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Growth Factor Receptor-Bound Protein 14 Undergoes Light-Dependent Intracellular Translocation in Rod Photoreceptors: Functional Role in Retinal Insulin Receptor Activation†

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ABSTRACT: Growth factor receptor-bound protein 14 (Grb14) is involved in growth factor receptor tyrosine kinase signaling. Here we report that light causes a major redistribution of Grb14 among the individual subcellular compartments of the retinal rod photoreceptor. Grb14 is localized predominantly to the inner segment, nuclear layer, and synapse in dark-adapted rods, whereas in the light-adapted rods, Grb14 redistributed throughout the entire cell, including the outer segment. The translocation of Grb14 requires photoactivation of rhodopsin, but not signaling through the phototransduction cascade, and is not based on direct Grb14−rhodopsin interactions. We previously hypothesized that Grb14 protects light-dependent insulin receptor (IR) activation in rod photoreceptors against dephosphorylation by protein tyrosine phosphatase 1B. Consistent with this hypothesis, we failed to observe light-dependent IR activation in $Grb14^{-/-}$ mouse retinas. Our studies suggest that Grb14 translocates to photoreceptor outer segments after photobleaching of rhodopsin and protects IR phosphorylation in rod photoreceptor cells. These results demonstrate that Grb14 can undergo subcellular redistribution upon illumination and suggest that rhodopsin photoexcitation may trigger signaling events alternative to the classical transducin activation.

Phosphoinositide 3-kinase kinase (PI3K) is at the heart of one of the major signal transduction pathways (*1*−*4*). The signals mediated by this enzyme influence a wide variety of cellular functions, including cell growth, differentiation and survival, glucose metabolism, and cytoskeletal organization. The PI3K is expressed in photoreceptor cells and is regulated through the light-induced tyrosine phosphorylation of the insulin receptor (IR)[1] *in vivo* (*5*, *6*). We have reported that light-induced tyrosine phosphorylation of IR requires the photoactivation of rhodopsin but not transducin signaling (*7*). We also found that photoreceptor-specific deletion of IR resulted in stress-induced photoreceptor degeneration, suggesting the importance of IR in the survival of photoreceptor neurons (*8*).

The molecular mechanism behind the light-induced activation of retinal IR is not known. Retinal IR has a high basal level of autophosphorylation compared to liver IR (*9*), and retinal IR autophosphorylation is light-dependent (*5*). These observations led us to hypothesize that retinal IR phosphorylation could be modulated by soluble factor(s) in the retina. To identify the regulators of IR, yeast two-hybrid screening of a bovine retinal cDNA library with the cytoplasmic domain of retinal IR (*10*) identified growth factor receptor-bound protein 14 (Grb14) (*11*, *12*), which binds to various tyrosine kinase receptors including IR (*13*−*16*). The crystal structure of the tyrosine kinase domain in complex with the IR-interacting domain of Grb14 has been resolved and revealed that Grb14 acts as a pseudosubstrate inhibitor that binds in the peptide binding groove of the kinase and thus functions as a selective inhibitor of insulin signaling (*17*). *In vitro* experiments have shown that Grb14 impairs the tyrosine kinase activity of the IR toward exogenous substrates and protects the tyrosine phosphorylation from dephosphorylation by protein tyrosine phosphatase 1B (PTP1B) (*18*). In liver, Grb14 deletion resulted in decreased IR phosphorylation due to increased dephosphorylation of the IR by PTP1B (*19*). The precise functional role of Grb14 in the retina is not known.

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[1]Abbreviations: Grb14, growth factor receptor-bound protein 14; ROS, rod outer segments; IR, insulin receptor; Tdα, alpha subunit of transducin; Rpe, retinal pigment epithelium; PTP1B, protein tyrosine phosphatase 1B.

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Here we report that Grb14 undergoes light-dependent intracellular redistribution upon illumination of rod photoreceptor cells. In the dark, Grb14 was found to occupy all subcellular compartments of the rod, except for the outer segment. Following 30 min of light exposure, Grb14 was found evenly distributed throughout the entire rod cell, including the outer segment. This process is triggered by the photoexcitation of rhodopsin but is not mediated by transducin signaling. Ablation of Grb14 in the retina resulted in the loss of light-dependent activation of retinal IR, and our studies suggest Grb14 translocates to photoreceptor outer segments following photobleaching of rhodopsin and protects the IR phosphorylation in rod photoreceptors cells. These findings demonstrate that Grb14 can undergo subcellular redistribution upon illumination and suggest that rhodopsin may have additional previously uncharacterized signaling functions in photoreceptors.

EXPERIMENTAL PROCEDURES

Materials. Polyclonal anti-transducin-alpha (Td) subunit and anti-cytochrome C antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-myc tag and monoclonal anti-IRβ antibodies were obtained from Cell Signaling (Danvers, MA). X-press antibody was obtained from Invitrogen. The monoclonal anti-opsin antibody (Rho 4D2) was a gift from Dr. Robert Molday (University of British Columbia). The anti-arrestin antibody was a gift from Dr. Paul Hargrave (University of Florida). Grb14 antibody was from Chemicon International Inc. The TNT-Quick-coupled transcription and translation kit was purchased from Promega (Madison, WI). All other reagents were of analytical grade from Sigma (St. Louis, MO).

Animals. All animal work was in strict accordance with the *NIH Guide for the Care and Use of Laboratory Animals*, and the Association for Research in Vision and Ophthalmology on the Use of Animals in Vision Research. All of the protocols were approved by the IACUC of the University of Oklahoma Health Sciences Center and the Dean McGee Eye Institute. Transducin alpha ($Td\alpha^{-/-}$) knockout mice were derived on a BALB/cx129/SvJ background at Tufts University (*20*). Mice lacking retinal pigment epithelium 65 protein ($Rpe65^{-/-}$) were derived at the National Institutes of Health, Bethesda, MD (*21*). Wild-type controls were obtained from breeding pairs established with C57BL/6-DBA F1s. The generation of photoreceptor-specific conditional insulin receptor knockout (*8*) and Grb14 knockout mice have been reported previously (*19*). We carried out all experiments with 6−8-week-old mice. A breeding colony of albino Sprague-Dawley rats is maintained in our vivarium in cyclic light (12 h on; 12 h off; ~300 lx). Experiments were carried out on both male and female rats (150−200 g). All animals were born in 60 lx cyclic light (12 h on/off) in the animal facility and maintained under these lighting conditions until they were used in experiments.

