Tyrosine Phosphorylation of the α Subunit of Transducin and Its Association with Src in Photoreceptor Rod Outer Segments

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Abstract: Recent evidence indicates that tyrosine phosphorylation may play important roles in retinal photoreceptor rod outer segments (ROS). We investigated the tyrosine phosphorylation of endogenous proteins in isolated bovine ROS. Several proteins with apparent molecular masses of 31, 39, 60, 83, 90, 97, 120, 140, and 180 kDa were tyrosine-phosphorylated in ROS incubated with Mg^{2+}, ATP, and orthovanadate. Several tyrosine kinase inhibitors significantly inhibited tyrosine phosphorylation of these proteins in ROS. The 39- and 60-kDa tyrosine-phosphorylated proteins were identified as the α subunit of the G protein transducin (Tα) and the tyrosine kinase Src, respectively. The presence of Src and tyrosine kinase activity in bovine ROS was confirmed by their cofractionation with rhodopsin and Tα on continuous sucrose gradients. Several tyrosine-phosphorylated proteins, including Src, coimmunoprecipitated with Tα. The association of Src with Tα was detected in the absence of tyrosine phosphorylation, but was enhanced with increased tyrosine phosphorylation of ROS. Moreover, tyrosine kinase activity also associated with Tα was sevenfold higher under tyrosine-phosphorylating conditions. The recovery of transducin by hypotonic GTP extraction from tyrosine-phosphorylated ROS was significantly less than that from nonphosphorylated ROS. We localized the site on Tα phosphorylated by Src to the amino-terminal half by limited tryptic digests, and further mapped it by ion trap mass spectrometry to Tyr^{142} in the helical domain of Tα. Tα was also tyrosine-phosphorylated in vivo in rat retina, but this phosphorylation was not affected by light. **Key Words:** Transducin—Src—Retina—Rod outer segments—Tyrosine phosphorylation—G proteins.
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Tyrosine phosphorylation of cellular proteins has been shown to play an important role in the proliferation, differentiation, and survival of cells in nonocular tissues. Following ligand binding, receptor tyrosine kinases (e.g., growth factor receptors) stimulate signaling pathways leading to the activation and/or tyrosine phosphorylation of a number of common signaling proteins, such as phosphatidylinositol 3-kinase, phospholipase Cγ, SHP-2 tyrosine phosphatase, and Src tyrosine kinases (for re-

view, see Fantl et al., 1993). Tyrosine phosphorylation of proteins can modulate their enzymatic activity and/or promote their interaction with other proteins (Pawson, 1995), leading to the assembly of signaling complexes and activation of downstream mitogen-activated protein kinase pathways (Marshall, 1995). Several G protein-coupled receptors (GPCRs) have also been shown to stimulate tyrosine kinases and activate mitogen-activated protein kinase pathways within a variety of cell types (Malarkey et al., 1995; Gutkind, 1998). Activation of Src by GPCRs stimulates the tyrosine phosphorylation of numerous signaling proteins, such as the Shc adapter protein (Cazaubon et al., 1994; Linseman et al., 1995; van Biesen et al., 1995) and the epidermal growth factor receptor (Daub et al., 1996; Luttrell et al., 1997), suggesting that cross-talk may exist between tyrosine kinase signaling pathways stimulated by GPCRs and receptor tyrosine kinases. Recently, Src has even been found to form a signaling complex with activated β-adrenergic receptors, a GPCR, through the use of arrestin as an adapter protein (Luttrell et al., 1999).

The role of tyrosine phosphorylation in rod photoreceptor cells of the retina is unclear, but several lines of evidence indicate that tyrosine kinase signaling pathways play an active role in photoreceptors. Our laboratory recently demonstrated that light stimulates tyrosine phos-

phorylation in photoreceptor rod outer segments (ROS) (Ghalayini et al., 1998*a*). In addition, activated 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 have also been shown to stimulate tyrosine phosphorylation in photoreceptors. Several nonreceptor tyrosine kinases, including Src (Ghalayini and Anderson, 1998) and Lck (Omri et al., 1998), have been localized to photoreceptor cells, and our laboratory has also identified several putative tyrosine kinase substrates in ROS, including phosphatidylinositol 3-kinase (Guo et al., 1997), phospholipase Cγ1 (Ghalayini et al., 1998*b*), and SHP-2 (Bell et al., 1999), that could be regulated by tyrosine phosphorylation. Molokanova et al. (1997, 1999) have shown that the rod photoreceptor cyclic GMP-gated channel is modulated by tyrosine phosphorylation and proposed that tyrosine phosphorylation of one of its subunits or regulators may be an important mechanism for controlling the photoresponse. The α subunit of rod transducin (Tα) has also been shown to be tyrosine-phosphorylated in reconstitution assays with several different tyrosine kinases (Zick et al., 1986, 1987; Krupinski et al., 1988; Waldbillig et al., 1988; Hausdorff et al., 1992), suggesting that Tα is a substrate for an endogenous tyrosine kinase in ROS.

To understand further the role of tyrosine phosphorylation in photoreceptors, we investigated the identity of endogenous proteins that are tyrosine-phosphorylated in isolated bovine ROS. We show that rod Tα is phosphorylated by an endogenous tyrosine kinase in ROS and associates with Src, which was also found to be tyrosine-phosphorylated. We also mapped a site on Tα phosphorylated by Src to a tyrosine residue in the helical domain, which is in the proximity of the guanosine nucleotide-binding pocket.

MATERIALS AND METHODS

Materials

Bicinchoninic acid protein assay reagent kit and GelCode Coomassie stain were from Pierce (Rockford, IL, U.S.A.). Nonfat dry milk, nitrocellulose sheets, Coomassie Brilliant Blue (R-250), 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.). Tyrosine kinase inhibitors genistein, tyrophostin A25, and PP1 were from Calbiochem (San Diego, CA, U.S.A.). Monoclonal anti-phosphotyrosine PY20 and monoclonal anti-Tα (Gαt; recognizes amino acids 282–300) were from Transduction Laboratories (Lexington, KY, U.S.A.). Polyclonal anti-phosphotyrosine was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Monoclonal anti-phosphotyrosine PY69, monoclonal anti-phosphotyrosine PY99, horseradish peroxidase (HRP)-conjugated anti-PY99-HRP, agarose-conjugated anti-PY99, polyclonal anti-Tα (K-20; recognizes amino acids 90–109), polyclonal anti-Src (SRC2), normal mouse IgG, and normal rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Protein tyrosine kinase assay kit (QIA28), Raytide EL tyrosine kinase substrate, purified c-Src, and polyclonal anti-Tα (Ab-6; recognizes amino acids 340–349) were from

Oncogene Research Products (Cambridge, MA, U.S.A.). HRP-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.). 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.). *N*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)–trypsin and trypsin inhibitor were from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). Bovine serum albumin (BSA), ATP, GTP, sodium orthovanadate (Na_3VO_4), Triton X-100, and all other reagents were from Sigma Chemical Company (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 4.0.

Preparation and tyrosine phosphorylation of ROS

ROS were isolated and fractionated on continuous sucrose gradients as previously described (Bell et al., 1999). ROS (1 mg/ml) were incubated in 50 mM Tris-HCl (pH 7.4), 1 mM ATP, 1 mM EGTA, 5 mM MgCl$_2$, and 1 mM Na$_3$VO$_4$ at 37°C for 30 min. Incubations were terminated by the addition of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and the tyrosine-phosphorylated ROS (PY-ROS) were subjected to SDS-PAGE and immunoblot analysis as described below. When necessary, ROS were preincubated with tyrosine kinase inhibitors or vehicle [1% dimethyl sulfoxide (DMSO)] for 5 min at 37°C prior to the addition of ATP and Na$_3$VO$_4$.

SDS-PAGE and immunoblot analysis

Samples were resolved by 8 or 10% SDS-PAGE and stained with 0.1% Coomassie Blue or transferred to nitrocellulose. After transferring, blots were washed for 2 × 10 min in TTBS [0.1% Tween 20 in 20 mM Tris-HCl (pH 7.4) and 410 mM NaCl] and blocked with either 10% BSA in TTBS or 1% milk and 1% BSA in TTBS (for anti-PY99 immunoblots) for 2 h at room temperature or overnight at 4°C. Blots were incubated with either anti-PY69 (0.25 µg/ml), anti-PY20 (1:1,000), anti-PY-poly (1:2,000), anti-PY99-HRP (0.1 µg/ml), anti-PY99 (0.1 µg/ml), anti-Src (0.5 µg/ml), anti-Tα (K-20, 90–109) (0.1 µg/ml), anti-Tα (Gαt, 282–300) (1:1,000), or anti-Tα (Ab-6, 340–349) (1:1,000) for 2 h at room temperature. Following primary antibody incubations, blots were washed 3 × 5 min with TTBS, incubated for 1 h with HRP-linked secondary antibodies (1:3,000), washed 4 × 10 min with TTBS, and developed by ECL. In some instances, membranes were stripped by incubation in stripping buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol] for 30 min at 50°C.

