Published in final edited form as:
Biochemistry. 2009 June 23; 48(24): 5563–5572. doi:10.1021/bi9000062.

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

Ammaji Rajala[1,3], **Roger J. Daly**[5], **Masaki Tanito**[1,3,*], **Dustin T. Allen**[1,3], **Lowenna J. Holt**[5], **Ekaterina Lobanova**[4], **Vadim Y. Arshavsky**[4], and **Raju V.S. Rajala**[1,2,3]

[1]Department of Ophthalmology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104

[2]Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104

[3]Dean A. McGee Eye Institute, Oklahoma City, OK 73104

[4]Department of Ophthalmology and Neurobiology, Duke University Medical Center, Durham, NC 27710

[5]Cancer Research Program, Garvan Institute of Medical Research, Sydney, NSW 2010, Australia

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) *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

Address Correspondence to: Raju V.S. Rajala, University of Oklahoma Health Sciences Center, 608 Stanton L. Young Blvd., Oklahoma City, OK 73104. Telephone: 405-271-8255, Fax: 405-271-8128, raju-rajala@ouhsc.edu.
*Present address: Department of Ophthalmology, Shimane University Faculty of Medicine, Izumo, Japan 693-8501.

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 pseudo-substrate 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 towards 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.

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 thirty minutes 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. 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 Use of Laboratory Animals*, and the Association for Research in Vision and Ophthalmology on the Use of Animals in Vision Research. All 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 (12h on; 12 h off; ~ 300 lux). Experiments were carried out on both male and female rats (150–200 g). All animals were born in 60-lux cyclic light (12h 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 (Fig. 1A). Bovine ROS, mouse and rat retina lysates were subjected to Western blot analysis with Grb14 anti-peptide antibody and the results indicate the expression of Grb14 in all species (Fig. 1B). Detection of the Grb14 immunoreactivity was prevented by co-incubating the anti-peptide antibody with peptide to which the antibody was generated (Fig. 1C). This blot was reprobed with Chemicon antibody and the results clearly indicate the re-appearance of the immunoreactivity of Grb14 in bovine, mouse and rat tissues (Fig. 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 (Fig. 1E) whereas chemicon antibody recognizes only full length, but not truncated Grb14 (Fig. 1F). These experiments further confirm the specificity of the Grb14 antibodies. We used the peptide antibody for all the experiments described in this manuscript.

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 lux). ROS were prepared from either rat or mouse retinas using discontinuous sucrose gradient centrifugation as previously described (5;7). Eight retinas from 4 mice or 4 retinas from 2 rats were homogenized in 1.25 ml of ice-cold 47% sucrose solution containing 100 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl 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 82,000 × 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 27,000 × 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% isopropanol, 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 disc 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, 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 were 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) ROS membranes from dark- or light-adapted rat retinas in a total volume of 50 μl 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 15,000 rpm and the supernatant was decanted. The ROS were washed 3 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 lux) 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 phenylmethylsulfonylfluoride)]. Insoluble material was removed by centrifugation at 17,000 × g for 20 min, and the solubilized proteins were pre-cleared 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 14,000 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 14,000 rpm for 1 min, and 15 μl of supernatant was spotted on Whatman p81 phosphocellulose paper discs. Filter paper discs 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 non-fat 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 (1:10,000) 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 (Fig. 2A). We observed significantly more Grb14 immunoreactivity associated with light-adapted than dark-adapted ROS membranes (Fig. 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) (Fig. 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 (Fig. 2B and 2C) and outer plexiform layer (Fig. 2B). However, following 30 min of light exposure, the Grb14 immunoreactivity was reduced in rod inner segments and increased in outer segments (Fig. 2B and 2C). 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 (Fig. 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 (Fig. 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. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 5). The Grb14 immunostaining decreased in the RIS and appeared in the ROS in wild-type and $Td^{-/-}$ mice (Fig. 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 Methods and subjected to Western blot analysis with anti-myc antibody (Fig 6 A and 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 (Fig. 6A). On the other hand, arrestin bound only to LROS or to DROS exposed to light, *in vitro*, but not to DROS (Fig. 6A). The blots were also reprobed with anti-arrestin antibodies to confirm the light- and dark-adapted localization of endogenous arrestin (Fig. 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 (Fig. 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 (Fig. 2 and Fig 3), but its binding is not (Fig. 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 (Fig. 7A). The retinas appeared normal and ROS appeared to be well organized (Fig. 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 (Fig. 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 (Fig. 7C). The photopic cone b-wave amplitude was also found be normal (Fig. 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. In wild type mice, IR kinase activity was significantly

increased in light-adapted compared to dark-adapted retinas (Fig. 8C). The light-dependent activation of IR kinase activity was lost in Grb14$^{-/-}$ mice (Fig. 8C) and the IR kinase activity was comparable to dark-adapted state. These results suggest that 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 (Fig. 8D).

DISCUSSION

The central observation of this study is that the IR-interacting protein, Grb14, undergoes light-dependent translocation within 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 non-receptor protein tyrosine kinase, to N-terminal domain-interacting receptors (Nir) is activated by GPCRs (32). Nir proteins are human homologs 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 non-receptor 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 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 towards 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). Based on 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 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 (Fig. 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 Fig. 6, ROS may also contain Grb14 binding sites, but these sites alone can not drive the process of translocation because they do not undergo any appreciable light-induced changes. Fig. 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.

ACKNOWLEDGEMENTS

The authors acknowledge Dr. Jian-Xing Ma (University of Oklahoma, Oklahoma City) for providing Rpe65 and Dr. Janis Lem (New England Medical Center and Tufts University School of Medicine, Boston, MA) for providing rod specific transducin alpha knockout mice. The authors thank Dr. Michael H. Elliott for critical reading of this manuscript.

This work was supported by grants from the National Institutes of Health (EY016507; EY00871, EY10336), NEI Core grants (EY12190 and EY5722) and NCRR COBRE Core modules (P20-RR17703), and Research to Prevent Blindness, Inc.