Production and Characterization of Polyclonal Grb14 Antibody. To study the expression, immunolocalization, and interaction of Grb14 with other proteins, we generated a rabbit polyclonal anti-peptide antibody (amino acids 57-TRGCAADRRKKKDLDVLE-74 of the bovine sequence). We characterized its specificity and compared it with a commercially available polyclonal Grb14 antibody (raised to the N-terminus) from Chemicon (Figure 1A). Bovine ROS, mouse, and rat retina lysates were subjected to Western blot analysis with Grb14 anti-peptide antibody, and the results indicate the

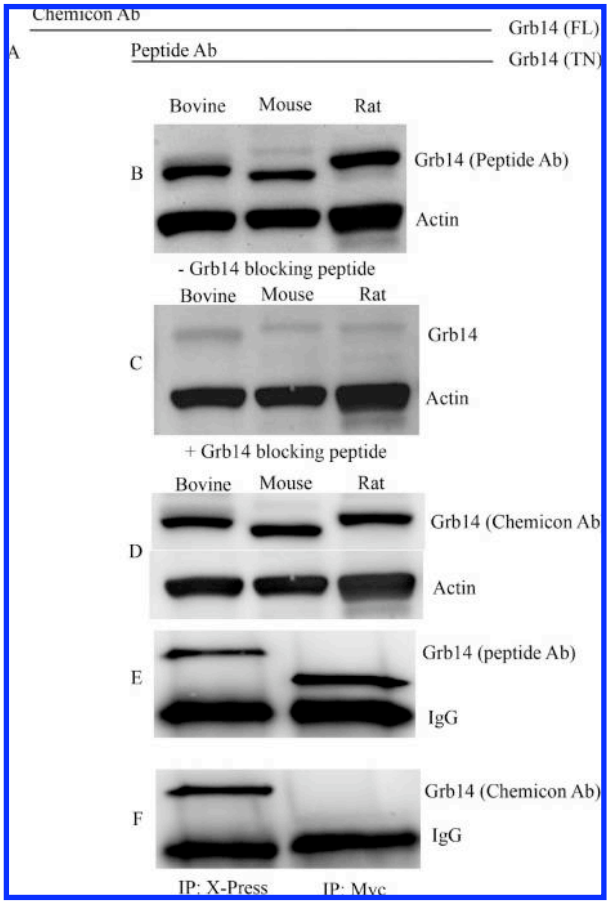

FIGURE 1: Characterization of Grb14 antibodies. Full-length and truncated versions of Grb14 are shown in (A). Twenty micrograms of bovine ROS, mouse, and rat retinal lysates was subjected Western blot analysis with Grb14 peptide antibody in the absence (B) and presence (C) of Grb14 blocking peptide to which the antibody was generated. The blot treated with blocking peptide was reincubated with Grb14 antibody from Chemicon (D). X-press-tagged full-length and truncated myc-tagged Grb14 were expressed *in vitro* using the *in vitro* coupled transcription and translation system, and the proteins were subjected to immunopreciptaion with respective tags. The immune complexes were run on SDS−PAGE followed by Western blot analysis with either Grb14 peptide antibody (E) or Grb14 antibody from Chemicon (F).

expression of Grb14 in all species (Figure 1B). Detection of the Grb14 immunoreactivity was prevented by coincubating the anti-peptide antibody with peptide to which the antibody was generated (Figure 1C). This blot was reprobed with Chemicon antibody, and the results clearly indicate the reappearance of the immunoreactivity of Grb14 in bovine, mouse, and rat tissues (Figure 1D). To further demonstrate the specificity of these antibodies, we expressed truncated myc-tagged bovine Grb14 (missing the N-terminus) and full-length X-press-tagged mouse Grb14 *in vitro* using an *in vitro* coupled transcription and translation system. The expressed proteins were immunoprecipitated with either X-press-tag or myc-tag antibodies followed by Western blot analysis with anti-Grb14 antibodies. The results indicate that the peptide antibody recognizes both full-length and truncated Grb14 (Figure 1E) whereas Chemicon antibody recognizes only full-length but not truncated Grb14 (Figure 1F). These experiments further confirm the specificity of the Grb14 antibodies. We used the peptide antibody for all of the experiments described in this paper.

Light Conditioning of the Animals and Preparation of Rod Outer Segments. Albino rats or mice were dark adapted

overnight and sacrificed by CO_2 asphyxiation either under dim red light or following 30 min of light exposure (300 lx). ROS were prepared from either rat or mouse retinas using discontinuous sucrose gradient centrifugation as previously described (*5*, *7*). Eight retinas from four mice or four retinas from two rats were homogenized in 1.25 mL of ice-cold 47% sucrose solution containing 100 mM NaCl, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10 mM Tris-HCl (pH 7.4). Retinal homogenates were transferred to 4.5 mL centrifuge tubes and sequentially overlaid with 1.5 mL of 37% and 1.0 mL of 32% sucrose dissolved in buffer A. The gradients were centrifuged at 82000*g* for 90 min at 4 °C. The 32%/37% interfacial sucrose band containing ROS membranes was harvested and diluted with 10 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and 1 mM EDTA and centrifuged at 27000*g* for 30 min. The ROS pellets were resuspended in 10 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and 1 mM EDTA and stored at −20 °C. Protein concentrations were determined using the BCA reagent from Pierce (Pierce, Rockford, IL) following the manufacturer's instructions.

Preparation of Retinal Sections for Immunohistochemistry. Preparation of retinal tissue sections and methods of immunohistochemistry were described previously (*22*). Enucleated eyes were immersed in 4% paraformaldehyde (PFA) containing 20% 2-propanol, 2% trichloroacetic acid, and 2% zinc chloride for 24 h (mouse or rat eyes) at room temperature. The eyes were embedded in paraffin, and 4 μm thick sections containing the whole retina including the optic disk were cut along the vertical meridian of the eyeball. Endogenous peroxidase activity was inactivated with 3% H_2O_2 for 10 min. For paraffin-embedded sections, antigen was retrieved by microwaving in 10 mM citrate buffer for 8 min. The sections were blocked with serum-free blocking reagent (Dako, Carpenteria, CA) for 1 h at room temperature and incubated for 2 h at 37 °C with anti-Grb14 antibody diluted (1:200) with antibody diluent (Dako) and then with peroxidase-linked anti-rabbit IgG polymer (EnVision + System, Dako) for 1 h at 37 °C. The signals were developed with 3′,3′-diaminobenzidine (Dako) as chromogen. The sections were observed using a Nikon Eclipse E800 microscope.