Immunoprecipitation and immunodepletion

ROS (200 µg) were incubated for 1 h at 37°C in 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 5 mM MgCl$_2$, and in the absence [nonphosphorylated ROS (N-ROS)] or presence (PY-ROS) of 1 mM ATP and 1 mM Na$_3$VO$_4$ in a final volume of 0.2 ml. Incubated ROS were diluted to 0.5 mg/ml with solubilization buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, and 1% Triton X-100] and sonicated for 5 min. 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. When required, anti-Tα (K-20) antibodies were neutralized by incubating with a 10-fold excess of blocking peptide for 2 h at 4°C. Tyrosine-phosphorylated proteins (PY-proteins) and Tα were

immunoprecipitated by incubating precleared ROS with either 1 μg of anti-PY20, 2 μg of anti-Tα (K-20), 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 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100]. The immune complexes were assayed for tyrosine kinase activity using Raytide EL as described below or subjected to SDS-PAGE and immunoblot analysis as follows. 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 mM phenyl phosphate in TNGT for 2 h at 4°C with gentle shaking, and resolved by SDS-PAGE. In some experiments, solubilized ROS membranes (100 μg) were incubated with P99-agarose (40 μg) or glutathione S-transferase (GST)-agarose (control) for 2 h at 4°C with gentle mixing. After washing (4×) with TNGT, bound proteins were eluted by incubation in the presence of 25 mM phenyl phosphate for 1 h at 4°C with gentle mixing and subjected to SDS-PAGE and immunoblot analysis. For immunodepletion of Src, 250 μg of precleared ROS (2.5 mg/ml) was incubated with either 3 μg of anti-Src or nonimmune IgG (control) overnight at 4°C and subsequently incubated with 25 μl of protein A–Sepharose for 1 h. The immune complexes were pelleted by centrifuging at 2,000 g for 5 min, and the immunodepleted ROS were transferred to a fresh tube and mixed with SDS-PAGE sample buffer.

Tyrosine kinase assay using Raytide EL

Anti-PY20 and anti-Tα immune complexes were washed twice with kinase assay buffer [50 mM HEPES (pH 7.5), 0.1 mM EDTA, and 0.02% Brij 35], resuspended in 25 μl of kinase assay buffer, mixed with 5 μg of Raytide EL tyrosine kinase substrate or Raytide control substrate, and preincubated with either 10 μM PP1 or vehicle (1% DMSO) for 5 min at 30°C. The reaction was initiated by adding 15 μl of ATP mix (0.15 mM ATP and 30 mM MgCl$_2$) and 10 μCi of [γ-^{32}P]ATP, and incubated for 30 min at 30°C in a final volume of 45 μl. The reaction was terminated by adding 120 μl of 10% phosphoric acid, gently mixing, and centrifuging at low speed. The supernatant was applied to a 2.5-cm-diameter Whatman P81 filter paper, which was washed 4 × 10 min in 50 ml of 0.5% phosphoric acid, washed briefly in acetone, air-dried, and placed in 15 ml of scintillation cocktail, and radioactivity was quantified by scintillation counting.

Washing of ROS and preparation of transducin

ROS (1 mg/ml) were suspended in 100 mM Tris-HCl (pH 7.4), 1 mM ATP, 10 mM MgCl$_2$, 0.1 mM EGTA, and either with or without 1 mM orthovanadate, 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 "unwashed ROS," and the remaining ROS were centrifuged at 45,000 g for 30 min. The supernatant (isotonic wash no. 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 mM Tris-HCl (pH 7.4), 3 mM MgCl$_2$, and 1 mM dithiothreitol], twice in hypotonic wash buffer [10 mM Tris-HCl (pH 7.4), 1 mM MgCl$_2$, and 1 mM dithiothreitol], twice in GTP buffer [100 μM GTP (or 10 μM GTPγS) in hypotonic buffer], and once in hypertonic buffer [10 mM Tris-HCl (pH 7.4), 1 mM MgCl$_2$, 1 mM dithiothreitol, and 1 M NH$_4$Cl]. The final washed ROS membranes were resuspended in isotonic wash buffer, and all washes were centrifuged again

to remove contaminating membranes. Transducin was extracted from ROS membranes in the GTP-hypotonic wash as previously described (Kuhn, 1982). Transducin prepared for limited tryptic proteolysis was extracted from ROS membranes that had *not* been incubated previously under tyrosine-phosphorylating conditions. Transducin prepared under these conditions had no kinase activity when incubated in kinase buffer in the absence of exogenously added Src (data not shown). Alternatively, for comparison of the effect of tyrosine phosphorylation of Tα on its association with ROS membranes, ROS were incubated under standard phosphorylation conditions with or without 1 mM ATP, and the washing procedure was performed as above with the final hypertonic wash omitted.

Limited tryptic proteolysis of tyrosine-phosphorylated Tα

Five micrograms of transducin was incubated for 2 h at 30°C with 5 units of purified c-Src in a buffer containing 50 mM Tris-HCl (pH 7.4), 50 μM ATP, 500 μCi/ml [γ-^{32}P]ATP, 10 mM MgCl$_2$, 0.1 mM EGTA, 0.1 mM Na$_3$VO$_4$, and 0.05% β-mercaptoethanol. The samples were subsequently prechilled for 10 min at 10°C and subjected to trypsinization essentially as described by Mazzoni et al. (1991). TPCK-trypsin (0.5 μg) and 100 mM NaCl were added, and the samples were incubated at 10°C for either 0 or 15 min. The reactions were terminated by the addition of 5 μg of trypsin inhibitor, precipitated using 10% trichloroacetic acid and 0.1 mg/ml BSA, and centrifuged at 15,000 g for 30 min. The supernatants were removed, and the pellet was air-dried for 20 min, resuspended in SDS-PAGE sample buffer, and boiled for 5 min. The proteolytic fragments were resolved on a 10–20% gradient Tris–tricine gel, transferred to a polyvinylidene difluoride membrane, and subjected to autoradiography and immunoblot analysis.

Sequencing of proteolytic tryptic fragments from Tα phosphorylated by Src

Four micrograms of transducin was phosphorylated by incubating with 15 units of purified recombinant c-Src for 2 h at 30°C in a buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM ATP, 10 mM MgCl$_2$, 0.1 mM EGTA, and 0.05% β-mercaptoethanol. The tyrosine-phosphorylated Tα was precipitated using 10% trichloroacetic acid and centrifuged at 15,000 g for 30 min. The supernatants were removed, and the pellet was air-dried for 20 min, resuspended in SDS-PAGE sample buffer, boiled for 10 min, and resolved on a 10% gel. The gel was stained for 10 min using GelCode Coomassie stain and washed extensively in dH$_2$O. The Tα band was excised, washed 2 × 3 min with 50% acetonitrile (HPLC grade), frozen at −20°C, and sent to the Harvard Microchemistry Facility for in-gel tryptic digestion and sequencing. Sequence analysis was performed by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry on a Finnigan LCQ DECA quadrupole ion trap mass spectrometer as previously described (Chittum et al., 1998). The tandem mass spectrometry spectra of multiple peptides were acquired and correlated to the known sequence of bovine Tα from the National Center for Biotechnology protein database using the algorithm SEQUEST (Eng et al., 1994). The sequences were reviewed and confirmed manually using the program FuzzyIons (Chittum et al., 1998).

Preparation and immunoprecipitation of rat retinas

Albino rats were dark-adapted overnight and killed in the dark or after 5, 15, 30, or 60 min in room light (∼300 lux). Retinas were dissected, and each pair/animal was placed immediately in 0.5 ml of solubilization buffer [50 mM Tris-HCl

(pH 7.4), 150 m*M* NaCl, 1 m*M* EGTA, 0.1% SDS, 1% Triton X-100, 10 μ*M* PP1, and 1 m*M* orthovanadate], homogenized using a glass/Teflon homogenizer, and centrifuged at 17,000 *g* for 20 min. Total protein concentration of the rat retinas was determined using the bicinchoninic acid protein assay according to the manufacturer's protocol. Retinal homogenates (500 μg) were precleared with 20 μl of protein A–Sepharose prior to incubation with 20 μg of agarose-conjugated anti-PY99 overnight at 4°C. Following exposure of the rats to light, all subsequent steps were performed in the dark or under dim red light. Immune complexes were washed three times with TNGT, heated for 3 min at 85°C in 25 μl of SDS-PAGE sample buffer, and subjected to immunoblot analysis as previously described.