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.

REFERENCES

1. Barber AJ, Nakamura M, Wolpert EB, Reiter CE, Seigel GM, Antonetti DA, Gardner TW. Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol 3-kinase/Akt-mediated mechanism that reduces the activation of caspase-3. J. Biol. Chem 2001;276:32814–32821. [PubMed: 11443130]
2. Datta SR, Ranger AM, Lin MZ, Sturgill JF, Ma YC, Cowan CW, Dikkes P, Korsmeyer SJ, Greenberg ME. Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. Dev. Cell 2002;3:631–643. [PubMed: 12431371]
3. D'Mello SR, Borodezt K, Soltoff SP. Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI 3-kinase in IGF-1 signaling. J. Neurosci 1997;17:1548–1560. [PubMed: 9030615]
4. Spear N, Estevez AG, Barbeito L, Beckman JS, Johnson GV. Nerve growth factor protects PC12 cells against peroxynitrite-induced apoptosis via a mechanism dependent on phosphatidylinositol 3-kinase. J. Neurochem 1997;69:53–59. [PubMed: 9202293]
5. Rajala RV, McClellan ME, Ash JD, Anderson RE. In vivo regulation of phosphoinositide 3-kinase in retina through light-induced tyrosine phosphorylation of the insulin receptor beta-subunit. J. Biol. Chem 2002;277:43319–43326. [PubMed: 12213821]
6. Rajala RV, Anderson RE. Light regulation of the insulin receptor in the retina. Mol. Neurobiol 2003;28:123–138. [PubMed: 14576451]
7. Rajala A, Anderson RE, Ma JX, Lem J, Al Ubaidi MR, Rajala RV. G-protein-coupled Receptor Rhodopsin Regulates the Phosphorylation of Retinal Insulin Receptor. J. Biol. Chem 2007;282:9865–9873. [PubMed: 17272282]
8. Rajala A, Tanito M, Le YZ, Kahn CR, Rajala RV. Loss of neuroprotective survival signal in mice lacking insulin receptor gene in rod photoreceptor cells. J. Biol. Chem 2008;283:19781–19792. [PubMed: 18480052]

9. Reiter CE, Sandirasegarane L, Wolpert EB, Klinger M, Simpson IA, Barber AJ, Antonetti DA, Kester M, Gardner TW. Characterization of insulin signaling in rat retina in vivo and ex vivo. Am. J. Physiol Endocrinol. Metab 2003;285:E763–E774. [PubMed: 12799319]
10. Rajala RV, McClellan ME, Chan MD, Tsiokas L, Anderson RE. Interaction of the Retinal Insulin Receptor beta-Subunit with the P85 Subunit of Phosphoinositide 3-Kinase. Biochemistry 2004;43:5637–5650. [PubMed: 15134438]
11. Rajala RV, Chan MD. Identification of a NPXY Motif in Growth Factor Receptor-Bound Protein 14 (Grb14) and Its Interaction with the Phosphotyrosine-Binding (PTB) Domain of IRS-1. Biochemistry 2005;44:7929–7935. [PubMed: 15924411]
12. Rajala RV, Chan MD, Rajala A. Lipid-protein interactions of growth factor receptor-bound protein 14 in insulin receptor signaling. Biochemistry 2005;44:15461–15471. [PubMed: 16300394]
13. Jones N, Master Z, Jones J, Bouchard D, Gunji Y, Sasaki H, Daly R, Alitalo K, Dumont DJ. Identification of Tek/Tie2 binding partners. Binding to a multifunctional docking site mediates cell survival and migration. J. Biol. Chem 1999;274:30896–30905. [PubMed: 10521483]
14. Daly RJ, Sanderson GM, Janes PW, Sutherland RL. Cloning and characterization of GRB14, a novel member of the GRB7 gene family. J. Biol. Chem 1996;271:12502–12510. [PubMed: 8647858]
15. Reilly JF, Mickey G, Maher PA. Association of fibroblast growth factor receptor 1 with the adaptor protein Grb14. Characterization of a new receptor binding partner. J. Biol. Chem 2000;275:7771–7778. [PubMed: 10713090]
16. Kasus-Jacobi A, Perdereau D, Auzan C, Clauser E, Van Obberghen E, Mauvais-Jarvis F, Girard J, Burnol AF. Identification of the rat adapter Grb14 as an inhibitor of insulin actions. J. Biol. Chem 1998;273:26026–26035. [PubMed: 9748281]
17. Depetris RS, Hu J, Gimpelevich I, Holt LJ, Daly RJ, Hubbard SR. Structural basis for inhibition of the insulin receptor by the adaptor protein Grb14. Mol. Cell 2005;20:325–333. [PubMed: 16246733]
18. Bereziat V, Kasus-Jacobi A, Perdereau D, Cariou B, Girard J, Burnol AF. Inhibition of insulin receptor catalytic activity by the molecular adapter Grb14. J. Biol. Chem 2002;277:4845–4852. [PubMed: 11726652]
19. Cooney GJ, Lyons RJ, Crew AJ, Jensen TE, Molero JC, Mitchell CJ, Biden TJ, Ormandy CJ, James DE, Daly RJ. Improved glucose homeostasis and enhanced insulin signalling in Grb14-deficient mice. EMBO J 2004;23:582–593. [PubMed: 14749734]
20. Calvert PD, Krasnoperova NV, Lyubarsky AL, Isayama T, Nicolo M, Kosaras B, Wong G, Gannon KS, Margolskee RF, Sidman RL, Pugh EN Jr, Makino CL, Lem J. Phototransduction in transgenic mice after targeted deletion of the rod transducin alpha -subunit. Proc. Natl. Acad. Sci. U. S. A 2000;97:13913–13918. [PubMed: 11095744]
21. Redmond TM, Yu S, Lee E, Bok D, Hamasaki D, Chen N, Goletz P, Ma JX, Crouch RK, Pfeifer K. Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. Nat. Genet 1998;20:344–351. [PubMed: 9843205]
22. Tanito M, Kwon YW, Kondo N, Bai J, Masutani H, Nakamura H, Fujii J, Ohira A, Yodoi J. Cytoprotective effects of geranylgeranylacetone against retinal photooxidative damage. J. Neurosci 2005;25:2396–2404. [PubMed: 15745966]
23. Sokolov M, Lyubarsky AL, Strissel KJ, Savchenko AB, Govardovskii VI, Pugh EN Jr, Arshavsky VY. Massive light-driven translocation of transducin between the two major compartments of rod cells: a novel mechanism of light adaptation. Neuron 2002;34:95–106. [PubMed: 11931744]
24. Gurevich VV, Benovic JL. Cell-free expression of visual arrestin. Truncation mutagenesis identifies multiple domains involved in rhodopsin interaction. J. Biol. Chem 1992;267:21919–21923. [PubMed: 1400502]
25. Gurevich VV, Benovic JL. Visual arrestin interaction with rhodopsin. Sequential multisite binding ensures strict selectivity toward light-activated phosphorylated rhodopsin. J. Biol. Chem 1993;268:11628–11638. [PubMed: 8505295]
26. Gurevich VV, Chen CY, Kim CM, Benovic JL. Visual arrestin binding to rhodopsin. Intramolecular interaction between the basic N terminus and acidic C terminus of arrestin may regulate binding selectivity. J. Biol. Chem 1994;269:8721–8727. [PubMed: 8132602]

