Accelerated Publications

Melanopsin Forms a Functional Short-Wavelength Photopigment†

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ABSTRACT: Recently, melanopsin has emerged as the leading candidate for the elusive photopigment of the mammalian circadian system. This novel opsin-like protein is expressed in retinal ganglion cells that form the retinohypothalamic tract, a neuronal connection between the retina and the suprachiasmatic nucleus. These hypothalamic structures contain the circadian pacemaker, which generates daily rhythms in physiology and behavior. In mammals, proper synchronization of these rhythms to the environmental light−dark cycle requires retinal input. Surprisingly, rod and cone photoreceptors are not required. Instead, the melanopsin-containing ganglion cells are intrinsically sensitive to light, perhaps responding via a melanopsin-based signaling pathway. To test this hypothesis, we have characterized melanopsin following heterologous expression in COS cells. We found that melanopsin absorbed maximally at 424 nm after reconstitution with 11-*cis*-retinal. Furthermore, melanopsin activated the photoreceptor G-protein, transducin, in a light-dependent manner. In agreement with the measured absorbance spectrum, melanopsin was most efficiently excited by blue light (420−440 nm). In contrast, published action spectra suggest that the photopigment underlying the intrinsic light sensitivity of SCN-projecting RGCs has an absorption maximum near 484 nm. In summary, our experiments constitute the first direct demonstration that melanopsin forms a photopigment capable of activating a G-protein, but its spectral properties are not consistent with the action spectrum for circadian entrainment.

Light entrainment of the circadian clock requires input from the retina, which communicates with the SCN[1] via the axonal projections of a small subset of retinal ganglion cells (RGCs) (*1*). Surprisingly, traditional rod and cone photoreceptors are not required (*2*). Instead, RGCs that project to the SCN seem to function as autonomous circadian photoreceptors. In striking contrast to RGCs involved in vision, the SCN-projecting RGCs exhibit light responses independent of rod- and cone-driven synaptic input (*3*), and the action spectrum of the light-evoked depolarization of these photosensitive RGCs is well described by templates for an A1 retinal-based photopigment with a λ_{max} of 484 nm (*3*). This action spectrum is also similar to those measured for photoentrainment of circadian rhythms in mice and for the pupillary light reflex in rodless, coneless mice (*3*−*5*). These results suggest that the photopigment mediating these nonvisual photic responses would have an absorbance spectrum with a similar shape, peaking near 480 nm.

Recently, melanopsin, a novel opsin-like protein expressed in the SCN-projecting RGCs, has been proposed to be the elusive circadian photopigment on the basis of several lines of indirect evidence: (1) the primary structure of melanopsin resembles that of an invertebrate-type opsin protein (*6*), (2) melanopsin is selectively expressed in RGCs whose axons form the retinohypothalamic tract (*7*, *8*), and (3) RGCs that contain melanopsin are intrinsically sensitive to light (*9*). More recently, several groups have produced transgenic mice in which the melanopsin gene has been disrupted (*10*−*14*). These melanopsin knockout mice displayed a decreased ability to shift their circadian phase in response to pulses of light and a decreased pupillary light reflex. More importantly, perhaps, the SCN-projecting RGCs in these mice were no longer intrinsically photosensitive (*12*). Recent work by K.-W. Yau and colleagues has demonstrated that disabling the transduction mechanism of both rod and cone photoreceptors in melanopsin knockout mice eliminates all photoentrainment, indicating that all accessory visual functions in mice are mediated by both the melanopsin and rod−cone photoreceptor systems (*14*). However, these elegant experiments do not directly address the question of whether melanopsin is a photopigment responsible for generating the light response or an isomerase required to regenerate the retinal chromophore. In this paper, we provide the first direct

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[1] Abbreviations: SCN, suprachiasmatic nucleus; RGC, retinal ganglion cells; RGR, retinal G-protein-coupled receptor; RPE, retinal pigment epithelium; LWS, long-wavelength sensitive.

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evidence that melanopsin does indeed form a functional photopigment. However, its in vitro absorbance spectrum and wavelength dependence of G-protein activation do not match the spectrum predicted by the nonvisual photic responses.

