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A Molecular Pathway for Light-Dependent Photoreceptor Apoptosis in *Drosophila*

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Summary

Light-induced photoreceptor apoptosis occurs in many forms of inherited retinal degeneration resulting in blindness in both vertebrates and invertebrates. Though mutations in several photoreceptor signaling proteins have been implicated in triggering this process, the molecular events relating light activation of rhodopsin to photoreceptor death are yet unclear. Here, we uncover a pathway by which activation of rhodopsin in *Drosophila* mediates apoptosis through a G protein–independent mechanism. This process involves the formation of membrane complexes of phosphorylated, activated rhodopsin and its inhibitory protein arrestin, and subsequent clathrin-dependent endocytosis of these complexes into a cytoplasmic compartment. Together, these data define the pro-apoptotic molecules in *Drosophila* photoreceptors and indicate a novel signaling pathway for light-activated rhodopsin molecules in control of photoreceptor viability.

Introduction

The apoptotic death of neurons is an essential process in the development of the nervous system in all animals. Apoptosis typically occurs late in the developmental program and results in the pruning of functionally unnecessary "extra" cells that are often produced during differentiation of adult structures (Miller and Cagan, 1998; Bangs and White, 2000). For surviving adult cells, a consequence of the presence of this potential cell death mechanism is the requirement for efficient regulatory processes to suppress apoptosis. Not surprisingly, cell death due to many neurodegenerative diseases or in response to inappropriate stimuli appears to result from the activation of the apoptotic machinery (Cotman and Anderson, 1995; Kim et al., 1997; Namura et al., 1998; Banasiaka et al., 2000). In the visual system, essentially all of the most common inherited degenerative diseases that cause blindness in humans, such as retinitis pigmentosa (RP), age-related macular degeneration, cone dystrophy, Oguchi's disease, and others, involve the loss of photoreceptor cells through apoptosis (Portera-Cailliau et al., 1994; Adler, 1996).

Unlike necrosis or other forms of cell death, apoptosis shows typical features of a highly regulated cellular signaling process. A dedicated and well-conserved cascade of proteins mediates the discrimination and amplification of appropriate triggering stimuli and, ultimately, the execution of the apoptotic program (Bergmann et al., 1998; Thornberry and Lazebnik, 1998). The molecular events in apoptosis can be divided into three phases: (1) a cell type–specific proapoptotic phase that determines the triggers for cell death, (2) a commitment phase mediated by a canonical core cascade of proteases known as capases that amplifies the triggering signal, and (3) cell breakdown and phagocytosis. Though much is known about the biochemistry and mechanisms of the core caspase cascade and the cell disassembly process (Thornberry and Lazebnik, 1998), a current challenge is to understand the molecular players that are sufficient to trigger the normally quiescent apoptotic machinery and to identify the mechanism by which a wide variety of molecular lesions in neuronal signaling pathways funnel into generation of the apoptotic trigger. For example, the retinal degeneration diseases listed above are associated with mutations in several proteins involved in visual signaling, but likely share a common final pathway for activation of apoptosis (van Soest et al., 1999).

An excellent experimental system in identifying the mechanisms of triggering apoptosis in photoreceptor cells is the *Drosophila* compound eye. The eye is not required for viability of the organism in a laboratory setting, and photoreceptor cell loss produces easily scorable behavioral defects. These features have enabled genetic screens designed to isolate mutants in which the morphogenesis of the eye is normal, but in which photoreceptor cells degenerate starting with the onset of adult visual function (Hotta and Benzer, 1970; Harris et al., 1976; Harris and Stark, 1977; Steele and O'Tousa, 1990). These studies revealed two classes of molecular lesions that produce degeneration phenotypes. Mutations in genes required for the biosynthesis and maturation of the light receptor rhodopsin or for the normal structure of the rhabdomere, the specialized microvillar organelle that is the site of phototransduction lead to a light-independent form of photoreceptor degeneration (Pak, 1994; Lee et al., 1996). This class of mutants has mainly provided insight into proteins required to maintain the structural integrity of the light-sensitive membrane. The second class consists of mutations in a number of visual signaling molecules that result in light-dependent photoreceptor cell death through one of two mechanistically distinct processes. First, missense mutations in several key components of the signaling cascade (e.g., rhodopsin, the Trp ion channel) and hypomorphic mutations in regulatory molecules (e.g., Arrestin2, the RdgA diacylglyercol kinase) lead to excessive activation of the phototransduction system and result in a rapid necrotic death of photoreceptor cells (Dolph et al., 1993; Bentrop, 1998; Raghu

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et al., 2000; Yoon et al., 2000). This is likely due to the pathologically elevated levels of intracellular calcium that result from unregulated signaling activity. Second, an interesting class of retinal degeneration mutants were identified that absolutely require light-activation of rhodopsin, but do not require activity of the phototransduction cascade (Meyertholen et al., 1987; Stark et al., 1989; Steele and O'Tousa, 1990). These mutants, some of which paradoxically are null alleles in genes required for the light activation pathway, cause light-dependent degeneration due to triggering photoreceptor apoptosis through an unknown mechanism.