RESULTS

Tyrosine phosphorylation of ROS proteins

Isolated bovine ROS were incubated in a buffer with or without 5 m*M* MgCl$_2$, 1 m*M* ATP, and/or 1 m*M* orthovanadate, and probed with a monoclonal antibody to phosphotyrosine, anti-PY69 (Fig. 1B, left). Several proteins were tyrosine-phosphorylated in the presence of both ATP and MgCl$_2$. The addition of orthovanadate (a tyrosine phosphatase inhibitor) along with ATP and MgCl$_2$ caused a dramatic increase in tyrosine phosphorylation of ROS proteins. No tyrosine phosphorylation was observed in ROS incubated in the absence of either ATP or MgCl$_2$. The specificity of anti-PY69 immunoreactions in ROS was verified using two other phosphotyrosine antibodies. Immunoreactions with monoclonal anti-PY20 (Fig. 1B, center) and polyclonal anti-PY (Fig. 1B, right) were very similar to immunoreactions with anti-PY69 in ROS that had been incubated with ATP, MgCl$_2$, and orthovanadate. The effect of increasing concentrations of ATP (0–1.0 m*M*) on the tyrosine phosphorylation of ROS proteins was also tested (Fig. 1C). In the presence of 1.0 m*M* orthovanadate, tyrosine phosphorylation of several proteins was highest at 1.0 m*M* ATP, diminished below 500 μ*M* ATP, and not detectable below 100 μ*M* ATP. Several tyrosine kinase inhibitors were also tested for their effect on tyrosine phosphorylation in ROS (Fig. 1D). PP1 significantly reduced tyrosine phosphorylation in ROS at concentrations as low as 10 μ*M*, and virtually abolished tyrosine phosphorylation at 100 μ*M*. Similar concentrations of PP1 have previously been shown to be required for inhibition of tyrosine phosphorylation in intact human T cells (Hanke et al., 1996). Tyrphostin A25 also significantly reduced tyrosine phosphorylation in ROS at a concentration of 100 μ*M*, whereas genistein was less effective at the same concentration.
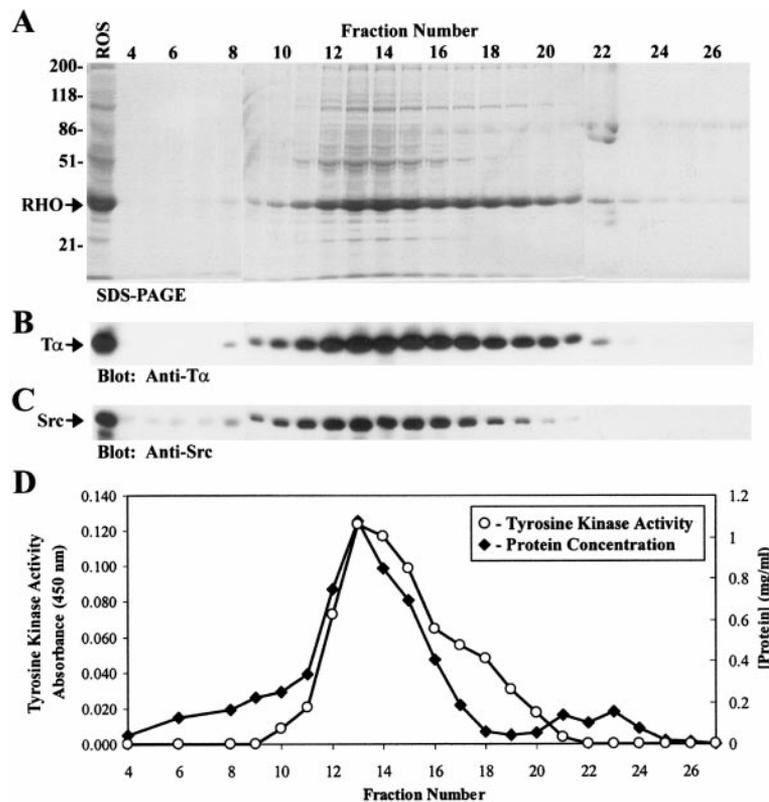
Identification of tyrosine kinase activity and Src in ROS

We identified the tyrosine kinase Src in isolated bovine ROS by immunoblot analysis using an antibody that recognizes three members (c-Src, Fyn, and Yes) of the Src family (data not shown). To ensure that the presence of Src in isolated bovine ROS was not due to cytosolic contamination from other cell types, ROS were purified on two successive continuous sucrose gradients, and the
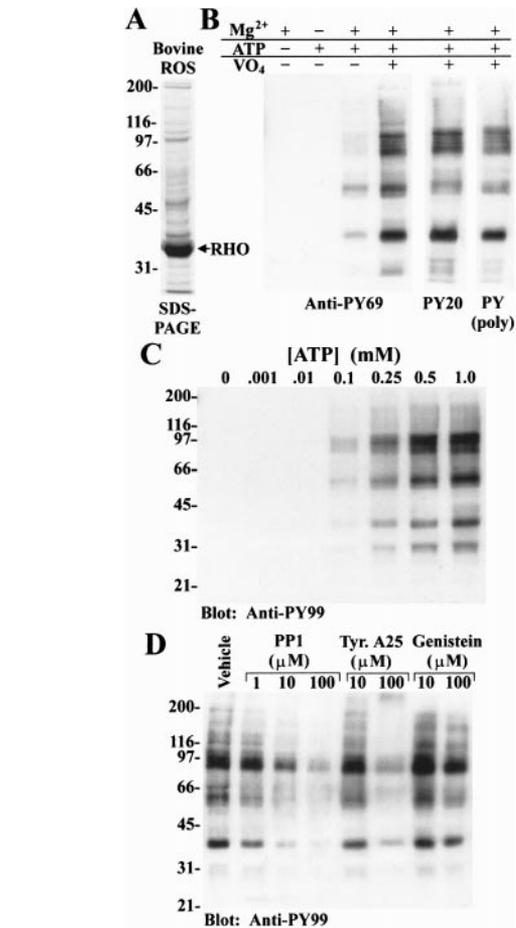
FIG. 1. Tyrosine phosphorylation of ROS proteins in the presence of MgCl$_2$, ATP, and orthovanadate. **A:** ROS (20 μg) were resolved on an 8% gel and stained with Coomassie Blue. The migration of rhodopsin (RHO) is indicated. **B:** ROS (10 μg) were incubated for 30 min at 37°C in a buffer containing either 5 m*M* MgCl$_2$ only, 1 m*M* ATP only, ATP and MgCl$_2$, or ATP, MgCl$_2$, and 1 m*M* orthovanadate (VO$_4$). The samples were subjected to immunoblot analysis using monoclonal anti-phosphotyrosine PY69 (left), monoclonal anti-PY20 (center), and polyclonal anti-PY (right). **C:** ROS (10 μg) were incubated for 30 min at 37°C in a buffer containing 5 m*M* MgCl$_2$, 1 m*M* orthovanadate, and increasing concentrations of ATP (0–1 m*M*), and subjected to immunoblot analysis using anti-PY99. **D:** ROS (10 μg) were preincubated for 5 min at 37°C with vehicle (1% DMSO) or increasing concentrations of tyrosine kinase inhibitors PP1 (1–100 μ*M*), tyrphostin A25 (10–100 μ*M*), or genistein (10–100 μ*M*). Samples were then incubated with ATP, MgCl$_2$, and orthovanadate for 30 min at 37°C and subjected to immunoblot analysis using anti-PY69.

second gradient was fractionated into 1-ml aliquots. SDS-PAGE and immunoblot analysis using anti-Tα showed that gradient fractions 12–17, which represent the ROS fractions normally collected from the first gradient, were enriched in both rhodopsin and transducin (Fig. 2A and B). Fraction 13, which represents sealed ROS, had the highest rhodopsin and transducin content and protein concentration (Fig. 2D). Immunoblot analysis using anti-Src showed that Src content was also

FIG. 2. Identification of Src and tyrosine kinase activity in ROS. ROS purified on a second continuous sucrose gradient were fractionated into 1-ml aliquots. **A:** ROS (20 μg) and equal aliquots (20 μl) of gradient fractions 4–27 were resolved on a 10% gel and stained with Coomassie Blue. An arrow indicates migration of rhodopsin (RHO). ROS (10 μg) and equal aliquots (10 μl) of gradient fractions 4–27 were subjected to immunoblot analysis using (**B**) anti-Tα (K-20) and (**C**) anti-Src. Tα (39 kDa) and Src (60 kDa) are indicated by arrows. **D:** Equal aliquots (10 μl) of gradient fractions 4–27 were assayed for protein concentration (◆) and tyrosine kinase activity (○) as described in Materials and Methods.

enriched in fractions 12–17 and was highest in fraction 13 (Fig. 2C). Tyrosine kinase activity in isolated ROS was also enriched in fractions 12–17 and highest in fraction 13 (Fig. 2D).

