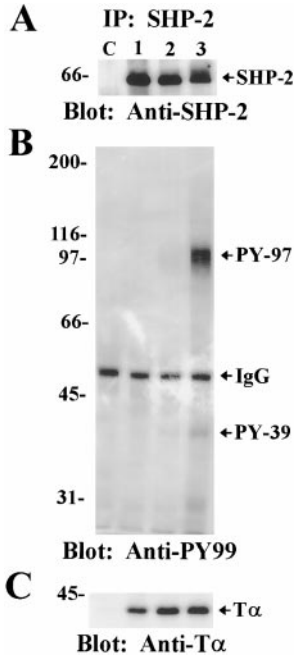

FIG. 7. Association of SHP-2 with Tα and a PY-97. ROS (500 μg) were incubated in buffers containing (lane 1) $MgCl_2$ only, (lane 2) $MgCl_2$ and ATP, or (lanes C and 3) $MgCl_2$, ATP, and 1.0 m*M* orthovanadate. The samples were solubilized and subjected to immunoprecipitation using either (lane C) nonimmune rabbit IgG (control) or (lanes 1–3) polyclonal anti-SHP-2. **B:** Immunoprecipitates (IP) were resolved on an 8% gel and subjected to immunoblot analysis using anti-PY99. The migrations of IgG, PY Tα (PY-39), and the coimmunoprecipitating PY-97 are indicated. The immunoblot was stripped and reprobed with (**A**) monoclonal anti-SHP-2 and (**C**) anti-Tα.

Tyrosine phosphorylation of SHP-2, activated receptors, and/or other signaling proteins has been shown to promote their association with each other and regulate, both positively and negatively, various signaling pathways and the phosphatase activity of SHP-2 (reviewed by Tonks and Neel, 1996). We stimulated tyrosine phosphorylation of endogenous ROS proteins by incubating isolated bovine ROS in a buffer containing Mg^{2+}, ATP, and orthovanadate. Although SHP-2 corresponded in size to a 66-kDa PY protein in anti-PY immunoprecipitates from ROS, its tyrosine phosphorylation was not evident in anti-SHP-2 immunoprecipitates. However, two other PY proteins with molecular masses of 39 and 97 kDa coimmunoprecipitated with SHP-2 from PY ROS, suggesting that the association of SHP-2 with these proteins may not involve its own tyrosine phosphorylation. The appearance of a doublet on immunoblots with SHP-2 could reflect truncation of the enzyme or a modification, e.g., phosphorylation, that might alter its mobility on SDS-PAGE. We were unable to determine if the association of SHP-2 with these proteins affected its phosphatase activity because the ROS had been previously incubated with the PTP inhibitor orthovanadate to enhance phosphorylation.

We identified Tα in anti-SHP-2 immunoprecipitates by immunoblot analysis using an antibody specific for

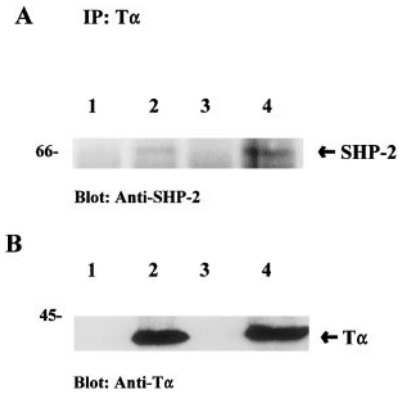

FIG. 8. Association of SHP-2 with Tα. ROS (250 μg) were incubated in buffers containing either (lanes 1 and 2) $MgCl_2$ only or (lanes 3 and 4) $MgCl_2$, ATP, and orthovanadate. The samples were solubilized and subjected to immunoprecipitation using either (lane 1 and 3) 2 μg of nonimmune rabbit IgG (control) or (lanes 2 and 4) 2 μg of anti-Tα. The immunoprecipitates (IP) were resolved on a 10% gel and subjected to immunoblot analysis using (**A**) polyclonal anti-SHP-2 or (**B**) polyclonal anti-Tα. The migrations of SHP-2 (66 kDa) and Tα are indicated by arrows.

rod Tα. The association of SHP-2 with Tα was further confirmed by identifying SHP-2 in anti-Tα immunoprecipitates, which also showed that tyrosine phosphorylation might enhance the association of SHP-2 with Tα. In other experiments, we have shown that Tα is phosphorylated on tyrosine in ROS incubated under these same conditions (authors' manuscript in preparation). The comigration of Tα with the PY-39 in SHP-2 immunoprecipitates suggests that they are the same protein. Tβγ was not present in the SHP-2 immunoprecipitates (data not shown), indicating that Tβγ is not involved in the association of Tα with SHP-2. In other nonocular cells, G_i proteins have been shown to link the thrombin receptor to the tyrosine phosphorylation of the closely related SHP-1 (Li et al., 1995). Although the association of Tα with SHP-2 in ROS occurred in the absence of phosphorylation, evidence from anti-Tα immunoprecipitates indicates that the association of Tα with SHP-2 may be enhanced by tyrosine phosphorylation. These results suggest that Tα interacts directly or indirectly with SHP-2 and may be a substrate for the tyrosine phosphatase in photoreceptors.

We have not identified the PY-97 associating with SHP-2 in ROS. Several other investigators have characterized a novel 97–100-kDa PY protein that associates with the SH2 domains of SHP-2 in hematopoietic cells and rat brain (Suzuki et al., 1995; Carlberg and Rohrschneider, 1997; Craddock and Welham, 1997; Gu et al., 1997, 1998; Gesbert et al., 1998; Zhang and Broxmeyer, 1999). It is not clear whether this 97-kDa protein associated with SHP-2 in ROS is related to any of those identified by other investigators, but it could play a significant role in the regulation of SHP-2 in ROS or the tight association of SHP-2 with ROS membranes, and identifying it will be important for further elucidating the function of SHP-2 in ROS.

The role that SHP-2 might play in ROS remains to be elucidated. However, our recent observation of light-induced tyrosine phosphorylation in rat ROS in vivo (Ghalayini et al., 1998*b*) and the effect of growth factors on tyrosine phosphorylation of photoreceptor cells in culture (Fontaine et al., 1998) suggest that the state of tyrosine phosphorylation of photoreceptor proteins is likely to play an important role in photoreceptor cells. In the current study, light had no effect on tyrosine phosphatase activity or the tyrosine phosphorylation of SHP-2 under the conditions used. Recent reports from our laboratory and others have identified several putative substrates for the phosphatase activity of SHP-2, including phospholipase Cγ (Ghalayini et al., 1998*a*) and phosphatidylinositol 3-kinase (Guo et al., 1997), in ROS and epidermal growth factor and fibroblast growth factor receptors in cultured photoreceptor cells (Fontaine et al., 1998). Reports on other tissues have demonstrated that SHP-2 associates with growth factor receptors (Bennett et al., 1994; Li et al., 1994) and regulates signaling proteins such as phospholipase Cγ (Arkinstall et al., 1998) and phosphatidylinositol 3-kinase (Welham et al., 1994). We propose that SHP-2 may play a similar role in photoreceptor cells by modulating the activity of the latter two enzymes and/or regulating downstream signals associated with growth factor activation.

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