A *Drosophila* mutant that is particularly promising for studying the causal relationship of rhodopsin activation and photoreceptor cell apoptosis is a loss-of-function allele in the *retinal degeneration C* gene (*rdgC*^{306}). *rdgC*^{306} mutants show light-dependent photoreceptor degeneration that requires the substantial activation of rhodopsin molecules (Steele and O'Tousa, 1990), but that is not suppressed by knockout of any signaling component downstream of rhodopsin. For example, neither elimination of G protein α subunit (*dgq*^{1}) (Lee et al., 1990) nor the phospholipase C molecule (Bloomquist et al., 1988) required for vision in *Drosophila* prevents the rhodopsin-dependent apoptosis in *rdgC*^{306} flies (Steele and O'Tousa, 1990; Vinos et al., 1997). These data suggest a novel signaling pathway independent of visual signaling that mediates control of the apoptotic machinery through rhodopsin.

Interestingly, cloning of the *rdgC* locus showed that it encodes a Ca^{2+}-dependent serine/threonine protein phosphatase (Steele et al., 1992) with substrate specificity for phosphorylated metarhodopsin (M-p) (Byk et al., 1993; Vinos et al., 1997); in wild-type photoreceptors, its efficient catalytic activity rapidly dephosphorylates M-p such that phosphorylated M is virtually absent at steady state under normal conditions. Light exposure in *rdgC*^{306} mutants results in accumulation of phosphorylated rhodopsin (Byk et al., 1993), suggesting that RdgC is the major mechanism for rhodopsin dephosphorylation and that the causative agent mediating photoreceptor death in *rdgC*^{306} mutants is a phosphorylated form of rhodopsin. Consistent with this proposal, deletion of the carboxy-terminal phosphorylation sites on rhodopsin suppresses degeneration in *rdgC*^{306} mutants (Vinos et al., 1997). Finally, the baculovirus antiapoptotic factor p35 also suppresses degeneration in *rdgC*^{306}, establishing that degeneration in *rdgC*^{306} proceeds through apoptosis (Davidson and Steller, 1998). The finding that this apoptotic process does not require known signaling components downstream of rhodopsin implicates signal transduction through other rhodopsin interacting proteins. Here we show that *Drosophila* visual arrestin (Arr2) mediates photoreceptor apoptosis in *rdgC*^{306} mutants and that long-lived internalized complexes of phosphorylated M-arrestin complexes are the triggering agents for apoptotic death of photoreceptor cells.

Results

Photoreceptor Degeneration in *rdgC*^{306} Requires Phosphorylated Metarhodopsin

To understand the process by which photoactivation of rhodopsin leads to cell death in *rdgC*^{306} mutants, we began by defining the molecular state of rhodopsin necessary for this phenotype. Each unit eye of the *Drosophila* compound eye contains eight photoreceptor neurons (R1–R8); of these, the R1–R6 cells comprise one functional class and express the major rhodopsin Rh1. Light absorption by inactive Rh1 (R, λ_{max} = 480 nm [blue]) triggers the isomerization of the molecule to the active state, metarhodopsin (M) (Figure 1A). Unlike vertebrate photopigments, light activation of invertebrate rhodopsins does not lead to chromophore bleaching; instead the active M state is thermally stable in vivo and can absorb another photon (λ_{max} = 580 nm [orange]) to isomerize back to the inactive R state (Minke, 1986). Like all activated G protein–coupled receptors (GPCRs), metarhodopsin is rapidly phosphorylated by a receptor-specific kinase (RK) to produce the M-p form. Both M-p and R-p can be dephosphorylated by the RdgC phosphatase, although R-p appears to be the preferred substrate (Byk et al., 1993; Vinos et al., 1997). The bistability and the distinct absorbance properties of the R and M states allows us to manipulate the quantity of rhodopsin in either of these states simply by varying the color of illumination. Thus, strong blue illumination drives the majority (around 85% [Kiselev and Subramaniam, 1997]) of rhodopsin in R1–R6 cells to the M form, and strong orange illumination predominantly drives M form back to the R form.

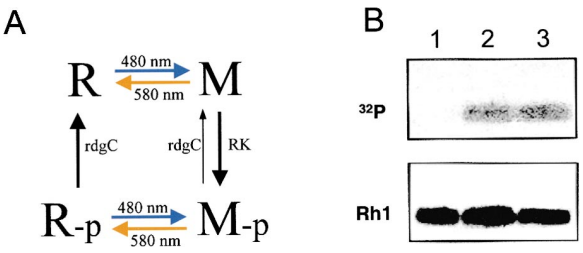

Figure 1. Generation of *rdgC*^{306} Flies Containing Distinct Structural States of Rhodopsin

(A) Diagram of the rhodopsin biochemical cycle in *Drosophila* photoreceptors. R, representing the inactive state of rhodopsin, is photoconverted to the active state (M) upon absorption of blue light (480 nm). Rhodopsin kinase (RK) phosphorylates metarhodopsin creating phosphorylated metarhodopsin (M-p). Absorption of orange light (580 nm) photoconverts phosphorylated metarhodopsin (M-p) to phosphorylated rhodopsin (R-p). The RdgC phosphatase dephosphorylates both R-p and M-p, but shows preferential substrate specificity for R-p.

(B) An autoradiograph (upper panel) and Western blot (lower panel) showing phosphorylation of Rh1 rhodopsin in dark-reared *rdgC*^{306} flies exposed to no light (1), 10 min of blue light (2), or 10 min of blue light followed by 10 min of orange light (3). Each lane was loaded with protein equal to one fly head. These three conditions represent three distinct states of rhodopsin: unphosphorylated rhodopsin (R), phosphorylated metarhodopsin (M-p), and phosphorylated rhodopsin (R-p).