27. Gurevich VV, Benovic JL. Visual arrestin binding to rhodopsin. Diverse functional roles of positively charged residues within the phosphorylation-recognition region of arrestin. J. Biol. Chem 1995;270:6010–6016. [PubMed: 7890732]

28. Strissel KJ, Sokolov M, Trieu LH, Arshavsky VY. Arrestin translocation is induced at a critical threshold of visual signaling and is superstoichiometric to bleached rhodopsin. J. Neurosci 2006;26:1146–1153. [PubMed: 16436601]

29. Li G, Rajala A, Wiechmann AF, Anderson RE, Rajala RV. Activation and membrane binding of retinal protein kinase Balpha/Akt1 is regulated through light-dependent generation of phosphoinositides. J. Neurochem 2008;107:1382–1397. [PubMed: 18823366]

30. Luttrell LM, van Biesen T, Hawes BE, Koch WJ, Krueger KM, Touhara K, Lefkowitz RJ. G-protein-coupled receptors and their regulation: activation of the MAP kinase signaling pathway by G-protein-coupled receptors. Adv. Second Messenger Phosphoprotein Res 1997;31:263–277. [PubMed: 9344257]

31. Luttrell LM, Daaka Y, Lefkowitz RJ. Regulation of tyrosine kinase cascades by G-protein-coupled receptors. Curr. Opin. Cell Biol 1999;11:177–183. [PubMed: 10209148]

32. Lev S, Hernandez J, Martinez R, Chen A, Plowman G, Schlessinger J. Identification of a novel family of targets of PYK2 related to Drosophila retinal degeneration B (rdgB) protein. Mol. Cell Biol 1999;19:2278–2288. [PubMed: 10022914]

33. Ghalayini AJ, Desai N, Smith KR, Holbrook RM, Elliott MH, Kawakatsu H. Light-dependent association of Src with photoreceptor rod outer segment membrane proteins in vivo. J. Biol. Chem 2002;277:1469–1476. [PubMed: 11705988]

34. Chang HY, Ready DF. Rescue of photoreceptor degeneration in rhodopsin-null Drosophila mutants by activated Rac1. Science 2000;290:1978–1980. [PubMed: 11110667]

35. Balasubramanian N, Slepak VZ. Light-mediated activation of Rac-1 in photoreceptor outer segments. Curr. Biol 2003;13:1306–1310. [PubMed: 12906790]

36. Calvert PD, Strissel KJ, Schiesser WE, Pugh EN Jr, Arshavsky VY. Light-driven translocation of signaling proteins in vertebrate photoreceptors. Trends Cell Biol 2006;16:560–568. [PubMed: 16996267]

37. Burns ME, Arshavsky VY. Beyond counting photons: trials and trends in vertebrate visual transduction. Neuron 2005;48:387–401. [PubMed: 16269358]

38. Fain GL. Why photoreceptors die (and why they don't). Bioessays 2006;28:344–354. [PubMed: 16547945]

39. Artemyev NO. Light-dependent compartmentalization of transducin in rod photoreceptors. Mol. Neurobiol 2008;37:44–51. [PubMed: 18425604]

40. Slepak VZ, Hurley JB. Mechanism of light-induced translocation of arrestin and transducin in photoreceptors: interaction-restricted diffusion. IUBMB. Life 2008;60:2–9. [PubMed: 18379987]

41. Nair KS, Hanson SM, Mendez A, Gurevich EV, Kennedy MJ, Shestopalov VI, Vishnivetskiy SA, Chen J, Hurley JB, Gurevich VV, Slepak VZ. Light-dependent redistribution of arrestin in vertebrate rods is an energy-independent process governed by protein-protein interactions. Neuron 2005;46:555–567. [PubMed: 15944125]