Serial Tangential Sectioning with Western Blotting. Serial tangential sectioning was performed according to the method described (*23*). Rat eyes were enucleated and dissected under dim red light. Retinas were placed in ice-cold Ringer's solution (130 mM NaCl, 3.6 mM KCl. 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.02 mM EDTA, 10 mM HEPES−NaOH (pH 7.4) adjusted to 313 mosM). Retinas were flat mounted by placing them between two glass slides separated by 0.5 mm spacers. The "sandwich" was then clamped with two small binder clips and immediately frozen on dry ice. The bottom side of the sandwich was in contact with the basal membrane of the retina. To ensure adhesion, the glass slide was roughened using sandpaper. The top side of the photoreceptors was covered with polytetrafluoroethylene spray to facilitate their subsequent separation from the retina. For sectioning, the clips and the glass slides were removed, and the bottom side of the attached retina was mounted on the cryomicrotome specimen holder. The retina was trimmed to remove any folded edges, and sequential, 5 μm tangential retinal sections were cut using a cryomicrotome. Each section was collected into 100 μL of SDS−PAGE sample buffer. Aliquots of each sample were subjected to SDS−PAGE followed by Western blot analysis with anti-Grb14, anti-rhodopsin, and anti-cytochrome C oxidase antibodies.

Plasmid Construction. Bovine Grb14 cDNA was amplified by PCR from pGAD10-Grb14 yeast two hybrid vector (*11*) and cloned into myc-tagged-pCDNA3 vector. This construct lacks the first 100 amino acids of the full-length protein. The cDNA encoding visual arrestin was cloned into myc-tagged pCDNA3 vector. Full-length X-pressed-tagged mouse Grb14 construct was a kind gift from Dr. Alexandra Newton (UCSD). All constructs that involved PCR were verified by DNA sequencing.

Grb14 Binding Assays. Binding experiments were carried out as described (*24*−*27*) with the following modifications. Myc-tagged Grb14, X-press-tagged Grb14, or myc-tagged arrestin was expressed *in vitro* using a TNT-coupled transcription and translation system. The reaction mixture containing 1 μg of plasmid DNA, 1 mM methionine, and 40 μL of TNT-T7 quick master mix was prepared in a total volume of 50 μL and incubated at 30 °C for 90 min. The binding experiments were carried out in dim red light. Twenty microliters of *in vitro* expressed product was incubated with 20 μg (total protein) of ROS membranes from dark- or light-adapted rat retinas in a total volume of 50 μL of binding buffer [50 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 150 mM potassium acetate, and 1.5 mM dithiothreitol]. The reaction was kept on ice and either exposed to light or kept in the dark for 5 min. At the end of the reaction, tubes were centrifuged for 5 min at 15000 rpm, and the supernatant was decanted. The ROS were washed three times with wash buffer (50 mM Tris-HCl, pH 7.5, and 100 mM NaCl) with repeated centrifugation. Sample buffer was added, and the ROS were subjected to SDS−PAGE followed by Western blot analysis with anti-myc antibody.

Insulin Receptor Kinase Activity. Wild-type and $Grb14^{-/-}$ mice were dark adapted overnight, and the next morning, half of the animals were exposed to normal room light (300 lx) for 30 min as described previously (*7*). Retinas were harvested and lysed in lysis buffer [1% NP 40, 20 mM HEPES (pH 7.4) and 2 mM EDTA] containing phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM $NaVO_3$, and 1 mM molybdate) and protease inhibitors (10 μM leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethanesulfonyl fluoride)]. Insoluble material was removed by centrifugation at 17000*g* for 20 min, and the solubilized proteins were precleared by incubation with 40 μL of protein A−Sepharose for 1 h at 4 °C with mixing. The supernatant was incubated with anti-IRβ antibody overnight at 4 °C and subsequently with 40 μL of protein A−Sepharose for 2 h at 4 °C. Following centrifugation at 14000 rpm for 1 min, immune complexes were washed three times with wash buffer [50 mM HEPES (pH 7.4) containing 118 mM NaCl, 100 mM sodium fluoride, 2 mM $NaVO_3$, 0.1% (w/v) sodium dodecyl sulfate, and 1% (v/v) Triton X-100]. The kinase reaction was performed at room temperature in kinase assay buffer [50 mM HEPES, pH 7.4, 12 mM $MgCl_2$, and 5 mM $MnCl_2$] containing 100 μM ATP, 3 mg/mL poly(Glu-Tyr) peptide (Sigma), and 10 μCi/mL [γ-^{32}P]ATP for 60 min. The reaction was briefly centrifuged at 14000 rpm for 1 min, and 15 μL of supernatant was spotted on Whatman p81 phosphocellulose paper disks. Filter paper disks were washed three times for 5 min in 0.75% *O*-phosphoric acid and once for 5 min in acetone before counting the radioactivity in a Beckman LS 6000SC scintillation counter (Beckman Instruments, Fullerton, CA).

SDS−PAGE and Western Blotting. Proteins were resolved by 10% SDS−PAGE and transferred onto nitrocellulose membranes. The blots were washed two times for 10 min with TTBS [20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1%

Tween-20] and blocked with either 5% bovine serum albumin or nonfat dry milk powder (Bio-Rad) in TTBS for 1 h at room temperature. Blots were then incubated with anti-Grb14 (1:1000), anti-Tdα subunit (1:8000), anti-arrestin (1:1000), and anti-opsin (1:10000) antibodies either overnight at 4 °C or 1 h at room temperature. Following primary antibody incubations, immunoblots were incubated with HRP-linked secondary antibodies (either anti-rabbit or anti-mouse) and developed by ECL according to the manufacturer's instructions.

RESULTS

Presence of Grb14 in Light-Adapted Rod Outer Segment (ROS) Membranes. ROS samples containing equal amounts of rhodopsin were collected from either dark- or light-adapted rat retinas and subjected to Western blot analysis with anti-Grb14, anti-Tdα subunit, anti-arrestin, and anti-opsin antibodies (Figure 2A). We observed significantly more Grb14 immunoreactivity associated with light-adapted than dark-adapted ROS membranes (Figure 2A). Arrestin and Tdα, well documented to undergo light-dependent translocation in rods, were used as positive controls for light and dark adaptation conditions, and as expected, light-adapted ROS (LROS) contained more arrestin and less transducin than the dark-adapted ROS (DROS) (Figure 2A).