We used this feature of *Drosophila* rhodopsin to test for the ability of each rhodopsin state to trigger degeneration in *rdgC*^{306} photoreceptors. Since RdgC is the rhodopsin phosphatase, we would expect three possible states for Rh1 rhodopsin in *rdgC* loss-of-function mutants: (1) unphosphorylated R (in dark-reared animals), (2) phosphorylated M (in blue-light stimulated animals), and (3) phosphorylated R (in animals stimulated by blue

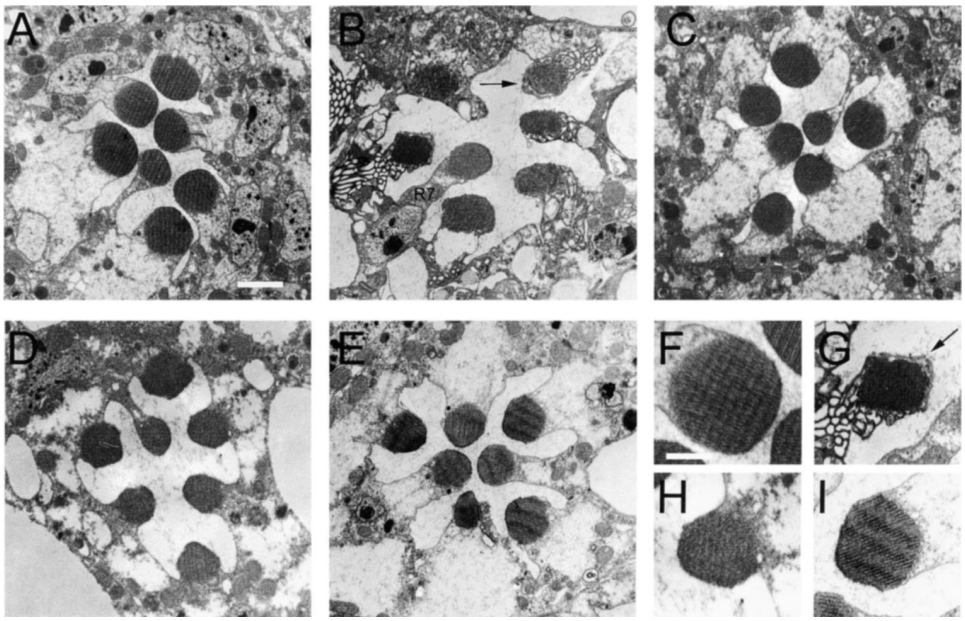

Figure 2. Photoreceptor Apoptosis in *rdgC*^{306} Requires Long-Lived Phosphorylated Metarhodopsin

Transmission electron micrographs (TEM) of sections from adult compound eyes showing either ommatidia (A–E) or individual rhabdomeres (F–I) in *rdgC*^{306} animals exposed to light stimuli as described below. All light exposures were 10 min pulses at maximal intensity (see Experimental Procedures). Each ommatidium shown contains seven rhabdomeres: six from the R1–R6 cells and one from the R7 cell. Unstimulated flies maintained in the dark for 6 days (containing R) show normal, healthy photoreceptor morphology (A and F), but flies exposed to blue light followed by incubation in the dark for 6 days (containing M-p) show extensive degenerative changes (B and G), including vesiculation of the photoreceptors cells and phagocytosis of rhabdomeres (arrow). Note that the R7 cell shows no signs degeneration (B). Flies exposed to blue light followed immediately by orange light and maintained in the dark for 6 days (containing R-p) show no degeneration, indicating complete suppression of the *rdgC*^{306} phenotype by photoconversion of M-p to R-p (C). Flies exposed to blue light followed by incubation in the dark for only 3 days have slightly reduced rhabdomere size but display no evidence of apoptosis (D and H). Flies exposed to blue light and maintained in the dark for 3 days (as in [D]), but then exposed to orange light followed by an additional 3 day dark incubation show complete suppression of apoptosis (compare [E] and [I] with [B] and [G]). Thus photoconversion of M-p to R-p even days after generation of M-p can suppress apoptosis. Scale bars, 2.5 μm in (A) and 1 μm in (F).

and then restimulated with orange light) (see Figure 1A). Figure 1B shows the results of an in vivo phosphorylation assay, demonstrating that *rdgC*^{306} flies maintained in the dark have unphosphorylated rhodopsin (R, lane 1), but that a 10 min pulse of blue light triggers the formation of phosphorylated metarhodopsin (M-p, lane 2). A subsequent 10 min pulse of orange light generates phosphorylated rhodopsin (R-p, lane 3).