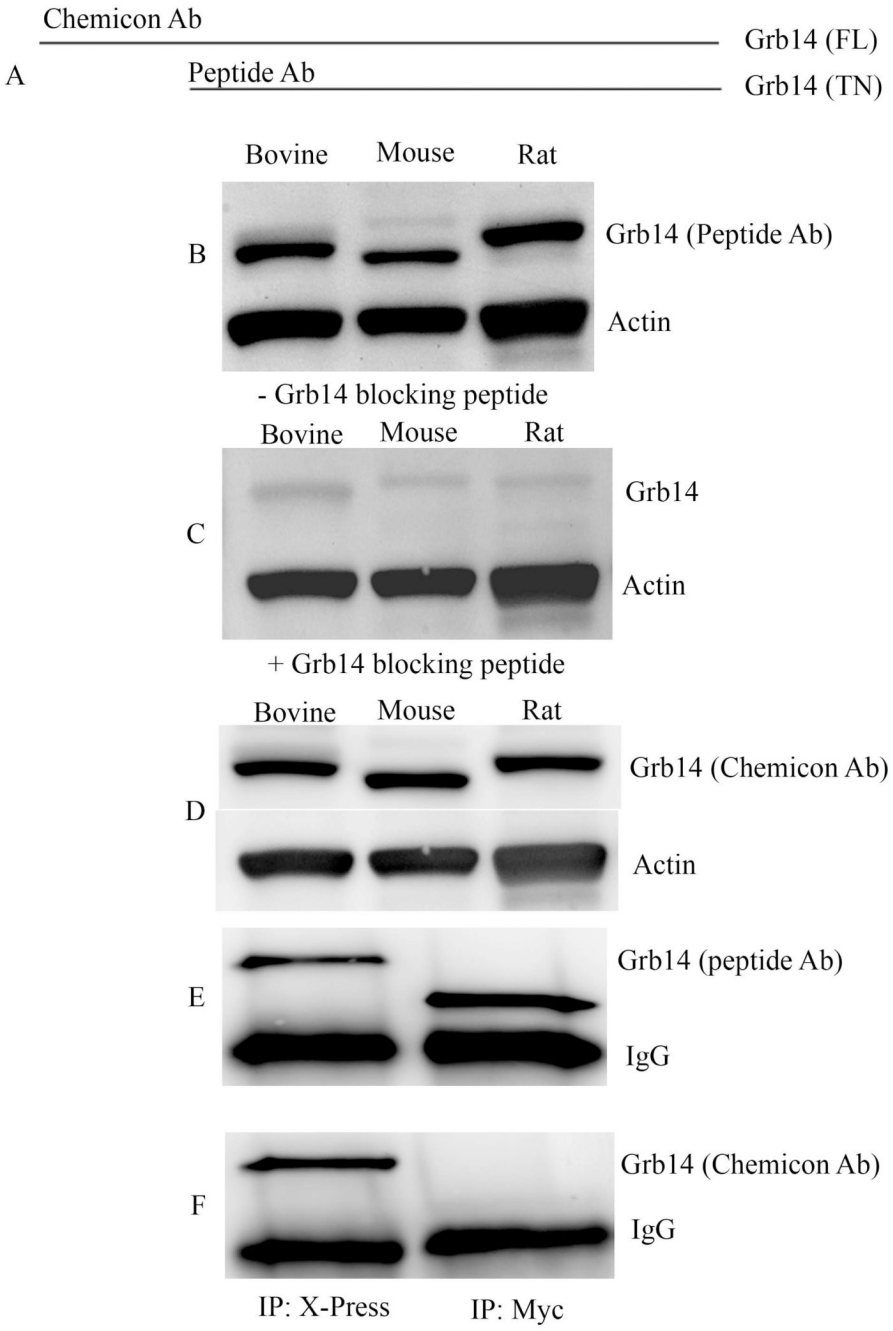

Figure 1. Characterization Grb14 antibodies

Full-length and truncated versions of Grb14 are shown in (A). Twenty micrograms of bovine ROS, mouse and rat retinal lysates were 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 re-incubated with Grb14 antibody from chemicon (D). X-press tagged-full length and truncated myc-tagged Grb14 were expressed in vitro using 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).