Identification of Src, Tα, and tyrosine kinase activity in anti-PY20 immunoprecipitates
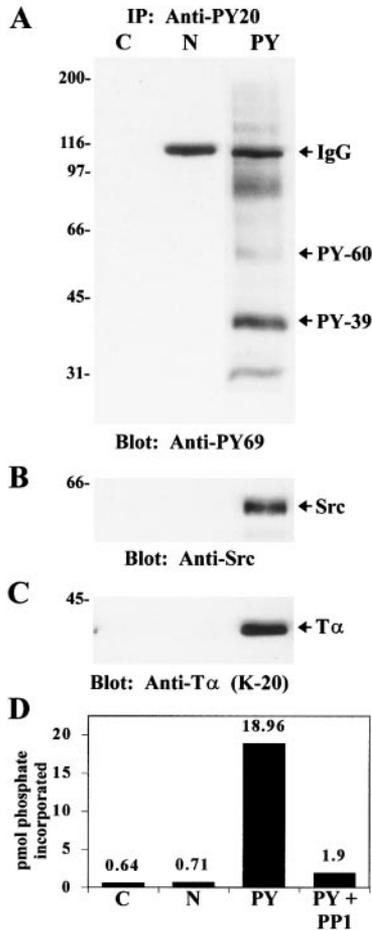
To identify proteins that are tyrosine-phosphorylated in ROS, anti-PY20 was used to immunoprecipitate PY-proteins from N-ROS (ROS incubated without ATP and orthovanadate) and PY-ROS (ROS incubated with ATP and orthovanadate). Several PY-proteins were observed in anti-PY20 immunoprecipitates from PY-ROS by immunoblot analysis using anti-PY69 (Fig. 3A). Two of the more prominent PY-proteins, which had apparent molecular masses of 60 and 39 kDa (designated PY-60 and PY-39), were identified as Src and Tα, respectively, by immunoblot analysis (Fig. 3B and C). PY-proteins, Src, and Tα were not present in either anti-PY20 immunoprecipitates from N-ROS or control immunoprecipitates from PY-ROS using normal (nonimmune) mouse IgG. Because the PY-proteins are eluted from the immune complex with phenyl phosphate and are *not* boiled, the IgG heavy chains of anti-PY20 do not separate and migrate at ~110 kDa (Fig. 3A).

Anti-PY20 immunoprecipitates from N-ROS and PY-ROS were assayed for tyrosine kinase activity using Raytide, a gastrin analogue peptide that contains a single tyrosine phosphorylation site (Fig. 3D). The tyrosine kinase activity in anti-PY20 immunoprecipitates from PY-ROS was ~25-fold higher than in immunoprecipitates from N-ROS. Moreover, tyrosine kinase activity in anti-PY20 immunoprecipitates from PY-ROS was inhibited ~90% by 10 μM PP1. Control immunoprecipitates from PY-ROS using normal mouse IgG contained negligible tyrosine kinase activity. No activity was detected using a control peptide in which the single tyrosine residue was changed to leucine, thus confirming the specificity of the kinase(s) in the anti-PY20 immunoprecipitates for tyrosine residues (data not shown).

Association of Tα with Src, a 97-kDa PY-protein, and tyrosine kinase activity

Tα was immunoprecipitated from both N-ROS and PY-ROS and subjected to immunoblot analysis using a different antibody to Tα and anti-PY99-HRP. Tα was recovered in the anti-Tα immunoprecipitates from both N-ROS and PY-ROS (Fig. 4A); however, tyrosine-phosphorylated Tα was detected only in the anti-Tα immunoprecipitates from PY-ROS (Fig. 4B). Immunoblot analysis using anti-PY99-HRP showed that several PY-proteins were also coimmunoprecipitating with Tα. Two of the more prominent PY-proteins had apparent molecular masses of 60 and 97 kDa, designated PY-60 and PY-97, respectively. Immunoblot analysis with anti-Src identified PY-60 as Src, which was present in anti-Tα immunoprecipitates from both N-ROS and PY-ROS (Fig. 4C). We were unable to identify any of the other PY-proteins, including PY-97, that also coimmunoprecipitated with Tα from PY-ROS. Control immunopre-
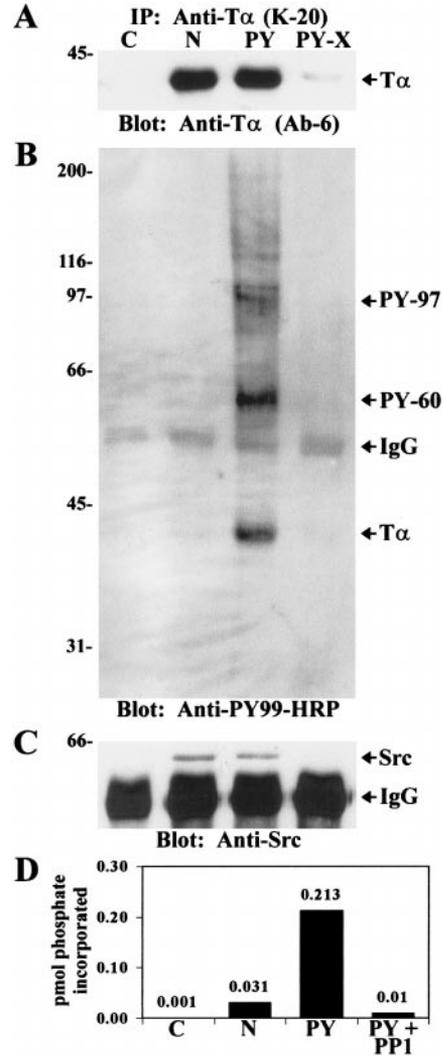
in anti-Tα immunoprecipitates from PY-ROS was inhibited >95% by 10 μM PP1. Virtually no activity was observed in control immunoprecipitates from PY-ROS using normal rabbit IgG.

FIG. 3. Identification of Src, Tα, and tyrosine kinase activity in anti-PY20 immunoprecipitates from PY-ROS. A 200 μg amount of either N-ROS (N) or PY-ROS (PY) was solubilized and subjected to immunoprecipitation (IP) using anti-PY20. For control (C), PY-ROS were subjected to immunoprecipitation using either (**A**) no antibody or (**B** and **C**) nonimmune mouse IgG. Immunoprecipitates were eluted from the immune complex with 40 mM phenyl phosphate and subjected to immunoblot analysis using (A) anti-PY69, (B) anti-Src, and (C) anti-Tα. The migrations of IgG, 39- and 60-kDa PY-proteins (PY-39 and PY-60), Src, and Tα are indicated. **D:** Nonimmune mouse IgG control (C) immunoprecipitates from PY-ROS and anti-PY20 immunoprecipitates from 200 μg of N-ROS (N) and PY-ROS (PY) were preincubated for 5 min at 30°C with either vehicle (1% DMSO) or 10 μM PP1 (PY + PP1), and then assayed for tyrosine kinase activity using [γ-^{32}P]ATP and the peptide substrate Raytide EL. Data are representative of three independent experiments.

cipitates from PY-ROS using either normal (nonimmune) rabbit IgG or anti-Tα neutralized with a peptide corresponding to the epitope recognized by anti-Tα verified the specificity of recovery of Tα, Src, and other PY-proteins in the immune complex.

Anti-Tα immunoprecipitates from both N-ROS and PY-ROS were assayed for tyrosine kinase activity (Fig. 4D). Although activity was present in anti-Tα immunoprecipitates from both N-ROS and PY-ROS, the tyrosine kinase activity was approximately sevenfold higher in immunoprecipitates from PY-ROS. The enzyme activity

FIG. 4. Tyrosine phosphorylation of Tα and its association with Src, a 97-kDa PY-protein, and tyrosine kinase activity. A 200-μg amount of either N-ROS (N) or PY-ROS (PY) was solubilized and subjected to immunoprecipitation (IP) using either nonimmune rabbit IgG (control, C), anti-Tα (N and PY), or anti-Tα neutralized with blocking peptide (PY-X). **B:** Immunoprecipitates were subjected to immunoblot analysis using anti-PY99-HRP. The migrations of tyrosine-phosphorylated Tα and the coimmunoprecipitating 60- and 97-kDa PY-proteins (PY-60 and PY-97, respectively) are indicated. The anti-PY99-HRP immunoblot was stripped and reprobed with (**A**) anti-Tα (Ab-6) and (**C**) anti-Src. C: The migration of Src is indicated just above the IgG heavy chain. **D:** Nonimmune rabbit IgG control (C) immunoprecipitates from PY-ROS and anti-Tα immunoprecipitates from 200 μg of N-ROS (N) and PY-ROS (PY) were preincubated for 5 min at 30°C with either vehicle (1% DMSO) or 10 μM PP1 (PY + PP1), and then assayed for tyrosine kinase activity using [γ-^{32}P]ATP and the peptide substrate Raytide EL. Data are representative of three independent experiments.
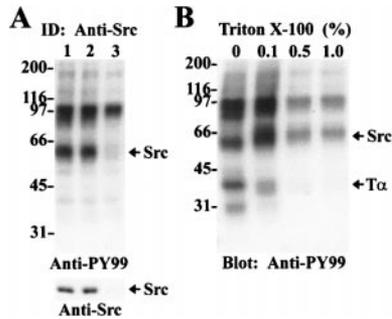
FIG. 5. Effect of solubilization and Src immunodepletion on tyrosine phosphorylation in ROS. **A:** ROS were solubilized with 1% Triton X-100 and subjected to immunoprecipitation with (lane 1) no antibody, (lane 2) nonimmune rabbit IgG, or (lane 3) anti-Src. The immunodepleted (ID) ROS (10 μg) were incubated for 30 min at 37°C in a buffer containing 5 mM MgCl$_2$, 1 mM ATP, and 1 mM orthovanadate, and subjected to immunoblot analysis with anti-PY99 (**top**). The immunoblot was stripped and reprobed with anti-Src (**bottom**). The migration of Src is indicated on both immunoblots. **B:** ROS (10 μg) were solubilized in increasing concentrations of Triton X-100 (0–1.0%), incubated with Mg^{2+}, ATP, and orthovanadate as described above, and subjected to immunoblot analysis using anti-PY99. The migrations of Src (PY-60) and Tα (PY-39) are indicated.