An investigation into the spectral and functional properties of melanopsin is also important in order to clarify its functional relationship to another recently discovered class of opsin proteins. These novel opsins are expressed in retinal cells other than rod and cone photoreceptors, as well as nonocular tissue. Some of these opsins appear to function as photopigments and may mediate light signals involved in nonvisual photic responses, such as circadian photoentrainment (see ref *5* for a recent review). On the other hand, at least one of these opsins, the retinal G-protein-coupled receptor (RGR), is not involved in sensory transduction but rather acts as a photoisomerase in the retinal pigment epithelium (RPE) (*15*). By analogy, it is not known if melanopsin could also act either in sensory transduction or as a photoisomerase. However, the possibility that melanopsin acts as an isomerase is particularly intriguing for the light-sensitive RGCs because these cells are located in the inner retina where they are quite distant from the RPE.

EXPERIMENTAL PROCEDURES

Materials. All reagents, except where indicated, were purchased from Sigma. GTP-γ-S was from Boehringer Mannheim, and [^{35}S]GTP-γ-S was from NEN. LipofectAMINE was from Invitrogen. Frozen retinas were obtained from Schenk Packing Co. (Stanwood, WA). The monoclonal antibody rhodopsin 1D4, which is specific for the C-terminus of rhodopsin, has previously been described (*16*). Peptide I (DEASTTVSKTETSQVAPA) was synthesized by Macromolecular Resources (Fort Collins, CO).

Modification of the Melanopsin cDNA Clone. The complementary DNA encoding mouse melanopsin (the kind gift of I. Provencio and M. Rollag, Uniformed Services University) was modified by the addition to the C-terminus of 15 amino acids (STTVSKTETSQVAPA) corresponding to the epitope for the monoclonal antibody 1D4. This tag has been used successfully to purify other transmembrane proteins (*17*). This modified melanopsin cDNA was subcloned into an altered version of the eukaryotic expression vector pMT-2 (*18*, *19*).

Construction of a Melanopsin Mutant. A mutation was introduced into the modified melanopsin gene using QuikChange (Stragene). The nucleotide sequence of the mutated melanopsin gene was confirmed by the dideoxy method of sequencing.

Expression, Purification, and Spectral Analysis of Melanopsin. The melanopsin clone was expressed in COS-1 cells following transfection with LipofectAMINE. Cells were harvested 48 h after transfection and stored at −80 °C. For reconstitution of melanopsin, cells were resuspended in PBS and incubated with 40 μM 11-*cis*-retinal in the dark. Proteins were solubilized from cell membranes as described by Weng et al. (*17*). Melanopsin was purified by immunoaffinity chromatography using the bovine rhodopsin monoclonal antibody 1D4 (*16*). The purified melanopsin was eluted in a PBS buffer containing 0.1% dodecyl maltoside and was analyzed using a Hitachi Model U-3300 dual path spectrophotometer. The same procedure was used for the expression, purification, and analysis of the melanopsin mutant K337A.

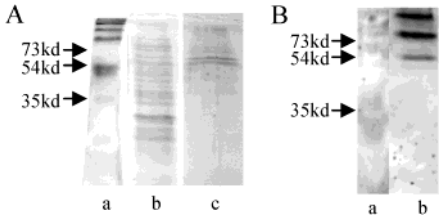

FIGURE 1: (A) SDS−PAGE silver stain analysis of immunoaffinity-purified melanopsin: (a) protein molecular mass gel standards; (b) solubilized melanopsin-transfected COS-1 supernatant; (c) immunoaffinity-purified melanopsin. (B) Anti-1D4 antibody western blot analysis of immunoaffinity-purified melanopsin: (a) protein molecular mass gel standards; (b) immunoaffinity-purified melanopsin.

GTP-γ-S Binding Assay. Melanopsin and bovine rhodopsin were assayed for the light-dependent activation of transducin using the procedure of Zhukovsky et al. (*20*). Briefly, each reaction mixture contained 2.1 μM transducin, 3 μM [^{35}S]-GTP-γ-S, and either COS cell membranes containing expressed melanopsin or bovine rhodopsin in a 10 mM Tris-HCl (pH 7.4) buffer with 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT.

SDS−PAGE. SDS−PAGE was performed according to Laemmli (*21*) using a 10% polyacrylamide gel. Proteins were visualized using silver staining according to the procedure of Bloom et al. (*22*).