Immunocytochemical Analysis of Grb14 in Dark- and Light-Adapted Rat Retina. The result obtained in Figure 2A has two potential interpretations. One is that illumination causes Grb14 to translocate to ROS from other parts of the photoreceptor cell. Alternatively, Grb14 may have increased affinity for light-adapted membranes and binds them upon homogenization of the retina. To determine whether Grb14 indeed undergoes light-driven translocation into ROS, we analyzed its subcellular localization in fixed cross sections of dark- and light-adapted rat retinas. In the dark, the Grb14 immunoreactivity was detected in the inner segment (Figure 2B,C) and outer plexiform layer (Figure 2B). However, following 30 min of light exposure, the Grb14 immunoreactivity was reduced in rod inner segments and increased in outer segments (Figure 2B,C). This result suggests that Grb14 undergoes a change in its intracellular localization upon illumination.

In addition to immunohistochemistry, we undertook an independent approach to analyze the intracellular distribution of Grb14 in light- and dark-adapted rods by measuring its content in serial 5 μm tangential cryosections from flat-mounted retinas (Figure 3). Grb14 in individual sections was visualized by Western blotting, and its distribution among the sections was compared to that of two intracellular markers, rhodopsin (a marker for ROS) and cytochrome C (a marker for the inner segment and synaptic terminal). These experiments indicate that in the dark Grb14 is present in the inner segment, the nuclear region, and the synaptic terminal, but not in ROS, whereas in the light it spreads rather evenly throughout the entire rod cell (Figure 3). Taken together, the results obtained by immunohistochemistry and serial sectioning with Western blotting establish that the intracellular localization of Grb14 in rods is light-dependent.

Transducin Signaling Is Not Required for Light-Dependent Translocation of Grb14. The light-dependent redistribution of Grb14 in rods could be driven by a mechanism linked to the activation of the phototransduction cascade. For example, such a dependency was previously shown to be important for arrestin translocation (*28*). To determine whether phototransduction has an effect on the light-dependent translocation of Grb14, we examined the translocation of Grb14 in rod transducin α-subunit ($Td^{-/-}$) knockout mice (*20*). These mice contain normal amounts of rhodopsin, but its photoexcitation does not initiate phototransduction due to the lack of transducin.

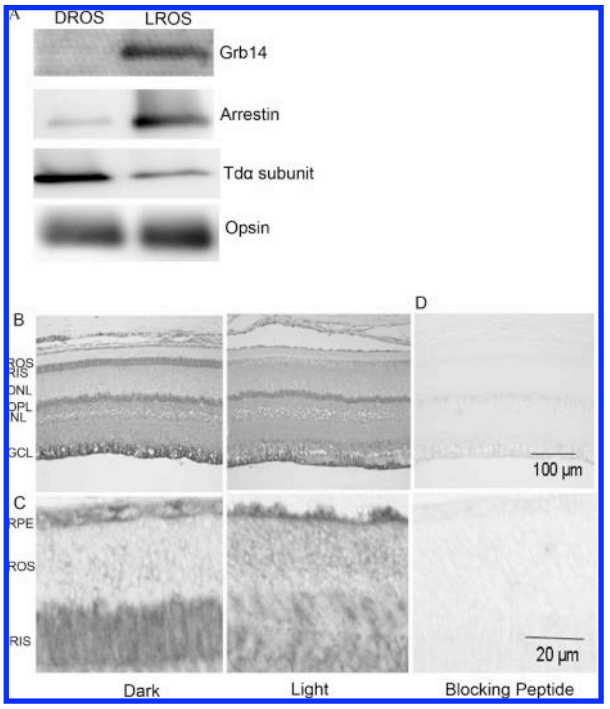

FIGURE 2: Light-dependent presence and ROS localization of Grb14. Mice were dark adapted overnight, and half were subjected to 300 lx for 30 min. Proteins from dark (DROS) or light (LROS) adapted ROS from wild-type mice were subjected to Western blot analysis with anti-Grb14, anti-arrestin, anti-Tdα subunit, and anti-opsin antibodies (A). Immunocytochemical analysis of Grb14 in dark- and light-adapted rat retinas. Paraffin-fixed sections of dark- and light-adapted (30 min) rat retinas were stained for Grb14. Complete view of retina (B) and region between rod inner segment (RIS) and rod outer segment (ROS) (C). Antibody staining was blocked with Grb14 blocking peptide from which the antibody was generated (D). Key: INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer; GCL, ganglion cell layer; RPE, retinal pigment epithelium.

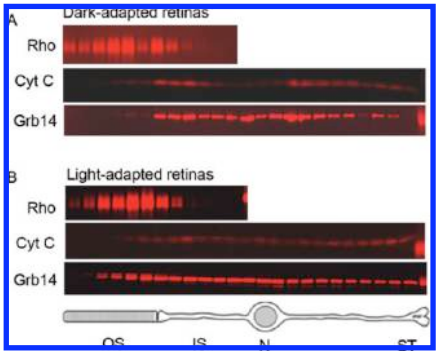

FIGURE 3: Determination of protein distribution throughout the photoreceptor layer of the rat retina by serial tangential cryosectioning with Western blotting. Western blots showing distribution of Grb14 and two marker proteins, rhodopsin (Rho) and cytochrome C (Cyt C), in the serial sections obtained from the retinas of either a dark-adapted rat (A) or a rat exposed to 30 min illumination (B) at normal room temperature. Each line of the gel represents the protein content of a single 5 μm section into the retina starting from the outer segment tips and progressing inward. Key: OS, outer segment; IS, inner segment; N, nucleus; ST, synaptic terminal.

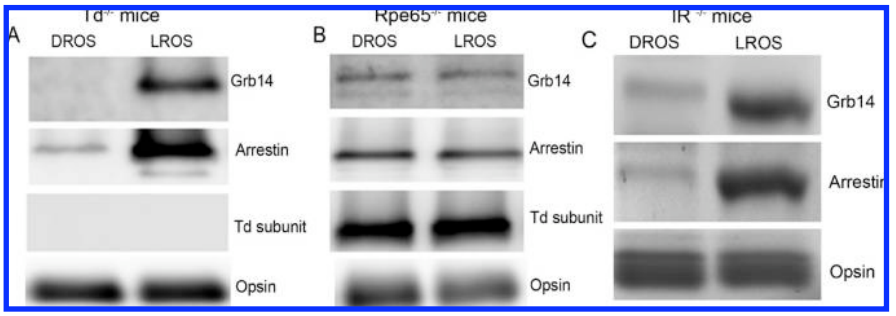

FIGURE 4: Binding of Grb14 to light- or dark-adapted rod outer segments of $Td^{-/-}$, $Rpe65^{-/-}$, and $IR^{-/-}$ mice. Mice were dark adapted overnight, and half were subjected to 300 lx for 30 min. Light (LROS) or dark (DROS) adapted ROS from $Td^{-/-}$ (A), $Rpe65^{-/-}$ (B), and $IR^{-/-}$ mice were subjected to Western blot analysis with anti-Grb14, anti-arrestin, anti-Td subunit, and anti-opsin antibodies.