Immunodepletion of Src from ROS prior to incubation with Mg^{2+}, ATP, and orthovanadate eliminated the 60-kDa immunoreaction with anti-PY99, verifying the identification of PY-60 as an Src tyrosine kinase (Fig. 5A). We were unable to determine if immunodepletion of Src affected the phosphorylation of Tα because solubilization of ROS with the detergent Triton X-100, which is required for immunodepletion, significantly inhibited phosphorylation of Tα (Fig. 5B). The tyrosine phosphorylation of Tα was slightly reduced at 0.1% Triton X-100 and virtually eliminated above 0.5% Triton X-100. The phosphorylation of Src was significantly diminished at 1% Triton X-100, indicating that it may be undergoing autophosphorylation.
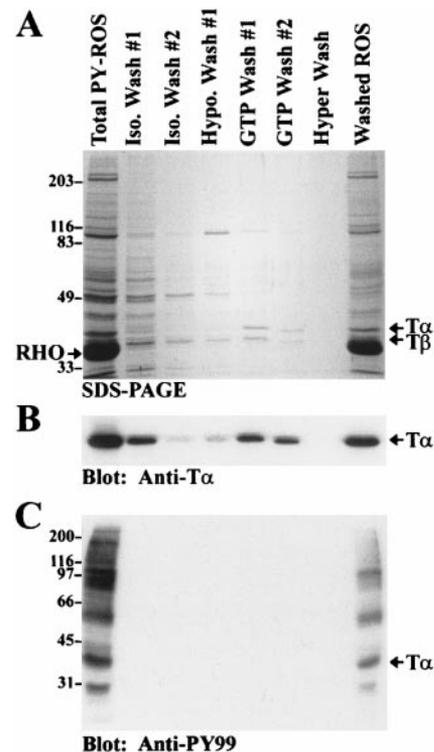
Association of tyrosine-phosphorylated Tα with ROS membranes

We attempted to purify tyrosine-phosphorylated Tα by extracting it from light-adapted ROS membranes in a hypotonic buffer containing GTP (Kuhn, 1980, 1982; Baehr et al., 1982). ROS were incubated in a buffer containing Mg^{2+}, ATP, and orthovanadate and then subjected to sequential washes in buffers of various ionic strengths. Equal volumes of the unwashed PY-ROS, supernatants from washes, and washed ROS membranes were subjected to SDS-PAGE and immunoblot analysis using anti-Tα and anti-PY99. Tα was present in the first isotonic wash, the GTP-hypotonic washes, and the washed ROS membranes (Fig. 6A and B). A very small amount of Tα was also present in the other washes. Incubation of ROS with ATP and orthovanadate prior to washing did not affect the amount of Tα recovered in the GTP-hypotonic washes (data not shown). Immunoblot analysis using anti-PY99 showed a 39-kDa PY-protein corresponding in size to Tα in the unwashed ROS and

washed ROS membranes (Fig. 6C). A faint 39-kDa PY-protein was also detected in the first isotonic wash upon longer exposure of the immunoblot (data not shown). Overexposure of the anti-PY99 immunoblot did not detect a 39-kDa PY-protein corresponding to Tα in the GTP-hypotonic washes, which contain transducin, indicating that tyrosine-phosphorylated Tα is not extracted from ROS membranes using GTP (data not shown). Tyrosine-phosphorylated Tα was also absent from hypotonic washes containing GTPγS instead of GTP (data not shown).

Tyrosine-phosphorylated Tα is resistant to extraction by GTP

To assess the effect of tyrosine phosphorylation on membrane association of Tα, we compared the extractability of transducin subunits from N-ROS and PY-ROS by GTP (Fig. 7). As shown, after several isotonic and hypotonic washes of N-ROS and PY-ROS, significantly more transducin was recovered in the first GTP wash (lane 6) from N-ROS than from PY-ROS. This was evident both on Coomassie Blue-stained gels (Fig. 7A)

FIG. 6. Association of tyrosine-phosphorylated Tα with ROS membranes. ROS (1 mg/ml) were incubated for 1 h at 37°C in buffers containing 10 mM MgCl$_2$, 1 mM ATP, and 1 mM orthovanadate. The PY-ROS were homogenized and subjected to sequential washes in buffers of various ionic strengths. Equal volumes of the total (unwashed) PY-ROS (20 μg), washes, and final washed ROS membranes were resolved on a 7.5% gel and (**A**) stained with Coomassie Blue or subjected to immunoblot analysis with (**B**) anti-Tα or (**C**) anti-PY99. The migrations of rhodopsin (RHO), Tα (PY-39), and the β subunit of transducin (Tβ) are indicated.

FIG. 7. Tyrosine-phosphorylated Tα in ROS membranes is resistant to GTP extraction. ROS (1 mg/ml) were incubated for 1 h at 37°C in buffers containing 10 mM MgCl$_2$ and 1 mM orthovanadate, with or without 1 mM ATP. N-ROS and PY-ROS (right panel) were homogenized and subjected to sequential washes in buffers of various ionic strengths (lanes 1 and 2, isotonic; lanes 3–5, hypotonic; lanes 6 and 7, hypotonic containing 0.2 mM GTP). Equal aliquots of the total (unwashed) PY-ROS (50 μg), washes, and final washed ROS membranes were resolved on a 10% gel and (**A**) stained with Coomassie Blue or subjected to immunoblot analysis with (**B**) anti-Tα or (**C**) anti-Tβ. Arrowheads show the amount of transducin recovered in first hypotonic GTP wash (lane 6) and the amount of transducin remaining in the final membranous pellet (lane 8). The migration of rhodopsin (RHO) is also indicated by arrow.
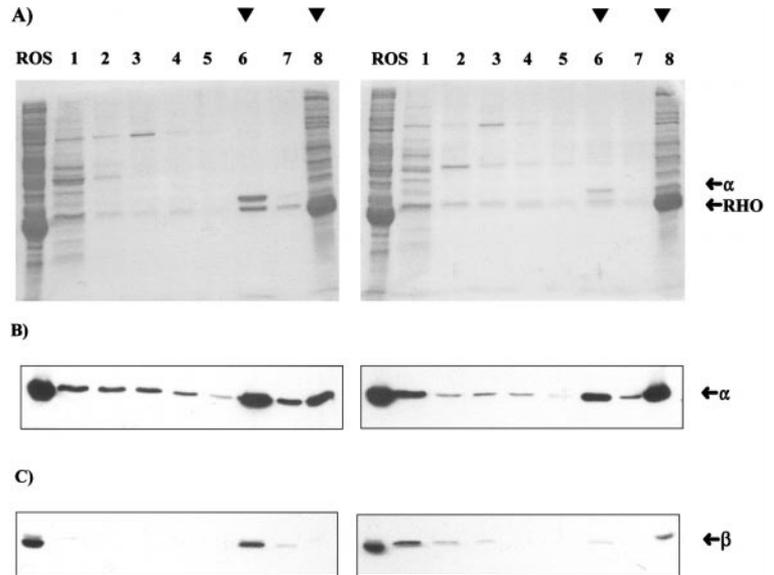
and on immunoblots of the same fractions with either anti-Tα (Fig. 7B) or anti-Tβ (Fig. 7C). Moreover, the amount of transducin subunits that were still membrane-bound was also significantly higher in PY-ROS than in N-ROS (lane 8), indicating that tyrosine phosphorylation enhances the binding of both Tα and Tβ to ROS membranes.
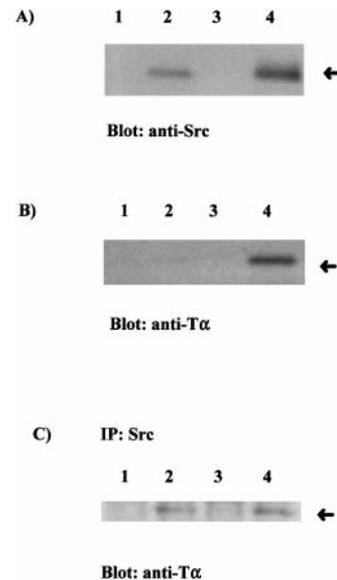
Tyrosine phosphorylation enhances the association of Tα with Src