Immunological Analysis. Immunoblots were analyzed according to the methods of Burnette (*23*). Rhodopsin and modified melanopsin were detected using the monoclonal antibody 1D4 and a secondary anti-mouse antibody conjugated to alkaline phosphatase. Antibody binding was visualized using a fluorescent alkaline phosphatase substrate (Attophos) from Promega Corp. Fluorescence was detected using the Storm 860 phosphorimaging system (Molecular Dynamics).

RESULTS

Complementary DNA encoding mouse melanopsin (the kind gift of I. Provencio and M. Rollag, Uniformed Services University) was fused at the carboxy terminus to DNA encoding the bovine rhodopsin 1D4 epitope tag. These additional eight amino acids allow for the purification of melanopsin using immunoaffinity chromatography. Melanopsin-1D4 was expressed in COS-1 cells and reconstituted with 11-*cis*-retinal. Membranes were solubilized in a buffer containing dodecyl maltoside and phosphatidylcholine, and melanopsin-1D4 was purified by immunoaffinity chromatography. The silver stain analysis of immunoaffinity-purified melanopsin following SDS−PAGE revealed a major band with an apparent molecular mass of 57 kDa (Figure 1A). This primary band was recognized by the anti-1D4 antibody in an immunoblot (Figure 1B), as were dimers and trimers.

The absorbance spectrum of purified melanopsin was determined using a dual beam spectrophotometer. The dark pigment was sensitive to bleaching by treatment with 50 mM hydroxylamine, which is known to cleave the Schiff's base linkage between the retinal chromophore and the opsin protein. The difference spectrum generated by this experiment is depicted in Figure 2, revealing the λ_{max} of 420 nm for the unilluminated pigment. In a separate experiment, the dark pigment was exposed to white light, which resulted in a small increase in the absorbance peak (Figure 3). The

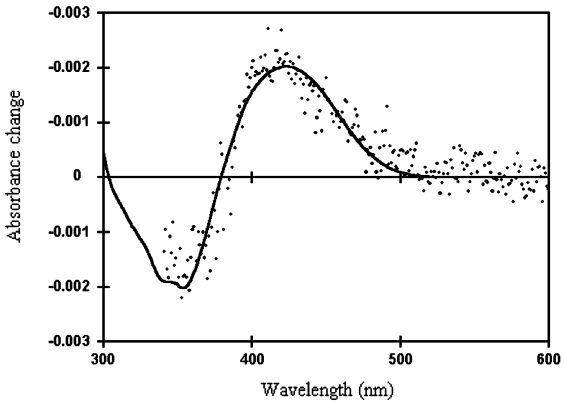

FIGURE 2: Difference spectrum of purified expressed melanopsin pigment. Melanopsin expressed in COS cells was reconstituted and purified by immunoaffinity chromatography (*25*). The purified melanopsin was solubilized in a PBS buffer containing 0.1% dodecyl maltoside. The spectrum (shown as filled circles) was obtained by subtracting the spectrum obtained following hydroxylamine treatment (50 mM hydroxylamine, pH 7.0) from that of melanopsin in the dark prior to treatment with hydroxylamine. The positive component of the spectrum reveals the dark pigment with a peak at 420 nm, which is susceptible to hydroxylamine attack. The smooth curve is a fit based upon the combination of a rhodopsin template (λ_{max} of 421 nm) with the spectrum of 11-*cis*-retinaldehyde oxime.