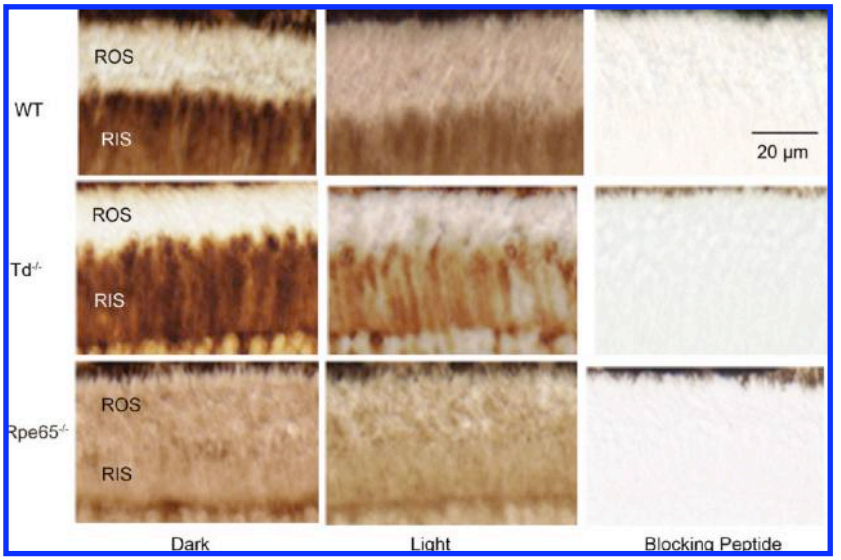

FIGURE 5: Immunocytochemical analysis of Grb14 in dark- and light-adapted retinas from wild-type, $Td\alpha^{-/-}$, and $Rpe65^{-/-}$ mice. Paraffin-fixed sections of dark- and light-adapted (30 min) mouse retinas were stained for Grb14. Antibody staining was blocked with Grb14 blocking peptide from which the antibody was generated. Key: ROS, rod outer segment; RIS, rod inner segment.

We isolated ROS from light- and dark-adapted $Td^{-/-}$ mice and found that light-dependent Grb14 enrichment in LROS was not affected by the knockout (Figure 4A). These experiments suggest that phototransduction is not necessary for the light-dependent redistribution of Grb14. However, we have not tested the light threshold of translocation of Grb14 in $Td^{-/-}$ mice as has been shown in the case of arrestin translocation in $Td^{-/-}$ mice (*28*).

Photoactivation of Rhodopsin Is Necessary for Light-Dependent Translocation of Grb14. To confirm that the light-dependent translocation of Grb14 is mediated by a signal originating from photoactivatable rhodopsin, we examined the translocation of Grb14 in retinas from $Rpe65^{-/-}$ mice that are deficient in 11-*cis*-retinal and therefore do not contain photobleachable rhodopsin. In these mice, Grb14 was found in both light- and dark-adapted ROS (Figure 4B), indicating that its localization is not light-regulated in $Rpe65^{-/-}$ mice. These data further suggest that opsin, but not the most active metarhodopsin II intermediate, is required for Grb14 localization to ROS. These results also suggest that photoactivation of rhodopsin reduces Grb14's affinity for a component or a hypothetical scaffolding complex in the inner segment.

ROS Binding of Grb14 Is Not IR Dependent. We previously identified Grb14 as a retinal IR-interacting protein by yeast two-hybrid screening (*11*, *12*) . To determine whether the IR in ROS provides the docking signal for Grb14 translocation, we examined the ROS localization of Grb14 in photoreceptor-specific $IR^{-/-}$ mice (*8*). We isolated ROS from light- and dark-adapted $IR^{-/-}$ mice and found that the light-dependent Grb14 enrichment in ROS was not affected by the lack of IR (Figure 4C). These experiments suggest that ROS localization of Grb14 does not require IR or its signaling.

Immunolocalization of Grb14 in Wild-Type, $Td^{-/-}$, and $Rpe65^{-/-}$ Mice. The patterns of Grb14 binding to isolated ROS from wild-type, $Td^{-/-}$, and $Rpe65^{-/-}$ mice were further substantiated by immunocytochemical analysis (Figure 5). Mouse retina sections were prepared from light- (30 min) and dark-adapted wild-type, $Td^{-/-}$, and $Rpe65^{-/-}$ mice and stained for Grb14. The results clearly indicate that Grb14 immunostaining was concentrated in the RIS in both wild-type and $Td^{-/-}$ dark-adapted mice (Figure 5). The Grb14 immunostaining decreased in the RIS and appeared in the ROS in wild-type and $Td^{-/-}$ mice (Figure 5) exposed to light. The localization of Grb14 in the RIS and ROS from dark- and light-adapted $Rpe65^{-/-}$ was the same. These results further confirm our biochemical findings that ROS localization of Grb14 requires the presence of either opsin or photoexcited rhodopsin intermediates.

Does Grb14 Bind to Photoactivated Rhodopsin?. To determine whether the translocation of Grb14 can be explained by its direct binding to rhodopsin, we expressed myc-tagged

Grb14 *in vitro* and incubated the product with LROS or DROS or DROS exposed to light for 5 min on ice. At the end of incubation, ROS were collected as described in Experimental Procedures and subjected to Western blot analysis with anti-myc antibody (Figure 6A,B). Control experiments were carried out with *in vitro* expressed myc-tagged arrestin. The results indicate that Grb14 binds to both LROS and DROS independent of light adaptation (Figure 6A). On the other hand, arrestin bound only to LROS or to DROS exposed to light, *in vitro*, but not to DROS (Figure 6A). The blots were also reprobed with anti-arrestin antibodies to confirm the light- and dark-adapted localization of endogenous arrestin (Figure 6C; note that the light exposure of DROS also resulted in an increase in arrestin binding, which can be explained by its direct interaction with photoactivated rhodopsin). To rule out the possibility that the lack of light dependency of Grb14 binding to ROS membranes could be explained by insufficient amounts of ROS membranes in the binding reaction, we carried out additional experiments with a 5-fold greater amount of ROS. We observed no light dependency in the Grb14 binding, whereas the light dependency of arrestin binding was preserved (Figure 6D). Collectively, the *in vitro* binding experiments indicate that Grb14 does not directly bind to photoactivated rhodopsin, but ROS contain binding sites for Grb14 that do not change their properties upon illumination. Collectively, these experiments suggest that Grb14 translocation is light-dependent (Figures 2 and 3), but its binding is not (Figure 6). These experiments further suggest that light might reduce the affinity of Grb14 binding in the inner segment.