To investigate further the specificity of association between Tα and Src, N-ROS and PY-ROS were enriched on a PY-99-agarose resin, followed by elution with phenyl phosphate. As shown in Fig. 8A and B, recovery of both Src and Tα was significantly increased from PY-ROS over N-ROS, indicating that the association between the two proteins is enhanced under conditions that favor tyrosine phosphorylation (lanes 2 and 4). Specificity of this interaction was verified by using GST-agarose instead of PY-99-agarose, where neither protein was detected in the phenyl phosphate eluates (lanes 1 and 3). In other experiments, N-ROS and PY-ROS were immunoprecipitated with anti-Src or normal rabbit IgG and probed on immunoblots with Tα (Fig. 8C). Anti-Src immunoprecipitates showed the presence of Tα in both N-ROS and PY-ROS; however, the amount of Tα recovered from PY-ROS (Fig. 8C, lane 4) was significantly increased over that from N-ROS (Fig. 8C, lane 2), indicating that tyrosine phosphorylation increases the association between Tα and Src. Tα was absent from control immunoprecipitates with nonimmune serum (lanes 1 and 3), indicating the specificity of association of Src and Tα.

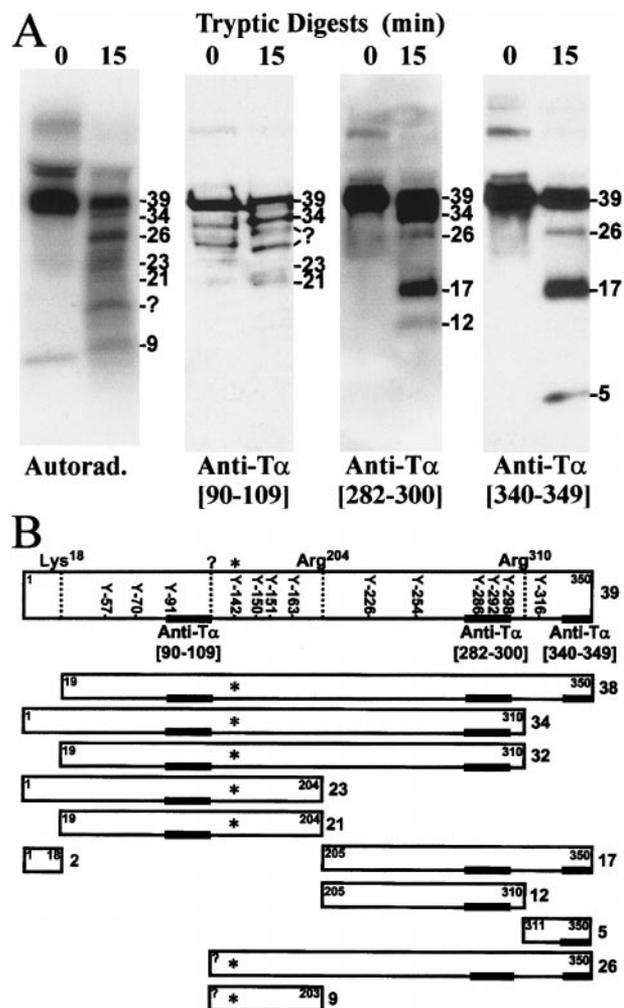## Tryptic proteolysis of tyrosine-phosphorylated Tα

In an attempt to identify the tyrosine phosphorylation site(s) on Tα, tyrosine-phosphorylated Tα-GDP labeled with [γ-^{32}P]ATP was subjected to limited tryptic proteolysis, and the proteolytic fragments were analyzed by

comparing autoradiograms and immunoblots using antibodies that recognize three different epitopes on Tα (Fig. 9A). Mazzoni et al. (1991) have shown that limited tryptic proteolysis cleaves Tα at Lys^{18}, Arg^{204}, and

FIG. 8. Tyrosine phosphorylation-dependent association of Src and Tα. Phenyl phosphate eluates (see Materials and Methods for details) of N-ROS or PY-ROS from PY99-agarose (lanes 2 and 4) or GST-agarose (lanes 1 and 3) were probed on immunoblots with (**A**) anti-Src or (**B**) anti-Tα. **C:** Identification of Tα in anti-Src immunoprecipitates (IP) of ROS. ROS (475 μg) incubated under phosphorylating (lanes 3 and 4) or nonphosphorylating conditions (lanes 1 and 2) were solubilized as previously described, precleared with protein A–agarose, and incubated with 5 μg of normal rabbit IgG (lanes 1 and 3) or 5 μg of anti-Src antibody overnight at 4°C. The immunoprecipitates were subjected to SDS-PAGE on 10% gels transferred to nitrocellulose and probed with anti-Tα at a dilution of 0.5 μg/ml.

FIG. 9. Limited tryptic proteolysis of Tα phosphorylated by c-Src. **A:** Purified transducin (5 μg) was phosphorylated by purified c-Src (5 units) and digested with TPCK-trypsin (0.5 μg) for either 0 or 15 min. The proteolytic fragments were resolved on a 10–20% gradient Tris-Tricine gel and subjected to autoradiography (far left) or immunoblot analysis with antibodies to Tα that recognize an epitope corresponding to amino acids 90–109 (left of center), 282–300 (right of center), or 340–349 (far right). The approximate molecular masses of the ^{32}P-labeled and immunoreactive tryptic fragments are indicated. **B:** Peptide map shows known cleavage sites (dashed lines) at Lys[18], Arg[204], and Arg[310] available during limited tryptic digestion of Tα-GDP. An additional cleavage site (?) may be present at Arg[96], Arg[101], Lys[103], Arg[125], or Lys[126]. The sites recognized by the three anti-Tα antibodies (thick bars), the positions of the 13 tyrosine (Y) residues, and the phosphorylation site at Tyr[142] (*) identified by ion trap mass spectrometry are indicated. The numbers to the right of the fragments indicate their approximate molecular masses.

Arg[310], and produces five transient proteolytic fragments of 38, 34, 32, 23, and 17 kDa and four final fragments of 21, 12, 5, and 2 kDa (Fig. 9B). The three antibodies to Tα recognize three different epitopes on the final 21-, 12-, and 5-kDa proteolytic fragments, allowing all of the transient and final fragments that contain tyrosine residues to be identified. Because tyrosine-phosphorylated

Tα could not be extracted from bovine ROS membranes, we labeled bovine Tα-GDP using [γ-^{32}P]ATP and purified recombinant c-Src, which previously has been shown to phosphorylate Gα$_i$ subunits, including Tα, in reconstitution assays with stoichiometries ranging from 0.3 to 0.9 mol of phosphate per mol of Gα (Hausdorff et al., 1992). Autoradiography and immunoblot analyses of undigested Tα identified a ^{32}P-labeled protein corresponding in size to Tα, indicating that Tα was phosphorylated by Src. ^{32}P-labeled tryptic fragments of 39, 34, 26, 23, 21, 15, and 9 kDa were identified by autoradiography of transducin digested for 15 min. The expected 38- and 32-kDa proteolytic fragments of Tα are likely to be comigrating with the 39-kDa protein and 34-kDa fragment, respectively. Anti-Tα [90–109], which recognizes an epitope corresponding to amino acids 90–109, immunoreacted with fragments of 39, 34, 23, and 21 kDa, which are the same size as several ^{32}P-labeled fragments, suggesting the presence of at least one phosphorylation site between Lys[18] and Arg[204]. Two additional bands at approximately 25 and 27 kDa did not correspond to any ^{32}P-labeled fragments. Anti-Tα [282–300] immunoreacted with the 34-, 17-, and 12-kDa fragments, but the 17- and 12-kDa fragments did not correspond to any ^{32}P-labeled fragments, suggesting that the tyrosine residues between Arg[204] and the carboxy terminus are *not* phosphorylated. The 17- and 5-kDa fragments recognized by anti-Tα [340–349] also did not correspond to any ^{32}P-labeled fragments. However, an unexpected 26-kDa proteolytic fragment was recognized by both anti-Tα [282–300] and anti-Tα [340–349] and corresponded exactly in size to a ^{32}P-labeled fragment, suggesting the presence of an additional cleavage site at Arg[125] or Lys[126] based on its estimated molecular weight. It is also possible that the potential cleavage site is located at Arg[96], Arg[101], or Lys[103]. The 26-kDa fragment is transient and disappears soon after 15 min of limited tryptic proteolysis (data not shown). A 9-kDa ^{32}P-labeled fragment, which is not recognized by any of the antibodies, corresponds in size to a Tα proteolytic fragment that would be cleaved at Arg[125]/Lys[126] and Arg[204]. If the 9- and 26-kDa ^{32}P-labeled fragments represent an additional cleavage site at Arg[125] or Lys[126], it suggests that the tyrosine phosphorylation site(s) on Tα are located at Tyr[142], Tyr[150], Tyr[151], and/or Tyr[163]. Upon longer exposure of the immunoblot with anti-Tα [90–109], we observed 14- and 12-kDa fragments, which would represent the amino-terminal fragments cleaved at Arg[125]/Lys[126] (data not shown). We were unable to collect enough of the 9-, 12-, 14-, and 26-kDa proteolytic fragments for sequencing, but except for the 14-kDa fragment, they do not correspond in size to Tβ, Tγ, or c-Src proteolytic fragments (data not shown). We were also unable to identify the 15-kDa ^{32}P-labeled fragment.