We generated *rdgC*^{306} flies containing R, M-p, or R-p forms of rhodopsin and followed photoreceptor degeneration in the dark using transmission electron microscopy. A cross section of a *Drosophila* ommatidium shows seven photoreceptor neurons (R1–R7) whose cell bodies are located peripherally around a central canal (Figure 2A). The rhabdomeres of the R1–R6 cells are arranged at the outer margin of the canal, and the rhabdomere of the UV-sensitive R7 cell is centrally located. The R7 cell rhabdomere provides a useful internal control in following stimulation-dependent cell death since the blue and orange light protocols for producing M-p and R-p only affect rhodopsin molecules in R1–R6 cells and do not significantly perturb the rhodopsin photoequilibrium in R7 cells. Thus, the morphology of the R7 cell should be largely unaffected in our experiments. *rdgC*^{306} animals kept in the dark for 6 days (containing R) show normal ommatidial structure and highly ordered rhabdomeric structure typical of healthy photoreceptors (Figures 2A and 2F), but animals 6 days after a single 10 min exposure to blue light (containing M-p) show extensive disorganization of ommatidial structure and morphological changes in photoreceptor cells that are characteristic of apoptosis (Figures 2B and 2G). Note the vesiculation and disassembly of the rhabdomeric membranes in the R1–R6 cells and, in some cases, encasement of the rhabdomere by phagocytic cells (Figure 2G). The rhabdomere of the UV-sensitive R7 cell is unaffected (Figure 2B), indicating that the apoptotic process is specific for Rh1 rhodopsin activation. In contrast to the blue light–stimulated animals, *rdgC*^{306} animals 6 days after 10 min blue immediately followed by 10 min orange light exposures (containing R-p) are indistinguishable from unstimulated animals (compare Figures 2C and 2A) showing complete suppression of the *rdgC*^{306} phenotype by photoconversion of M-p to R-p. Thus, phosphorylated metarhodopsin is necessary for mediating cell death in *rdgC*^{306} photoreceptors.

The finding that photoconversion of M-p to R-p by orange light 10 min after generation of M-p fully suppresses photoreceptor degeneration demonstrates that commitment to apoptosis cannot be a process that occurs with a time course of minutes. Indeed, photoreceptor cells in *rdgC*^{306} mutants stimulated by blue light and kept in the dark for 3 days show slightly smaller rhabdomeres, but little other evidence of photoreceptor pa-

thology (Figures 2D and 2H). In contrast, a full-blown picture of apoptosis is evident by 6 days (Figure 2B). Together, these data suggest that commitment to apoptosis upon generation of M-p is a slow process that may require long-lived signaling from a stable M-p state that continues over a time period of days. If so, destabilization of the M-p state, even long after its generation, should prevent execution of the apoptotic program. To test this hypothesis, we asked whether orange light (which photoconverts M-p to R-p) could suppress the *rdgC*^{306} phenotype 3 days after generation of M-p, at a time prior to the onset of discernable morphological effects of the degeneration process. Remarkably, a 10 min pulse of orange light 3 days after a blue light stimulus followed by 3 additional days in the dark shows complete suppression of the apoptosis in *rdgC*^{306} mutants (compare Figures 2E and 2I with Figures 2B and 2G), with no discernable differences in rhabdomeric organization or intracellular morphology from that of dark-reared animals. Thus, commitment to apoptosis is a slow process requiring signaling from a stable form of phosphorylated metarhodopsin for several days, and photoreceptors can be fully rescued during this phase by shutoff of this long-lived signaling state.

Arr2 Is Required for Photoreceptor Apoptosis in *rdgC*^{306}

What is the molecular mechanism by which the metarhodopsin state is stabilized for such a long period of time, and how does this state signal to the apoptotic machinery? Reasonable candidates are proteins known to interact with metarhodopsin, namely rhodopsin kinase, the G protein α subunit mediating downstream phototransduction (Gq_α), and arrestin. The finding that the *rdgC*^{306} phenotype does not require the Gq_α subunit (Vinos et al., 1997) rules out the involvement of this protein. Of the remaining molecules, arrestin emerges as an excellent candidate in mediating both stabilization of metarhodopsin and G protein–independent signaling from the metarhodopsin state. Arrestin is an abundant soluble protein that is implicated in the inactivation of many G protein–coupled receptors through high affinity binding to the active state (Wilden et al., 1986; Kuhn and Wilden, 1987; Baylor and Burns, 1998). In *Drosophila* photoreceptors, the major mechanism for stabilizing the M state appears to be the direct binding of arrestin molecules (Kiselev and Subramaniam, 1994); the average lifetime of M in the absence of bound arrestin is less than 2 min, and arrestin binding increases the lifetime by orders of magnitude. Also, recent work has demonstrated that arrestin is a remarkably multifunctional protein, which, in addition to mediating the inactivation of G protein–coupled receptors, also can act as an adaptor protein for clathrin and AP-2-dependent internalization of activated GPCRs (Laporte et al., 2000). The internalized GPCR-arrestin complexes can then recruit additional signaling components and confer novel signaling properties to desensitized GPCRs (Luttrell et al., 1999b). Together, these facts lead to a working hypothesis: the necessary causal steps in triggering apoptosis in *rdgC*^{306} photoreceptors may be the formation of stable M-p-arrestin complexes and the clathrin-dependent internalization of these complexes from rhabdomeric membranes.