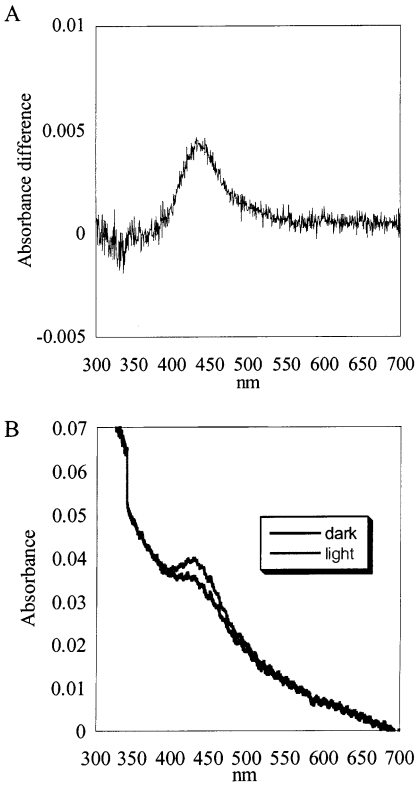

FIGURE 3: (A) Difference spectrum of purified expressed melanopsin pigment. The spectrum was obtained by subtracting the dark spectrum from that obtained following light treatment (a 10 min treatment of a 175 W fiberoptic white light source). The positive component of the spectrum reveals the 440 nm melanopsin species formed upon bleaching. (B) Raw spectra of dark and illuminated melanopsin pigment.

difference spectrum generated by subtracting the dark spectrum from that measured following illumination reveals the generation of a 440 nm photoproduct. Surprisingly, the photoproduct was insensitive to treatment with hydroxylamine and was thermally stable for many hours. Thus, melanopsin appears to manifest several properties that are unique for a vertebrate visual pigment.

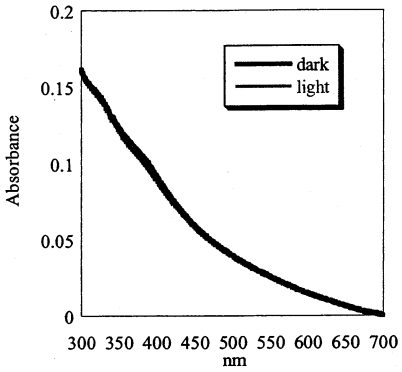

FIGURE 4: Spectra of the dark and illuminated melanopsin K337A mutant.

The spectrum of melanopsin observed in Figures 2 and 3 after reconstitution with 11-*cis*-retinal suggests that a specific Schiff base linkage is being formed between K337 (equivalent to position 296 in bovine rhodopsin) in the seventh transmembrane region of melanopsin and the chromophore. To test the specificity of this interaction, a mutant melanopsin K337A was constructed, expressed, and analyzed. The spectra depicted in Figure 4 demonstrate that in the absence of K337 no absorbance in the visible range is observed. This suggests that the visual pigment formed with wild-type melanopsin is the result of a Schiff base linkage between the chromophore and K337.

In an in vitro biochemical assay, melanopsin demonstrated a light-dependent ability to catalyze the uptake of [^{35}S]GTP-γ-S by the rod photoreceptor G-protein, transducin. COS cell membranes containing heterologously expressed melanopsin were reconstituted with 11-*cis*-retinal in the dark and mixed with purified transducin and [^{35}S]GTP-γ-S. One aliquot of membranes was assayed in the dark, and an identical sample was exposed to bright white light. The time course of [^{35}S]-GTP-γ-S uptake by transducin was determined using a filter-binding assay. Pigment concentrations were estimated by semiquantitative immunoblotting using the anti-1D4 antibody. As shown in Figure 5A, melanopsin reconstituted with 11-*cis*-retinal catalyzed transducin activation in a light-dependent manner as indicated by the increase in the rate of [^{35}S]GTP-γ-S binding. The activation of transducin is dependent on the presence of a G-protein-coupled receptor, either rhodopsin or melanopsin, as control membranes failed to catalyze [^{35}S]GTP-γ-S uptake (Figure 5A). Melanopsin proved to be a surprisingly efficient activator of transducin; the rate of transducin activation by melanopsin was only 3-fold lower than that achieved by the same concentration of bovine rhodopsin (Figure 5B). These results are somewhat surprising, in that melanopsin shows little conservation of the regions reported to be important for activation of transducin by rhodopsin (*24*). However, as previously reported, transducin can be activated by non-opsin G-protein receptors including the human retinal metabotropic glutamate receptor mGLUR6 (*17*).