Mass spectrometric sequencing of Tα tryptic phosphopeptides

To determine the specific tyrosine residue(s) on Tα phosphorylated by Src, peptides generated by in-gel tryp-
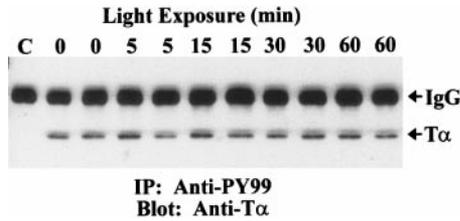
FIG. 10. Tyrosine phosphorylation of Tα in vivo in rat retina. Albino rats were dark-adapted overnight and killed in the dark (0 min) or after exposure to light for 5, 15, 30, or 60 min (2 rats/time point). Retinas from each rat were excised and solubilized together. Equal amounts (500 µg) of dark-adapted and light-exposed rat retinas were subjected to immunoprecipitation with agarose-conjugated anti-PY99. For control (C), dark-adapted retinas were subjected to immunoprecipitation with normal non-immune mouse IgG. The samples were subjected to immunoblot analysis with anti-Tα. The migrations of IgG and Tα are indicated.

tic digestion of Src-phosphorylated Tα were sequenced by microcapillary reverse-phase HPLC directly coupled to a custom nano-electrospray ionization source of an ion trap mass spectrometer. A phosphopeptide fragment representing amino acids Ala^{139}–Arg^{157} of Tα was sequenced and found to be phosphorylated at Tyr^{142}. There is a slight chance that the phosphopeptide was phosphorylated on Ser^{140} instead of Tyr^{142}; however, because Tα was phosphorylated by the Src tyrosine kinase in a reconstitution assay, the phosphopeptide was most likely phosphorylated at Tyr^{142}. The phosphopeptide was not phosphorylated at Tyr^{150} or Tyr^{151}. No other tryptic peptides that were sequenced were tyrosine-phosphorylated; however, we cannot rule out the presence of additional tryptic phosphopeptides that were either not recovered or not sequenced.

Tα is tyrosine-phosphorylated in vivo in rats

Rats were dark-adapted overnight and killed either in the dark or following exposure to light for 5, 15, 30, or 60 min. The retinas were dissected, solubilized, and subjected to immunoprecipitation using anti-PY99. Tα, which is expressed only in photoreceptor cells, was identified in anti-PY99 immunoprecipitates from dark-adapted and light-exposed rat retinas by immunoblot analysis using anti-Tα (Fig. 10). Approximately equal amounts of Tα were present in all of the immunoprecipitates, indicating that light did not affect the tyrosine phosphorylation of Tα. Tα was not present in control immunoprecipitates from rat retina using normal mouse IgG. Anti-Tα immunoprecipitates from dark- and light-adapted rat retinas contained very weak anti-PY99 immunoreactions corresponding in size to Tα, suggesting that the stoichiometry of tyrosine phosphorylation in vivo may be low (data not shown).

DISCUSSION

Tyrosine phosphorylation of cellular proteins plays a significant role in a variety of transmembrane and intra-

cellular signal transduction pathways (for review, see Hunter, 1995). In the current study, we investigated the tyrosine phosphorylation of endogenous proteins in retinal photoreceptors by incubating isolated bovine ROS in a buffer containing Mg^{2+}, ATP, and orthovanadate. Several PY-proteins were identified that had apparent molecular masses of approximately 31, 39, 60, 66, 83, 90, 97, 120, 140, and 180 kDa. The extent of tyrosine phosphorylation increased with increasing concentrations of ATP and was inhibited significantly by several tyrosine kinase inhibitors. We have previously shown that the addition of orthovanadate, a general tyrosine phosphatase inhibitor (Swarup et al., 1982), is essential to enhancing tyrosine phosphorylation in ROS, probably by inhibiting protein tyrosine phosphatase activity, which we have previously identified in ROS (Bell et al., 1999). Light did not have an effect on tyrosine phosphorylation in isolated bovine ROS under the conditions used in this report (data not shown). We do not know the specific mechanism by which light stimulates tyrosine phosphorylation in photoreceptors (Ghalayini et al., 1998a), but it may require an intact photoreceptor cell or retina.

Src is a ubiquitously expressed tyrosine kinase containing two Src homology 2 (SH2) domains and one SH3 domain that has been shown to be tyrosine-phosphorylated in response to activation of both growth factor receptors and GPCRs (for review, see Thomas and Brugge, 1997). We identified the 60-kDa PY-protein (PY-60) in anti-phosphotyrosine (anti-PY) immunoprecipitates from ROS as Src by immunoblot analysis using an antibody that recognizes three members (c-Src, Fyn, and Yes) of the Src family. PY-60 was removed from ROS by immunodepletion with anti-Src, further verifying the identification of PY-60 as Src in ROS. Immunodepletion of Src did not completely inhibit tyrosine phosphorylation in ROS, indicating that one or more other tyrosine kinases may also be active under these conditions. However, tyrosine phosphorylation in ROS was inhibited significantly by PP1, which specifically inhibits tyrosine phosphorylation by Src tyrosine kinases at similar concentrations in intact cells (Hanke et al., 1996), suggesting that Src may be partially responsible for the tyrosine phosphorylation of proteins in sealed ROS under our incubation conditions. To ensure that tyrosine kinase activity and the presence of Src in purified bovine ROS were not cytosolic contaminants from other retinal cells, osmotically intact ROS were purified a second time on a continuous sucrose gradient, and gradient fractions were analyzed for Src content and tyrosine kinase activity. Both were enriched in the same fractions that contained the highest amounts of rhodopsin and rod Tα, which are expressed only in rod photoreceptor cells and are significantly enriched in purified ROS (Zimmerman and Godchaux, 1982).

The 39-kDa PY-protein was identified as Tα by immunoblot analysis of anti-PY immunoprecipitates from ROS. Tyrosine phosphorylation of Tα was further confirmed by immunoblot analysis of anti-Tα immunoprecipitates from ROS with anti-PY, which showed immu-

noreactivity with a 39-kDa protein corresponding exactly in size to Tα. However, we cannot rule out that another PY-protein of a similar size to Tα may also be present. Several PY-proteins, including two with apparent molecular masses of 60 and 97 kDa, also coimmunoprecipitated with Tα. The 60-kDa PY-protein associated with Tα was identified as Src. Although the association of Tα with Src occurred in the absence of tyrosine phosphorylation of Tα, it was enhanced by conditions that increase tyrosine phosphorylation in ROS. We were unable to identify the 97-kDa PY-protein, but it is similar in size to a PY-protein that also coimmunoprecipitated with SHP-2 from ROS that were incubated under the same conditions used here (Bell et al., 1999). In addition, we also observed tyrosine kinase activity associated with Tα that was increased significantly in anti-Tα immunoprecipitates from PY-ROS compared with those from N-ROS, which may indicate stimulation of tyrosine kinase activity by tyrosine phosphorylation. The presence of Src in anti-Tα immunoprecipitates suggests that Src may be responsible for the tyrosine kinase activity in anti-Tα immunoprecipitates; however, we cannot rule out the presence of another tyrosine kinase in these immunoprecipitates. An earlier report has shown that purified preparations of Tα-GDP contain an endogenous kinase activity, but the kinase responsible for the activity was never characterized (Waldbillig et al., 1988). In another report, Src was shown to be present in highly purified preparations of G_i from brain (Neer and Lok, 1985). Taken together, our results and the previous observations suggest that Tα associates with Src in ROS either directly or indirectly through interactions involving an adaptor protein.