A straightforward prediction of this hypothesis is that arrestin must be required for degeneration in *rdgC*^{306} mutants. To investigate this, we designed a set of experiments to reveal genetic interactions between arrestin mutants and *rdgC*^{306}. *Drosophila* photoreceptor cells contain two isoforms of arrestin, arrestin1 (Arr1) and arrestin2 (Arr2) (LeVine et al., 1990; Smith et al., 1990), both of which contribute to metarhodopsin inactivation (Dolph et al., 1993). However, Arr2 is nearly 5-fold more abundant in the rhabdomere than Arr1 (LeVine et al., 1990; Matsumoto and Yamada, 1991). Consistent with its relative abundance, electrophysiological studies show that Arr2 is the primary inactivator of M (Dolph et al., 1993) and that it shuts off M through high-affinity 1:1 stoichiometric binding within a few milliseconds of its creation to produce the inactive metarhodopsin-Arr2 complex (M-Arr2) (Ranganathan and Stevens, 1995). Several mutant alleles of both arrestins exist; in these studies, we tested the ability of *arr2*^{3}, a severe loss-of-function allele, to suppress the photoreceptor degeneration in *rdgC*^{306}.

A complication in testing the requirement of Arr2 in mediating photoreceptor degeneration in *rdgC*^{306} is that *arr2*^{3} mutants themselves display light-dependent retinal degeneration. However, unlike the apoptotic death of photoreceptor cells in *rdgC*^{306}, *arr2*^{3} mutants show necrotic death of photoreceptor cells due to defective termination of metarhodopsin and unregulated signaling activity (Dolph et al., 1993). Thus, cell death in *arr2*^{3} (but not the apoptotic cell death in *rdgC*^{306}) is effectively suppressed in mutants that eliminate phototransduction (Dolph et al., 1993; Kurada and O'Tousa, 1995; Vinos et al., 1997). These observations provide a strategy for revealing the role of Arr2 in *rdgC*^{306} degeneration by carrying out these studies in a genetic background such as *dgq*^{1}, a loss-of-function mutant in the Gq_α molecule mediating vision. Light micrographs of eye sections from *dgq*^{1} (Figure 3A) or *dgq*^{1};*arr2*^{3} (Figure 3B) mutants raised in normal room light show normal ommatidial structure, but *dgq*^{1};*rdgC*^{306} (Figure 3C) mutants under identical conditions show profound degenerative changes including loss of photoreceptor cells and gross distortion of eye structure. As expected, *arr2*^{3} degeneration, but not that of *rdgC*^{306}, is suppressed by *dgq*^{1}. However, in the *dgq*^{1} background, apoptosis in *rdgC*^{306} is strongly suppressed by *arr2*^{3} (Figure 3D), demonstrating that Arr2 is a required component for photoreceptor death. Thus, both Arr2 and phosphorylated metarhodopsin are components of the proapoptotic trigger in *rdgC*^{306}.

Phosphorylation of Metarhodopsin Is Not Required for Arr2 Binding

The finding that Arr2 is required for the *rdgC*^{306} phenotype offers a simple potential explanation for why the cell death process might require the phosphorylated state of metarhodopsin. For most GPCRs, the phosphorylated, active form of the receptor is thought to be obligatory for high-affinity arrestin binding (Gurevich et al., 1995). Thus, the requirement for M-p may simply represent the fact that this is the only state capable of interacting with Arr2.

To test whether phosphorylation of metarhodopsin is necessary for Arr2 binding, we measured the associa-

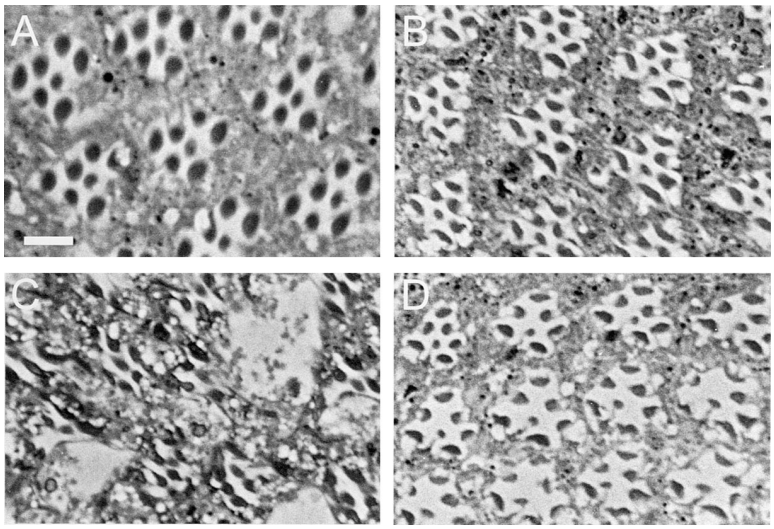

Figure 3. Arr2 Is Required for Photoreceptor Degeneration in *rdgC*^{306} Mutants

Cross sections (1 μm thick) of the adult compound eye in *dgq*^{1} (A), *dgq*^{1};*arr2*^{3} (B), *dgq*^{1};*rdgC*^{306} (C), and *dgq*^{1};*arr2*^{3} *rdgC*^{306} (D) maintained for 6 days in white light conditions (see Experimental Procedures). Light-dependent degeneration in *arr2*^{3} mutants, a hypomorphic allele of Arr2, is suppressed by *dgq*^{1}, a loss-of-function mutant in the G_{α} subunit mediating vision in *Drosophila* (B). In contrast, *dgq*^{1} does not suppress the apoptotic death of photoreceptor cells in *rdgC*^{306}, demonstrating that degeneration in *rdgC*^{306} is independent of visual signaling (C). However, *arr2*^{3} fully suppresses apoptosis in *rdgC*^{306} in a *dgq*^{1} background (D). Scale bar, 5 μm.