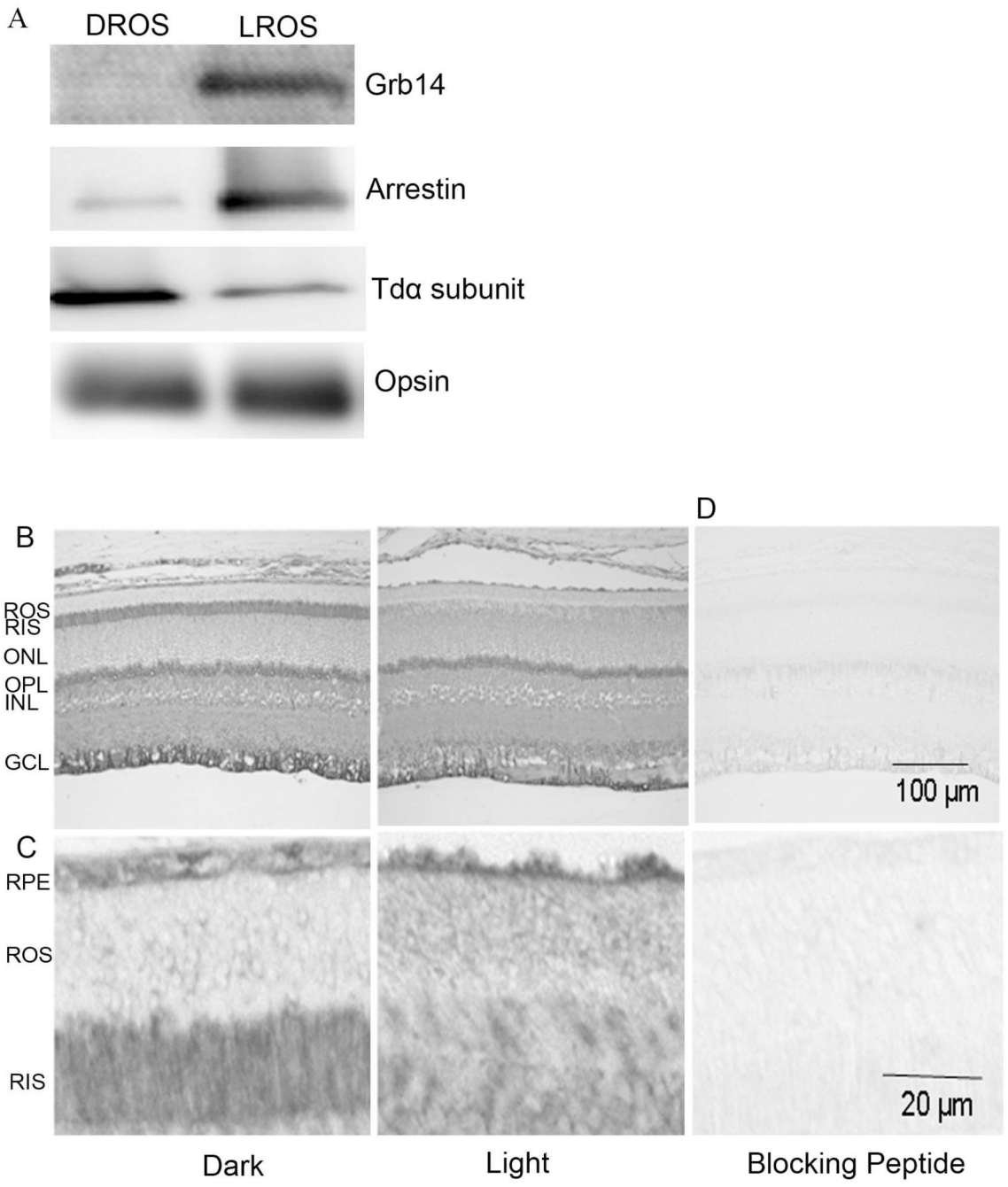

Figure 2. Light-dependent presence and ROS localization of Grb14

Mice were dark-adapted overnight and half were subjected to 300 lux for 30 min. Proteins form 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). ONL, outer nuclear layer; OPL, outer plexiform layer; GCL, ganglion cell layer; RPE, retinal pigment epithelium.

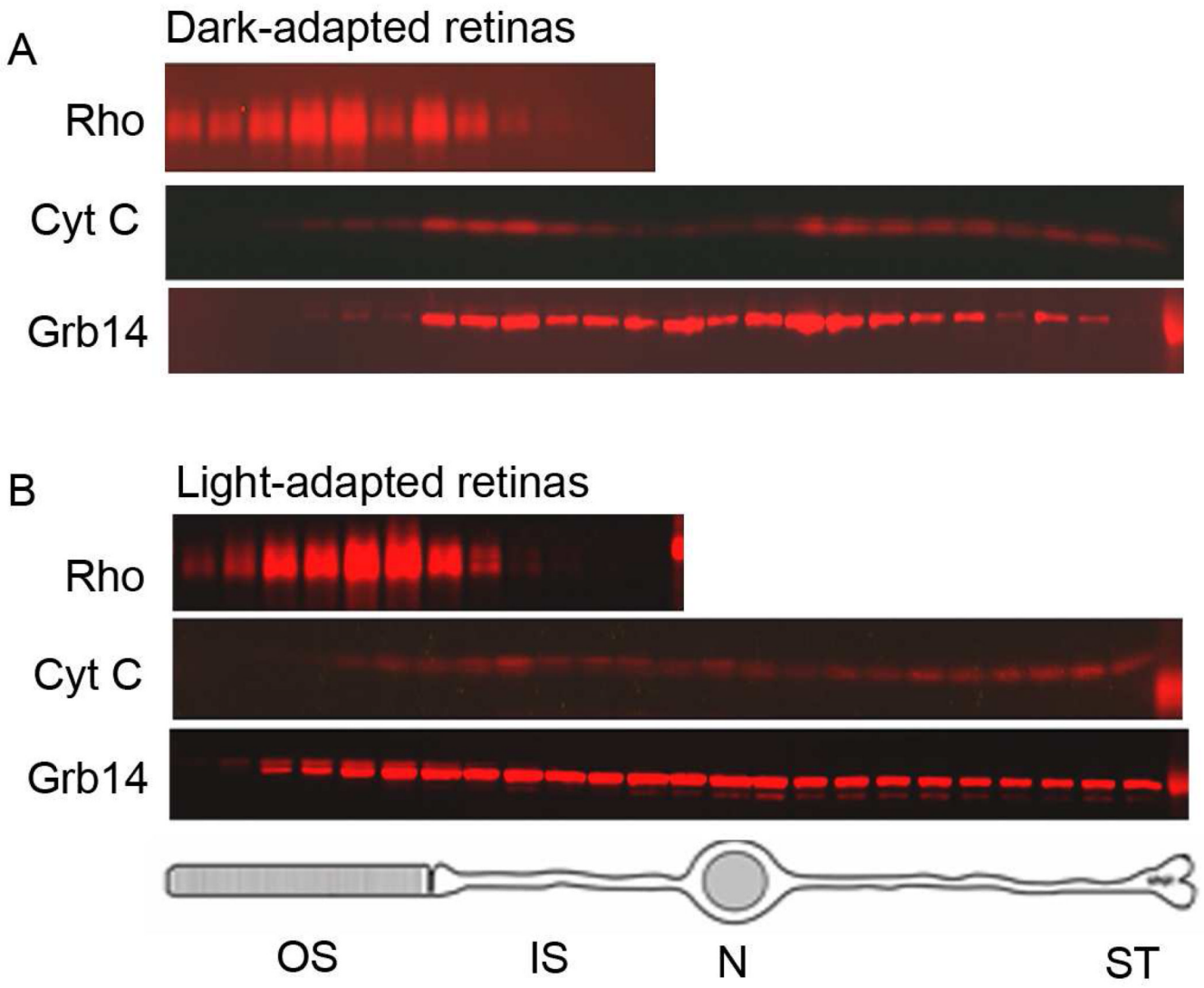

Figure 3. Determination of protein distribution throughout the photoreceptor layer of the rat retina by serial tangential cryosectioning with Western blotting

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. OS, outer segment; IS, inner segment; N, nucleus; ST, synaptic terminal.