Effect of Grb14$^{-/-}$ Deletion on Retinal Morphology and Function. Light microscopic examination of retinas from wild-type and Grb14$^{-/-}$ mice at 6−8 weeks of age showed no difference in retinal structure when each group was maintained in dim cyclic light (Figure 7A). The retinas appeared normal, and ROS appeared to be well organized (Figure 7A). There were 11 to 12 rows of photoreceptor nuclei in the outer nuclear layer (ONL), the number usually observed for rodents without retinal degeneration (*8*). Quantitative analysis of the superior and inferior regions of the ONL layer showed no significant differences in the average ONL thickness measured at 0.25 mm intervals from the ONL to the inferior and superior ora serrata among the two groups (Figure 7B), indicating that rod photoreceptor viability was not different among these mice. Thus, mice lacking Grb14 appeared to have no structural differences, and Grb14$^{-/-}$ mice did not exhibit any structural phenotype when maintained in dim cyclic light.

Electroretinography (ERG) was used to evaluate photoreceptor function in wild-type and Grb14$^{-/-}$ mice at 2 months of age. No significant differences were found in the amplitudes of the scotopic a-wave, which measures the response of rod photoreceptors to light stimuli, or the scotopic b-wave, which measures the response of the inner retinal cells (Figure 7C). The photopic cone b-wave amplitude was also found to be normal (Figure 7D). These results show that Grb14$^{-/-}$ mice at 2 months of age do not exhibit any functional phenotype when they are maintained in dim cyclic light.

Loss of Light-Dependent IR Activation in Grb14$^{-/-}$ Mice. We previously reported the light-dependent activation of IR in rod photoreceptor cells, and we hypothesized that outer segment localized Grb14 could protect IR phosphorylation against PTP1B (*7*). We directly tested this hypothesis in Grb14$^{-/-}$ mice. The IR kinase activity (which measures the phosphorylated state of IR) was measured from wild-type and Grb14$^{-/-}$ mice under dark- and light-adapted conditions.

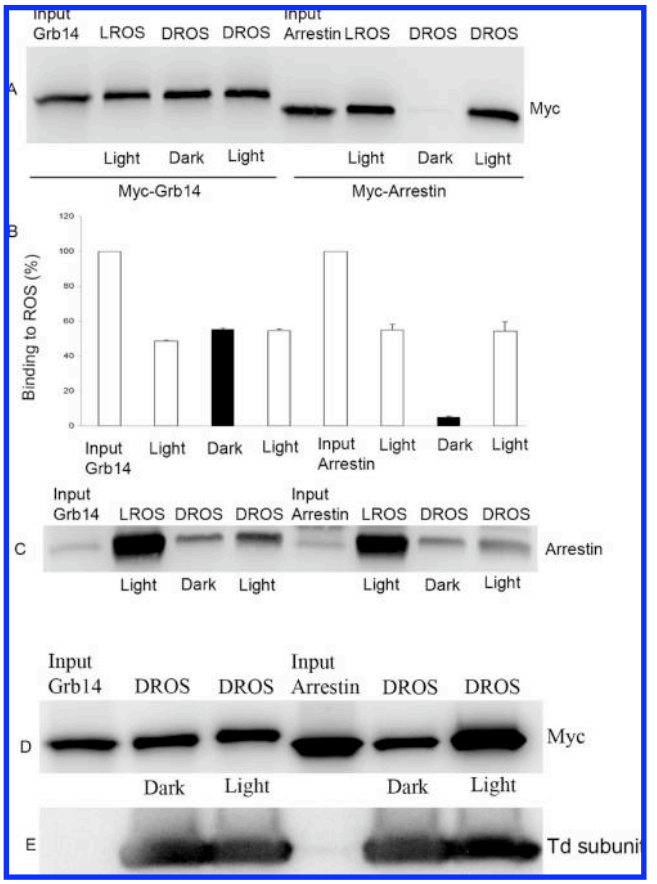

FIGURE 6: Binding of Grb14 to ROS membranes *in vitro*. Myc-tagged Grb14 or myc-tagged arrestin was expressed using *in vitro* coupled transcription and translation in a total volume of 50 μL as described in Experimental Procedures. Light- or dark-adapted ROS membranes containing 20 μg of protein were incubated with 20 μL of *in vitro* expressed myc-tagged Grb14 or arrestin. LROS were kept in the light, whereas DROS were either kept in the dark or exposed to light. The binding assay was conducted after 5 min incubation on ice as described in Experimental Procedures. (A) ROS proteins were subjected to Western blot analysis with anti-myc antibody. The input contained 10 μL of *in vitro* expressed protein products of myc-tagged Grb14 or myc-tagged arrestin. (B) Densitometric analysis of immunoblots was performed in the linear range of detection, and absolute values were expressed as percentage of the input (myc-Grb14 or myc-arrestin) normalized by the amount of loaded protein. Data are mean ± SD, $n = 3$. (C) The blot in (A) was reprobed with anti-arrestin antibody to detect endogenous arrestin. (D) Binding experiments were also carried out with 100 μg of DROS followed by Western blot analysis with anti-myc antibody. (E) The blot in (D) was reprobed with anti-Tdα subunit.

In wild-type mice, IR kinase activity was significantly increased in light-adapted compared to dark-adapted retinas (Figure 8A). The light-dependent activation of IR kinase activity was lost in Grb14$^{-/-}$ mice (Figure 8A), and the IR kinase activity was comparable to the dark-adapted state. These results suggest the loss of light-dependent IR activation in Grb14$^{-/-}$ mouse retinas. To determine whether IRs are functional in Grb14$^{-/-}$ mouse retinas, we added 100 nM insulin to light-adapted wild-type and Grb14$^{-/-}$ retinas and carried out the IR kinase activity. The results indicate increased IR kinase activity in Grb14$^{-/-}$ retinas, and activation is comparable to wild-type retinas (Figure 8B).