tion of Arr2 with either wild type or phosphorylation-deficient forms of Rh1 rhodopsin. We prepared head membrane homogenates from wild-type flies (*w*^{1118}) or from transgenic flies carrying a carboxy-terminal deletion (Rh1Δ356) or alanine point mutations (Rh1StoA) that remove all phosphorylation sites on the receptor. Neither rhodopsin mutant shows any detectable phosphorylation upon light activation (Vinos et al., 1997). Since arrestin shows substrate specificity for the metarhodopsin form, illumination of the membrane homogenates by blue light should cause binding of Arr2 and consequently, depletion from the soluble fraction. Reillumination of the homogenate with orange light should then trigger release of Arr2 and enrichment in the soluble fraction. Figure 4A shows that the quantity of blue light–dependent binding and orange light–dependent release of Arr2 from membranes was indistinguishable in homogenates from all genotypes tested, indicating that neither the ability to bind metarhodopsin nor the specificity of Arr2 for the active state of rhodopsin requires phosphorylation. These results are consistent with previous observations that Rh1Δ356 flies show no functional defects in the rate of deactivation of the light response (Vinos et al., 1997) and that the rate of binding of Arr2 to M is faster than the rate of M phosphorylation (Plangger et al., 1994).

To independently confirm these results, we used a quantitative spectroscopic assay that measures the quantity of metarhodopsin-Arr2 complexes formed in head membrane extracts (Kiselev and Subramaniam,

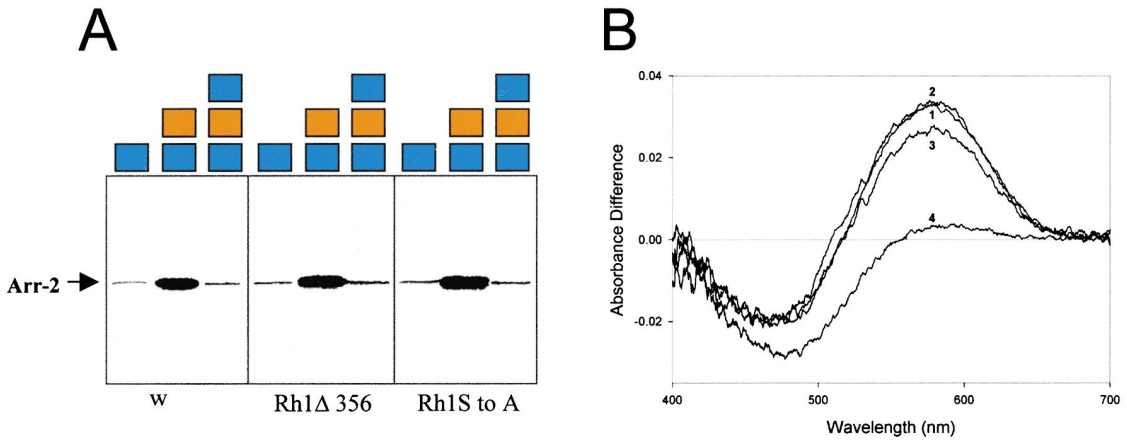

Figure 4. Metarhodopsin Phosphorylation Is Not Required for Arr2 Binding

(A) Light-dependent binding and release of Arr2 from head membranes prepared from wild-type flies (w), transgenic flies expressing a carboxy-terminal truncated rhodopsin in the R1–R6 photoreceptors (Rh1Δ356), and transgenic flies expressing rhodopsin with Ser to Ala mutations at all phosphorylation sites (Rh1StoA). Shown are Western blots probed with anti-Arr2 antibody of supernatants from the same membrane prep for each fly genotype subjected. Each lane contains proteins extracted from one head. Flies were stimulated by blue light, blue then orange light, or blue then orange then blue light. In each case, arrestin2 is depleted equally from the soluble fraction upon blue light exposure (generating metarhodopsin) and released upon orange light exposure (regenerating rhodopsin).

(B) Difference spectra (blue illuminated minus dark) of total head homogenate in wild-type (1), Rh1Δ356 (2), Rh1StoA (3), and arrestin null mutants (*arr1*^{1};*arr2*^{3}) (4). Depletion of the 480 nm absorbing species represents the photoisomerization of rhodopsin to metarhodopsin. Generation of the 580 nm absorbing metarhodopsin species is dependent on arrestin binding and results from the formation of stable metarhodopsin-arrestin complexes (1). Arrestin null mutants show no stabilized metarhodopsin as expected (4), but phosphorylation-deficient rhodopsin mutants show similar levels of stable metarhodopsin as wild-type (2 and 3), indicating normal arrestin binding.