Transducin is a peripherally associated protein that can be eluted from ROS disk membranes in a hypotonic buffer containing GTP (Kuhn, 1980, 1982; Baehr et al., 1982). PY-ROS were subjected to sequential washes in buffers of various ionic strengths. Transducin was eluted in the GTP-hypotonic wash, but none of the Tα in this wash was tyrosine-phosphorylated, suggesting that tyrosine-phosphorylated Tα is not eluted with GTP. A significant amount of Tα remained bound to disc membranes even after extensive washing in the GTP-hypotonic buffer. Although tyrosine phosphorylation of Tα could potentially increase its affinity for ROS membranes, or cause a conformational change that prevents its dissociation from ROS membranes, several other factors could also be preventing the dissociation of Tα from ROS membranes. Vanadate has previously been shown to inhibit the GDP–GTP exchange of Tα (Kancho et al., 1988), which could subsequently inhibit the elution of transducin from the membranes using GTP. In carefully controlled experiments, we show that under tyrosine phosphorylation conditions less Tα and Tβ could be recovered in hypotonic GTP washes of ROS. Moreover, the amount of transducin that remains associated with the membranes after extensive washing is also significantly higher under phosphorylating conditions. These observations strongly suggest that tyrosine phosphorylation of

Tα strengthens its membrane association. However, we cannot rule out that other modifications, such as myristoylation of Tα (Neubert et al., 1992; Yang and Wensel, 1992) and/or its interactions with rhodopsin and Tβγ, could also strengthen its membrane association. It is also worth mentioning that Tβ was also detected in both Src immunoprecipitates and phenyl phosphate eluates from PY99-agarose (data not shown). However, we have not been able to determine conclusively if tyrosine phosphorylation of Tα affects its interaction with Tβ. Interaction of Tα with other membrane-associated proteins, such as Src, which is enhanced under tyrosine phosphorylation conditions (Fig. 8), may also contribute to its tight association with ROS membranes. Alternatively, the population of Tα that is tightly associated with the ROS membranes and is not eluted with GTP may be in a conformation that is more susceptible to tyrosine phosphorylation. Src association with Tα in N-ROS could involve its SH2, SH3, or a yet undefined domain in Src or Tα. As this association is increased in PY-ROS (Fig. 8), it would suggest that this may involve tyrosine-phosphorylated residues of Tα interacting with the SH2 domain of Src. The observation that Src activity is low in N-ROS may indicate that the increased activity of Src in PY-ROS may be a consequence of autophosphorylation on Tyr[419]. Our data with phenyl phosphate elution of Src from PY99-agarose would indicate an increase in tyrosine phosphorylation of Src in PY-ROS that is coincident with its increased association with Tα; however, the specific site of tyrosine phosphorylation is unknown. Based on other reports on Src activation (Hubbard et al., 1998), it is unlikely that this involves phosphorylation of the inhibitory Tyr[530]. Instead, dephosphorylation of this tyrosine residue (possibly by SHP-2) may precede and might be necessary for its observed activation in PY-ROS. The inhibition of tyrosine kinase activity by PP1 would also suggest that autophosphorylation of Src is required for its activation. These observations taken together strongly suggest that Src is the most likely tyrosine kinase involved in the phosphorylation of Tα in ROS.

Other investigators have previously demonstrated that Tα can be tyrosine-phosphorylated in reconstitution assays with either the insulin-like growth factor 1 receptor (Zick et al., 1987; Waldbillig et al., 1988), the insulin receptor (Zick et al., 1986), or Src (Hausdorff et al., 1992). In this report, we have shown that Src phosphorylates the amino-terminal half of Tα by comparing the expected tryptic peptide map from limited proteolysis of Tα to the autoradiogram and anti-Tα immunoblots of ^{32}P-labeled tryptic fragments from phosphorylated Tα. Our tryptic proteolysis of Tα also indicates that an additional cleavage site may be present at Arg[125], Lys[126], or another nearby site, allowing us to further narrow the phosphorylation site to between Arg[125] and Arg[204]. The additional cleavage site in Tα may be exposed by a conformational change caused by tyrosine phosphorylation; however, recovery and sequencing of the 9- and 26-kDa proteolytic fragments will be required to verify

their identity. The carboxy-terminal half of Tα did not appear to be phosphorylated by Src, but it could be phosphorylated by other tyrosine kinases, such as the insulin-like growth factor 1 receptor, which have also been shown to phosphorylate Tα.

We found that Src phosphorylates Tα at Tyr[142] by sequencing tryptic peptides from Src-phosphorylated Tα. Tyr[142] is located in the helical domain of Tα in an area between the αD and αE helices that is involved in binding the guanosine nucleotide (Noel et al., 1993). The helical domain of Gα proteins acts as a hinged lid covering the guanosine nucleotide-binding pocket and is intricately involved in the binding and hydrolysis of GTP (Markby et al., 1993; Noel et al., 1993; Coleman et al., 1994). A recent report indicates that the helical domain of Tα may also act synergistically to enhance Tα-GTPγS activation of cyclic GMP phosphodiesterase (PDE), yet attenuate activated PDE in the inactive Tα-GDP state through interactions with the PDE catalytic core (Liu and Northup, 1998; Liu et al., 1998). Tyr[142] of Tα is highly conserved in most Gα subunits, but has not previously been shown to be phosphorylated in any Gα protein. Other reports have shown that Gα$_s$ (Moyers et al., 1995; Liebmann et al., 1996; Poppleton et al., 1996), Gα$_{q/11}$ (Liu et al., 1996; Umemori et al., 1997), and Gα$_i$ (O'Brien et al., 1987; Krupinski et al., 1988; Hausdorff et al., 1992) can also be tyrosine-phosphorylated. Src phosphorylated Gα$_s$ at Tyr[37] and Tyr[377] (Moyers et al., 1995), and Gα$_{q/11}$ was phosphorylated at Tyr[356] (Umemori et al., 1997); however, these sites are not conserved in Tα.

All Gα proteins contain functional regions that are involved in either the binding and hydrolysis of GTP or interactions with either their specific receptors, Gβγ subunits, downstream effectors, or regulators of G-protein signaling (RGS) (for review, see Neer, 1995; Hamm, 1998; Berman and Gilman, 1998). It is unlikely that phosphorylation of Tα at Tyr[142] would affect its association with Tβγ or interactions with rhodopsin, PDEγ, or RGS-9, but it could affect its GTPase activity or its proposed interactions with the catalytic subunits of PDE (Liu and Northup, 1998; Liu et al., 1998). Hausdorff et al. (1992) have previously shown that Src preferentially phosphorylates the holomeric GDP-bound form of Tα and causes a 140% increase in the rate of GTP hydrolysis catalyzed by light-activated rhodopsin compared with unphosphorylated Tα. Due to its proximity to the guanosine-binding pocket, Tyr[142] may be more accessible for phosphorylation in the Tα-GDP form and its phosphorylation could affect the binding and hydrolysis of GTP. Alternatively, phosphorylation of Tα at this site could promote its interaction with other signaling proteins, such as Src, and promote the formation of signaling complexes. Mutational analysis of Tyr[142] in Tα/Gα$_{i1}$ chimeras (Skiba et al., 1996) in the future could allow us to understand the functional consequences resulting from the tyrosine phosphorylation of Tα.

The significance of the tyrosine phosphorylation of Tα in photoreceptors is unclear. The recent observations that light (Ghalayini et al., 1998a) and growth factors (Fontaine et al., 1998) stimulate tyrosine phosphorylation in photoreceptor cells suggest that the state of tyrosine phosphorylation of photoreceptor proteins is likely to play an important regulatory role. Although light did not appear to affect the phosphorylation of Tα in vivo in rat retina, tyrosine phosphorylation of Tα and its association with Src in isolated ROS suggest that this modification may occur in vivo in photoreceptors in response to a stimulus yet to be identified. In an earlier report, we have shown that the tyrosine phosphatase SHP-2 associates with Tα, as well as an unknown 97-kDa PY-protein, under experimental conditions similar to those in the current report (Bell et al., 1999). Although the functional significance of this association remains to be elucidated, we propose that Tα may be involved in the formation of a multimeric signaling complex containing Src, SHP-2, and an unknown 97-kDa protein. Subsequent phosphorylation/dephosphorylation of Tα at Tyr[142] by Src and SHP-2, respectively, could affect its GTPase activity and/or regulate its association with ROS membranes or the catalytic subunits of PDE. However, based on our in vitro studies, we hypothesize that tyrosine phosphorylation of Tα in vivo might enhance its association with disc membranes. This could ultimately modulate the sensitivity of the photoresponse in rod cells. Alternatively, if tyrosine phosphorylation in vivo does recruit or target Tα to the disc membranes, it could also play a role in the light-dependent distribution (translocation) of Tα within photoreceptor cells. The formation of a signaling complex containing Tα, Src, SHP-2, and the unknown 97-kDa protein may also suggest a role for Tα in a novel signal transduction pathway in photoreceptors that has yet to be elucidated.

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