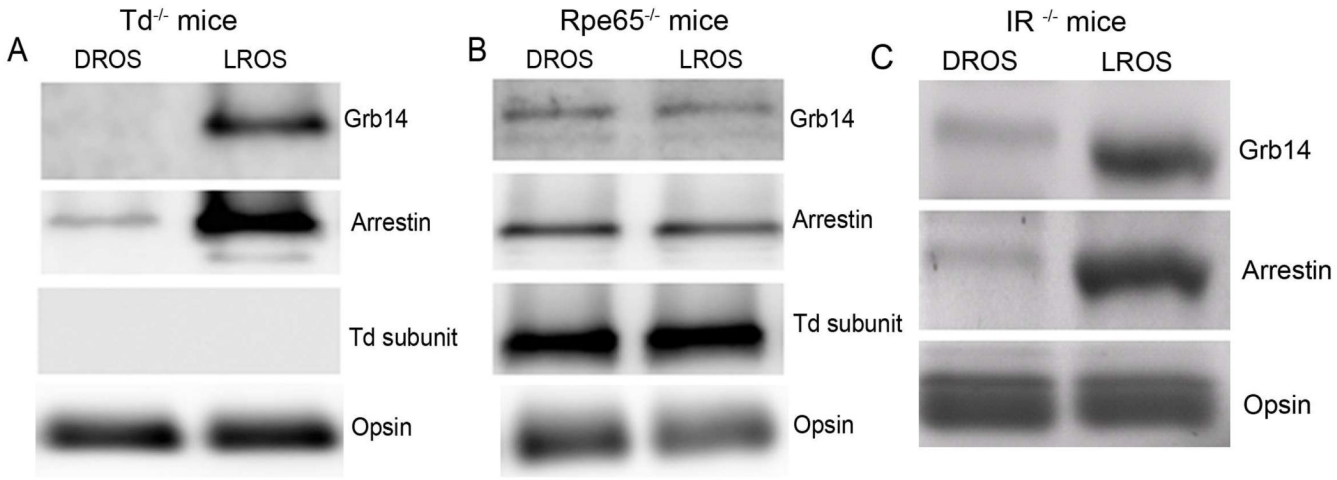

Figure 4. Binding of Grb14 to light- or dark-adapted rod outer segments of $Td^{-/-}$, $Rpe65^{-/-}$ and $IR^{-/}$-mice

Mice were dark-adapted overnight and half were subjected to 300 lux 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.

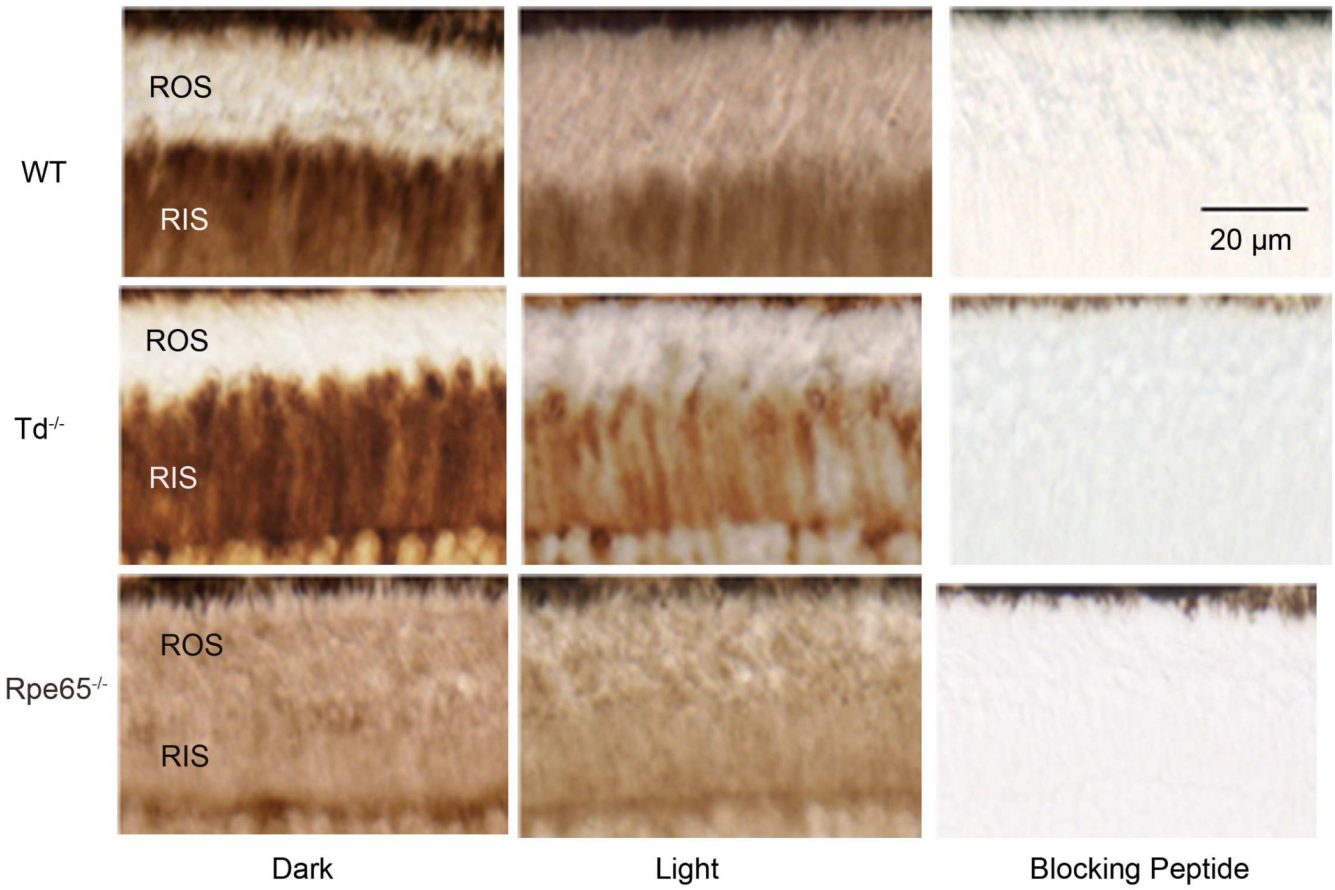

Figure 5. Immunocytochemical analysis of Grb14 in dark- and light-adapted retinas from wild type, Tdα$^{-/-}$ and Rpe65$^{-/-}$ mice

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. ROS, rod outer segment; RIS, rod inner segment.