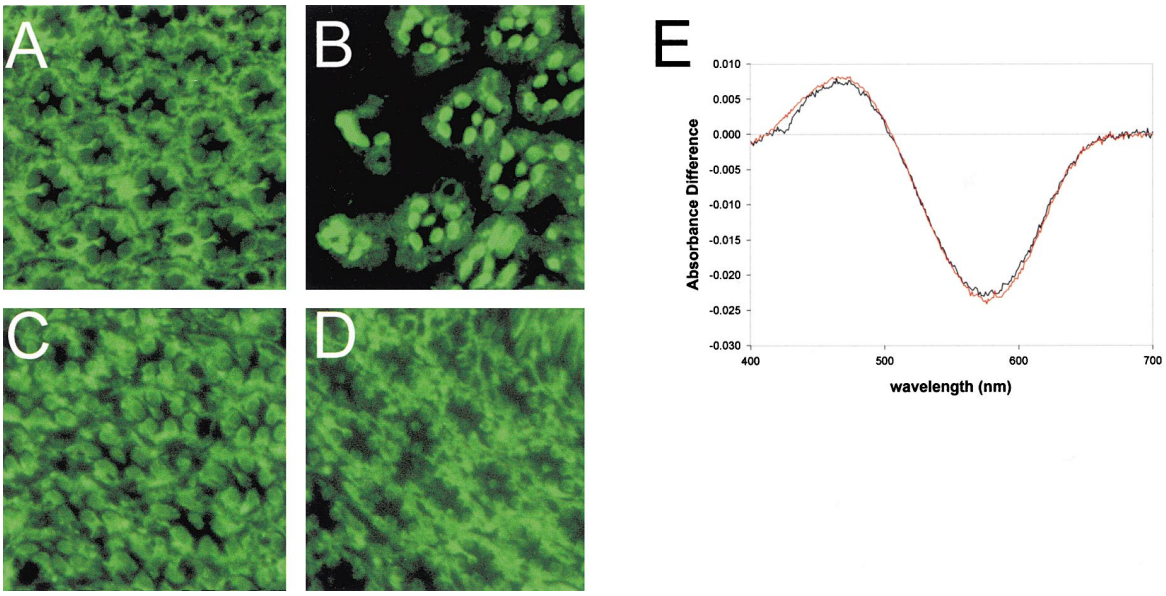

Figure 5. Light-Induced Arr2 Translocation to the Rhabdomere and Kinetics of Internalization

(A–D) Arr2 immunolocalization in cross sections (0.5 μm) of eyes from dark-reared wild-type animals (w^{1118}) stimulated as described. Arr2 is exclusively located in the cytosolic compartment in dark-reared animals (A), but is found localized to the rhabdomere membranes 1 hr after exposure to a 10 min pulse of blue light (B). Stimulation by blue light followed by dark incubations for 4 hr (C) or 3 days (D) shows translocation of Arr2 to the cytosol with a time course of hours.

(E) Difference spectra (orange illuminated minus dark) of detergent extracts prepared from wild-type heads at either 1 hr post blue illumination (black trace) or 1 day post blue illumination (red trace). The similar depletion of the 580 nm absorbing species (and the formation of the 480 nm absorbing species) indicates that the arrestin–metarhodopsin complex is stable throughout the process of internalization.

1997). The basis of this assay is that the lifetime of M in vitro is critically dependent on arrestin binding such that in the absence of arrestin, M decays to a nondetectable ultraviolet absorbing species within minutes. For example, a difference spectrum between blue-illuminated and dark-maintained membranes from wild-type (w^{1118}) animals shows depletion of R (λ_{max} = 480 nm) and formation of M (λ_{max} = 580 nm), but an identical experiment in membranes prepared from arrestin null mutants ($arr1^{1};arr2^{3}$) shows depletion of R but no formation of M (Figure 4B, traces 1 and 4). Thus, the formation of stable M is quantitatively proportional to the fraction of bound arrestin. Difference spectra (blue illuminated minus dark) from either Rh1Δ356 or Rh1StoA phosphorylation–deficient rhodopsin mutants show that the formation of stable metarhodopsin is similar to that of wild-type (Figure 4B, traces 2 and 3). Together, these data strongly argue that metarhodopsin phosphorylation is not required for its interaction with arrestin and that its requirement in triggering the apoptotic machinery is likely due to control of other molecular interactions in the apoptotic pathway.

Arr2 and Metarhodopsin Are Cointernalized upon Light Activation

To study the fate of M-Arr2 complexes formed upon light activation, we used immunofluorescence techniques to follow the intracellular distribution of Arr2 at several time points after stimulus conditions that generate M-Arr2. Dark-reared wild-type animals (containing R form) show a purely cytoplasmic distribution of Arr2 (Figure 5A), consistent with the fact the Arr2 has very low affinity for this state of rhodopsin. However, a 10 min exposure to blue light (generating M) followed by dark incubation for 1 hr shows a nearly exclusive localization of Arr2 to the rhabdomere (Figure 5B), consistent with formation of stable M-Arr2. An identical blue light stimulus, followed by 4 hr (Figure 5C) or 3 day (Figure 5D) dark incubations, shows that Arr2 is removed from the rhabdomere over a 1–4 hr time frame, ultimately returning to the cytosolic compartment. However, the cytoplasmically located Arr2 in Figures 5C and 5D is not free, soluble arrestin; it is still complexed with M, since difference spectra from head membranes isolated from animals 1 day after a 10 min blue stimulus show an identical quantity of stable metarhodopsin as that from animals 1 hr after the stimulus (Figure 5E). These data suggest that complexes of metarhodopsin and Arr2 are transported through an endocytic process to an internal compartment with a time course of hours and, once internalized, these complexes are potentially stable for days.