In the next set of experiments, we determined the wavelength dependence of melanopsin activation using the transducin [^{35}S]GTP-γ-S uptake assay. As described above, membranes containing reconstituted melanopsin were mixed

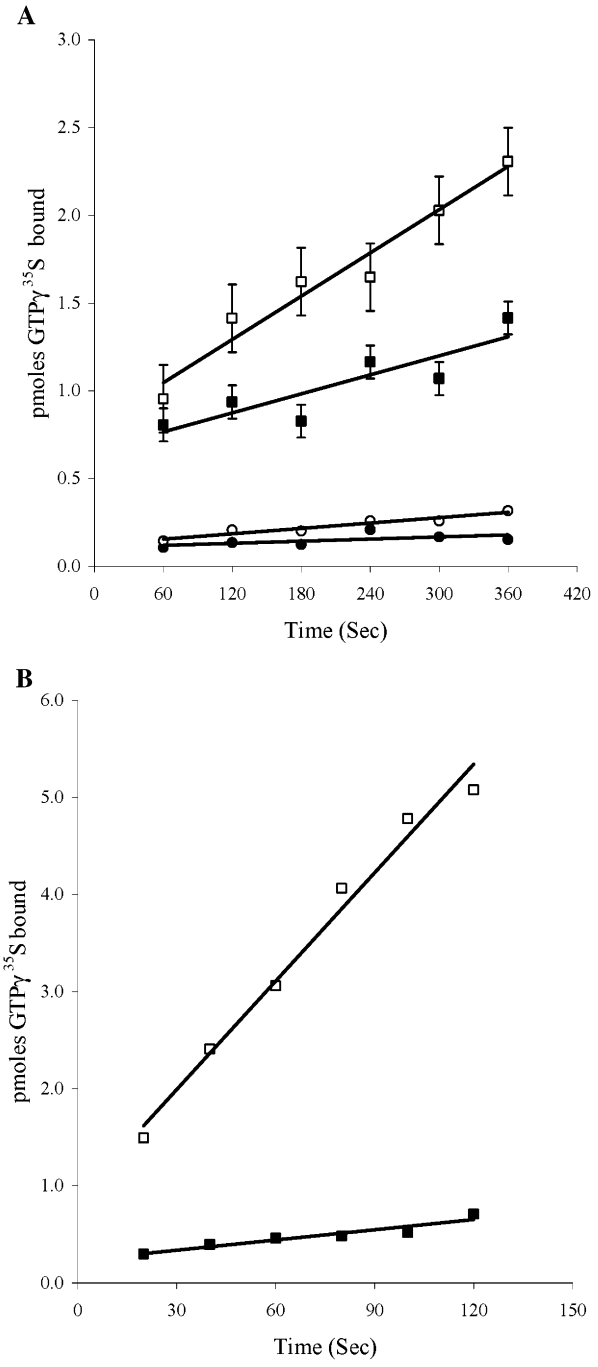

FIGURE 5: Melanopsin stimulates [^{35}S]GTP-γ-S uptake by transducin. (A) Melanopsin-mediated [^{35}S]GTP-γ-S binding in bright white light (□) and in the dark (■). Reaction rates were 0.04 pmol of [^{35}S]GTP-γ-S bound s^{-1} (pmol of receptor)$^{-1}$ for the light reaction and 0.02 pmol of [^{35}S]GTP-γ-S bound s^{-1} (pmol of receptor)$^{-1}$ for the dark reaction. In this assay, reaction mixtures contained 2.1 μM transducin, 3 μM [^{35}S]GTP-γ-S, and 9.14 nM melanopsin in a buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT. Circles represent [^{35}S]-GTP-γ-S binding by untransfected COS-1 membranes in bright white light (○) and in the dark (●). Reaction mixtures contained 2.1 μM transducin, 3 μM [^{35}S]GTP-γ-S, and untransfected COS-1 membranes in the Tris-HCl buffer described above. (B) Rhodopsin-mediated [^{35}S]GTP-γ-S binding in bright white light (□) and in the dark (■). Reaction rates were 0.126 pmol of [^{35}S]GTP-γ-S bound s^{-1} (pmol of receptor)$^{-1}$ in the light and 0.012 pmol of [^{35}S]-GTP-γ-S bound s^{-1} (pmol of receptor)$^{-1}$ in the dark. Reaction mixtures contained 2.1 μM transducin, 3 μM [^{35}S]GTP-γ-S, and 29.35 nM bovine rhodopsin in the Tris-HCl buffer described above.