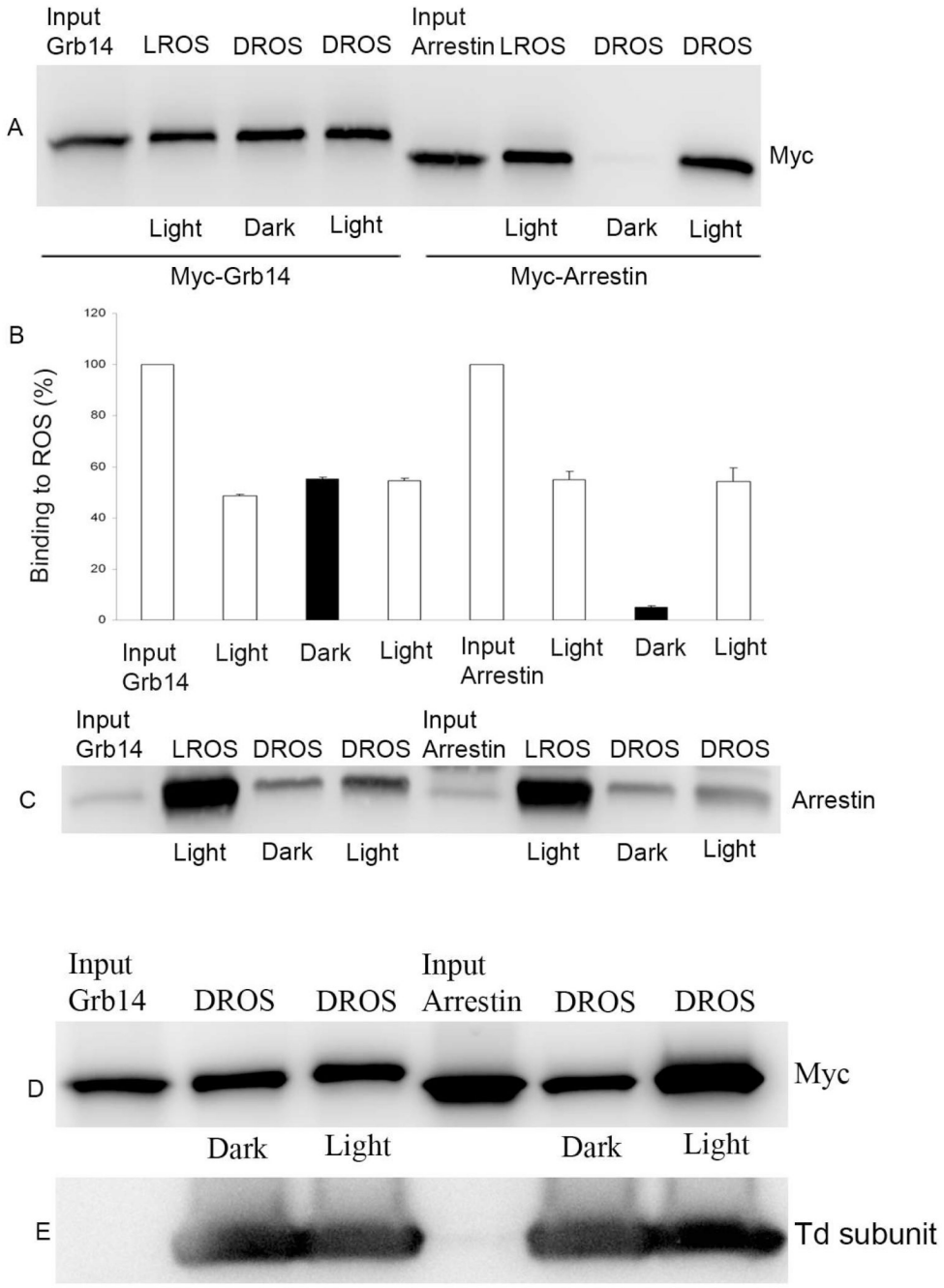

Figure 6. Binding of Grb14 to ROS membranes *in vitro*

Myc-tagged Grb14 or myc-tagged arrestin were expressed using *in vitro* coupled transcription and translation in a total volume of 50 μl as described in methods. Light- or dark-adapted ROS membranes containing 20 μg 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 the methods. (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 re-probed 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.