DISCUSSION

The central observation of this study is that the IR-interacting protein, Grb14, undergoes light-dependent translocation within

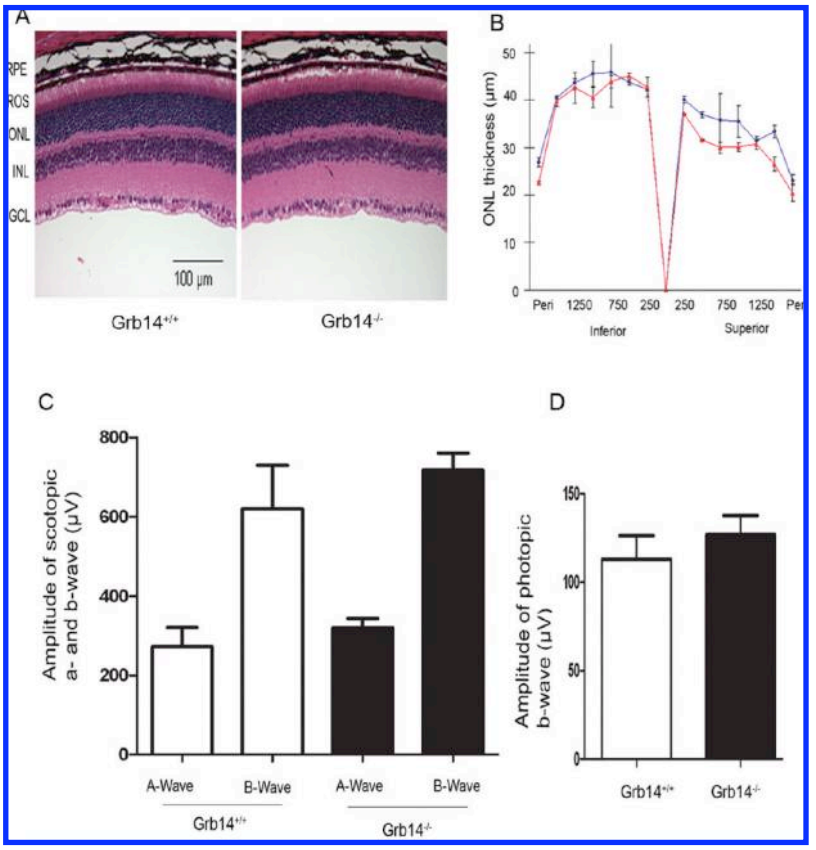

FIGURE 7: Morphological and functional characterization of Grb14$^{-/-}$ mice. Hematoxylin/eosin-stained retinal sections of wild-type and Grb14$^{-/-}$ mice at 6−8 weeks of age (A). Plots of total retinal thickness in the superior and inferior regions of the retinas of wild-type (blue line) and Grb14$^{-/-}$ (red line) mice (B). Values are mean ± SD from five mice in each group. Examination of retinas from each group did not reveal any structural differences in any of the retinal cells at the light microscope level. Key: RPE, retinal pigment epithelium; ROS, rod outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. The ERG responses were recorded from wild-type and Grb14$^{-/-}$ mice raised in dim cyclic light at 8 weeks of age. Average scotopic a- and b-wave amplitudes shown in (C) and average photopic b-wave amplitudes shown in (D) were measured from wild-type and Grb14$^{-/-}$ mice. The a-wave amplitude was measured from the resting level to the peak of the cornea-negative deflection, and the b-wave amplitude was measured from the trough of the a-wave to the crest of the cornea-positive response. Values are presented as mean ± SD, $n = 5$.

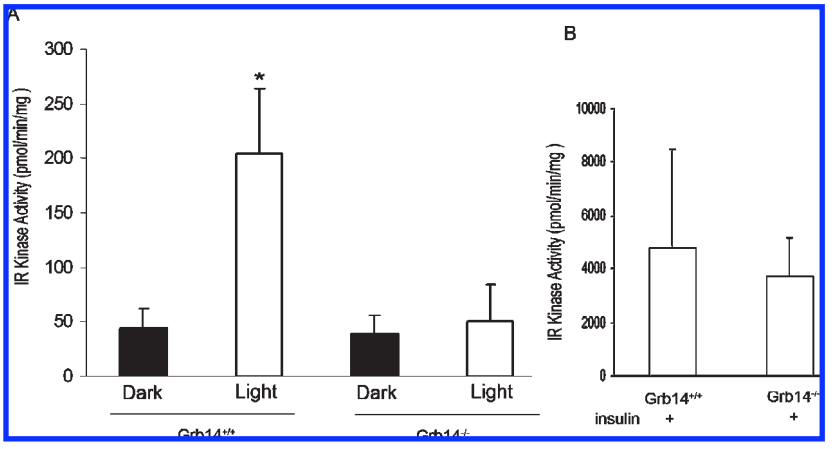

FIGURE 8: IR kinase activity in wild-type and Grb14$^{-/-}$ mice. IR kinase activity was measured from retinas harvested from dark- and light-adapted wild-type and Grb14$^{-/-}$ mice (A). The IR immunoprecipitates were subjected to IR kinase activity employing poly(Glu-Tyr) peptide as substrate. Retina lysates prepared from light-adapted wild-type and Grb14$^{-/-}$ mice were subjected to immunoprecipitation with anti-IRβ antibody and subjected to IR kinase activity in the presence of 100 nM insulin (B). Data mean ± SD, $n = 6$. *, $p < 0.034$.

rod photoreceptor cells. The outer segment localization of Grb14 required photoactivation of rhodopsin but did not require transducin signaling. Further, ablation of Grb14 resulted in the loss of light-dependent activation of retinal IR. Our results suggest that photoreceptors possess a novel pathway that uses rhodopsin-mediated Grb14 translocation to connect rhodopsin signaling with a tyrosine kinase signal transduction pathway.