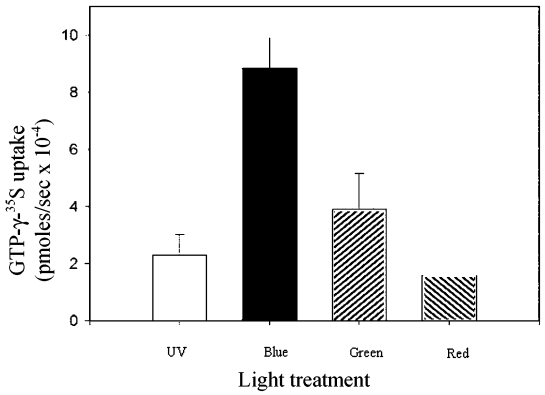

FIGURE 6: Melanopsin is activated most efficiently by blue light. Mean rates of melanopsin-catalyzed [^{35}S]GTP-γ-S binding following illumination with UV (381 ± 10 nm), blue (443 ± 19 nm), green (516 ± 30 nm), and red (>700 nm) light. Illumination was provided by a calibrated light source. The samples were illuminated for 10 s, and the rate of transducin activation was determined. The irradiance (photons s^{-1} cm^{-2}) was as follows: UV, 1.3×10^{13}; blue, 1.54×10^{13}; green, 2.72×10^{13}; red, 1.9×10^{15}. Wavelengths were selected using band-pass or long-pass filters. Intensities were chosen to provide clear discrimination among action spectra for candidate pigments that have absorption maxima in the spectral range from the UV to the green. The intensity of red light was 100-fold greater than the other stimuli to rule out any contribution of photoactivation by light outside the intended spectral bands. The [^{35}S]GTP-γ-S binding rate in the dark (4.8×10^{-4} pmol/s) was subtracted from raw rate values to generate the data shown (mean ± SD). Reaction mixtures contained 2.1 μM transducin, 3 μM [^{35}S]-GTP-γ-S, and 0.3 nM melanopsin.

in the dark with purified transducin and [^{35}S]GTP-γ-S. These mixtures were then illuminated with UV, blue, green, and red light, using band-pass filters and a calibrated light source providing approximately equal light intensity at each wavelength. The time course of [^{35}S]GTP-γ-S uptake by transducin was determined using a filter-binding assay. As shown in Figure 6, blue light stimulated the activation of transducin by melanopsin more efficiently than the other wavelengths tested. Calculations indicate that this pattern of activation is most likely derived from a photopigment with a λ_{max} of 420−440 nm. More to the point, a pigment with an absorption maximum of 484 nm (as predicted from the physiological measurements) would have been activated more effectively by the green light than the blue. Thus, the wavelength dependence of melanopsin activation matches the absorbance spectrum of the solubilized pigment, suggesting that the λ_{max} of 420 nm is not an artifact of solubilization but a bona fide property of the expressed melanopsin.

DISCUSSION

The experiments reported here constitute the first direct demonstration that melanopsin forms a photopigment capable of activating a G-protein. In our experiments, melanopsin was activated most efficiently by light in the mid-blue range (420−440 nm). The retinal signals that mediate circadian entrainment and the pupillary light reflex have been proposed to use a melanopsin-based pathway because they do not require rod or cone photoreceptors. However, the action spectra for both of these processes peak near 480 nm (*3*, *4*). Thus, the absorbance spectrum of melanopsin was surprising because the spectra of all other heterologously expressed vertebrate opsins have faithfully matched the spectral sensitivity of the photoreceptor from which they were

derived. Although unexpected, this discrepancy is quite intriguing and potentially significant. It is possible that native melanopsin uses an unusual chromophore or that its spectrum is shifted by interaction with endogenous proteins that are not present in COS cells. It is unlikely that melanopsin's wavelength is being modulated by chloride using a mechanism similar to that found in LWS opsins, since melanopsin does not have the chloride binding sites that exist in LWS opsins (*26*). Alternatively, photoactivation of the SCN-projecting RCGs may require other photopigments, in addition to melanopsin. Nevertheless, it is clear from our measurements that melanopsin forms a functional photopigment capable of activating G-protein-based intracellular signaling pathways. Further experiments will be needed to reconcile our measurements with the physiological data and to determine whether additional photopigments mediate the intrinsic light response of SCN-projecting RGCs.

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