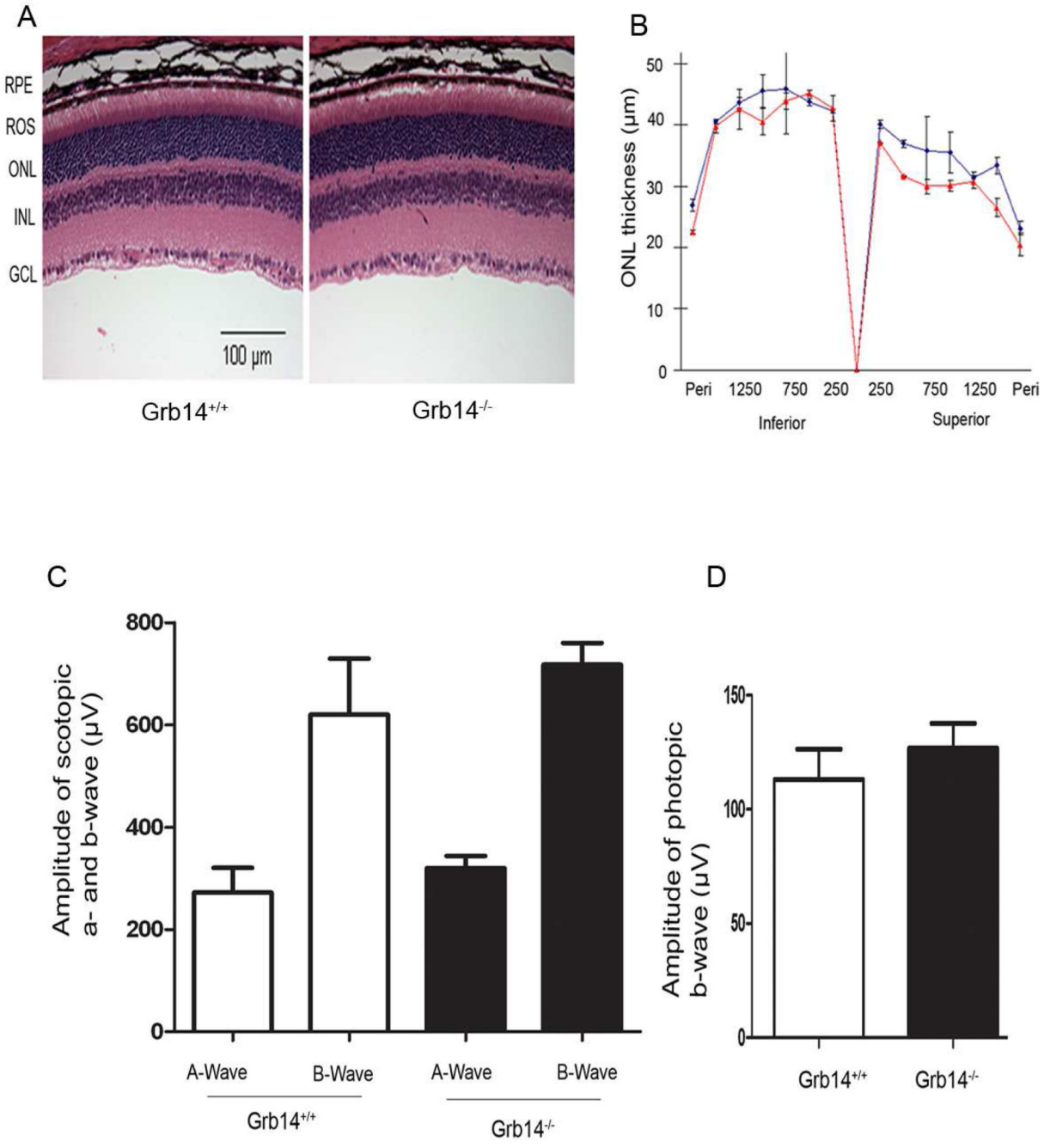

Figure 7. Morphological and functional characterization of Grb14$^{-/-}$ mice

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 5 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. 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.

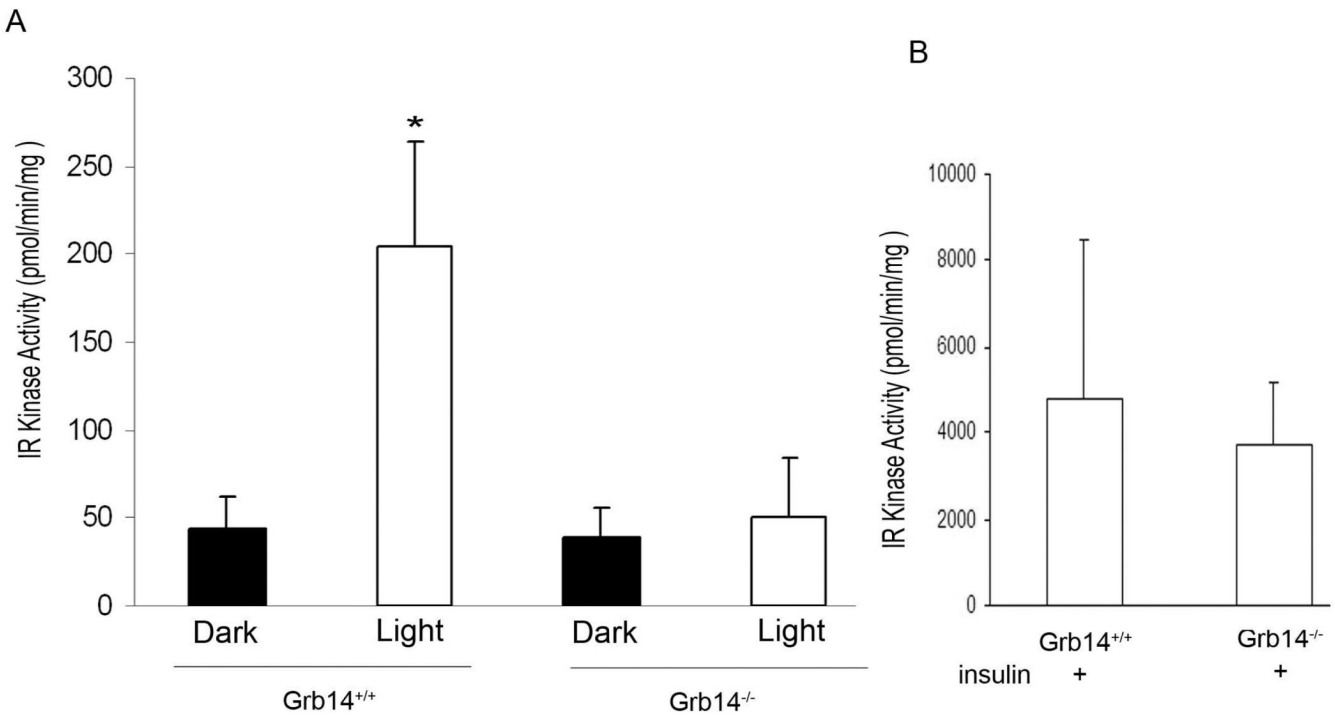

Figure 8. IR kinase activity in wild type and Grb14$^{-/-}$ mice

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.