Cross-Talk of GPCR and Tyrosine Kinase Signaling Pathways in Photoreceptors. We previously reported that the G-protein-coupled receptor rhodopsin regulates phosphorylation of the IR which leads to the activation of a downstream

survival pathway in rods (*5*, *29*). The significance of this pathway is underscored by the observation that deletion of the IR from rods resulted in stress-induced photoreceptor degeneration (*8*). These results suggest the existence of cross-talk between rhodopsin and tyrosine kinase-mediated signal transduction in photoreceptors. Such cross-talk has been shown in other cells where many tyrosine kinase cascades are regulated by GPCRs (*30*, *31*). For example, the binding of PYK2, a nonreceptor protein tyrosine kinase, to N-terminal domain-interacting receptors (Nir) is activated by GPCRs (*32*). Nir proteins are human homologues of the *Drosophila* retinal degeneration B protein (rdgB), a protein implicated in the visual transduction pathway (*32*). In vertebrate photoreceptors, light also induces the activation of nonreceptor tyrosine kinase Src and promotes its association with a complex containing bleached rhodopsin and arrestin (*33*). It is also proposed that the small G-protein Rac-1 may be regulated by rhodopsin in both *Drosophila* (*34*) and vertebrates (*35*). All of these studies suggest that rhodopsin photoexcitation may trigger signaling events alternative to classical transducin activation. Our current study clearly suggests that rhodopsin-mediated Grb14 translocation regulates the neuroprotective IR signaling pathway in rod photoreceptors.

Function of Grb14 in Photoreceptors. *In vitro* experiments have shown that Grb14 impairs the tyrosine kinase activity of the IR toward exogenous substrates; however, Grb14 has no effect on the autophosphorylated IR (*18*). Grb14 has also been shown to protect the autophosphorylated IR from dephosphorylation by PTP1B (*18*). We previously reported the light-dependent activation of IR in rod photoreceptor cells and that retinas lacking photoreceptors fail to show light-dependent IR activation (*5*, *6*). On the basis of our earlier studies, we hypothesized that outer segment localized Grb14 could protect the IR phosphorylation against PTP1B (*7*). Consistent with this hypothesis, we observed a loss of light-dependent retinal IR kinase activation in Grb14$^{-/-}$mice. The biochemical phenotype is similar to liver tissues as Grb14 deletion resulted in decreased IR phosphorylation due to increased dephosphorylation of the IR by PTP1B (*19*). In this study we also observed that addition of insulin to Grb14 knockout mouse retinas resulted in the activation of IR. These results indicate the existence of a rhodopsin-regulated, Grb14-dependent, light-mediated IR pathway in photoreceptors that is different from the known insulin-mediated pathway in non-neuronal tissues. The Grb14$^{-/-}$ mice did not show any structural or functional phenotype. This is not an unexpected finding as our photoreceptor-specific IR knockout mice did not show any functional or structural phenotype under normal lighting conditions. However, in response to light stress IR$^{-/-}$ mice exhibited photoreceptor degeneration (*8*). It is possible that Grb14$^{-/-}$ mice may also be more sensitive to light stress. Studies are underway in our laboratory to test this possibility. Our studies demonstrate that Grb14 translocates to photoreceptor outer segments following photobleaching of rhodopsin, resulting in enhanced IR activation in rod photoreceptors cells.

Putative Mechanism of Grb14 Translocation. The current observation that the IR interacting protein Grb14 changes its intracellular localization in response to light complements many previous reports that light exposure results in the massive translocation of three key signal transduction proteins, transducin, arrestin, and recoverin, into and out of ROS (*36*). It is believed that this phenomenon contributes to adaptation of photoreceptors to diurnal changes in ambient light intensity and may also contribute to protecting rods from damage imposed by bright illumination (*36*−*41*).

Here we demonstrate that the knockout of transducin, the only G-protein involved in vertebrate phototransduction, does not prevent light-triggered Grb14 translocation to ROS. This suggests that Grb14 translocation is mediated by neither the classical phototransduction cascade nor any other signaling downstream from transducin. However, the translocation of Grb14 requires the photoactivation of rhodopsin, which was established in experiments utilizing Rpe65 knockout mice. As discussed above, these data support the existence of cross-talk between rhodopsin and tyrosine kinase signaling in rods, although any specific details of this mechanism remain a challenge for future studies.

In Rpe65$^{-/-}$ mice, Grb14 lost its light-dependent compartmentalization and is present in the outer segment regardless of illumination. One can hypothesize that weak activity of opsin brings Grb14 to the ROS, and this possibility cannot be ruled out. However, recombinant myc-tagged Grb14 binds to DROS equally well as LROS suggest that Grb14 binding may not be due to interaction with bleached opsin. Under bright illumination, both Grb14 and arrestin move to the outer segment, and in this regard, both Grb14 and arrestin translocation are light-dependent. A major difference between Grb14 and arrestin is that arrestin binding to purified ROS is light-dependent whereas Grb14 binding is not (Figure 6). In addition, in bright light the majority of arrestin moves to the outer segment whereas only a fraction of Grb14 moves while a significant pool of Grb14 still remains in the inner segment. Our data suggest that in light we are not generating binding sites for Grb14 in the ROS but that light is inducing the release of Grb14 from the inner segment.

Strissel et al. (*28*) demonstrated that arrestin translocation is not dependent on the absolute amount of rhodopsin excited by light and proposed that light induces the release of arrestin from binding sites in the inner segment and that this released arrestin subsequently diffuses to all cellular compartments, including the outer segment. The same "light-induced release" mechanism may work for Grb14 as well. As shown in Figure 6, ROS may also contain Grb14 binding sites, but these sites alone cannot drive the process of translocation because they do not undergo any appreciable light-induced changes. Figure 6A also indicates that Grb14 does not bind directly to photoactivated rhodopsin but may bind to other ROS proteins or may bind to membrane phosphoinositides (*12*).

Our *in vitro* binding studies suggest that we are not generating any high-affinity binding sites for Grb14 in light as we see equal binding to both light- and dark-adapted ROS. Grb14 binding is unlike the binding of arrestin which is increased in the light in the *in vitro* binding studies. Further the light-independent binding of Grb14 to ROS is specific as the recombinant Grb14 did not bind to the COS-7 cell membranes (data not shown). Identification of binding partner(s) of Grb14 may provide additional clues as to how this protein is translocated in rod photoreceptor cells. Grb14 has several functional domains that may potentially interact with a variety of proteins involved in intracellular signaling (*14*). It may also be important to investigate the nature of the interaction of Grb14 with motor proteins or other components of the polarized transport machinery. However, it should be recognized that the entire Grb14 translocation phenomenon could be potentially explained by a combination of the light-dependent changes in the affinity of Grb14 for binding sites outside ROS and intracellular diffusion.

In summary, our results demonstrate that Grb14 can undergo subcellular redistribution upon illumination and suggest that rhodopsin photoexcitation may trigger signaling events additional to classical phototransduction.

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