Arr2 Binds to Clathrin in a Phosphorylation-Dependent Manner

A large body of work suggests that the likely mechanism for the transport of the Arr2-M complex from rhabdomere to the cytoplasmic compartment is clathrin dependent endocytosis. For several mammalian GPCRs, β-arrestin1 or β-arrestin2 acts as an adaptor protein that binds simultaneously to the activated receptors and to the globular terminal domain of the clathrin heavy chain (Lefkowitz, 1998). This activity triggers the clustering of activated receptors at coated pits and promotes their internalization as part of a mechanism for long-term

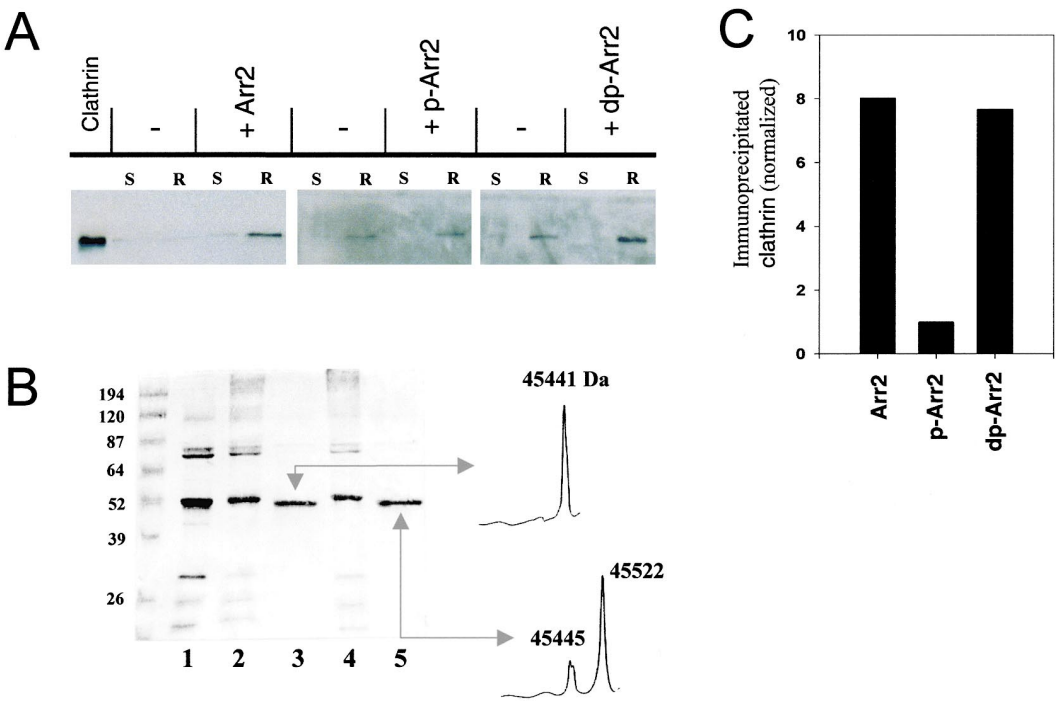

Figure 6. Recombinant Arr2 Interacts with Bovine Clathrin Cages in a Phosphorylation-Dependent Manner

(A) Coimmunoprecipitation of clathrin either in the presence or absence of Arr2, phosphorylated Arr2 (p-Arr2), or subsequently dephosphorylated Arr2 (dp-Arr2). Immunoprecipitation was carried out with an anti-Arr2 antibody, and blots were probed with an anti-clathrin heavy chain antibody.

(B) SDS-PAGE showing steps in the purification of recombinant Arr2 and phosphorylated Arr2. His_6-Arr2 expressed in *E. coli* was partially purified through Ni-NTA chromatography (1) and incubated in arrestin-deficient head homogenates either with (4) or without (2) ATP. The resulting Arr2 protein was repurified through Ni-NTA chromatography and cleavage of the histidine tag (3 and 5). Mass spectrophotometric analysis shows that addition of ATP results in the majority of Arr2 shifted in mass by 81 Da, consistent with single-site phosphorylation of Arr2.

(C) Quantitation of specific immunoprecipitated clathrin through densitometry of the immunoblots shown in (A), showing reversible phosphorylation-dependent binding of Arr2 and clathrin. The data are normalized to the signal of phosphorylated Arr2.

desensitization of receptor activity. The interaction of β-arrestin1 with clathrin appears to be subject to regulation by phosphorylation of β-arrestin1 at a serine located near the carboxyl terminus; phosphorylation destabilizes the interaction with clathrin (Lin et al., 1997).

To determine whether Arr2 could also act as an adaptor protein for clathrin-dependent M internalization in *Drosophila* photoreceptors, we examined the interaction of purified recombinant Arr2 with intact clathrin cages prepared from bovine brain (Kibbey et al., 1998). The bovine clathrin heavy chain, which contains the arrestin interaction domain, displays more than 90% amino acid similarity with that of *Drosophila* (Bazinet et al., 1993). We incubated clathrin cages with or without Arr2, immunoprecipitated with an anti-Arr2 antibody, and visualized coprecipitating clathrin heavy chain by Western blot analysis (Figure 6A, first panel). This experiment demonstrates that like the β-arrestins, Arr2 interacts strongly with clathrin cages. Interestingly, the consensus sequence motif identified in mammalian arrestins for clathrin interaction is not evident in Arr2, suggesting either that a homologous structural motif is encoded by a highly dissimilar sequence or that a distinct structural interaction mediates the binding.

The findings that carboxy-terminal phosphorylation of β-arrestin1 regulates its interaction with clathrin (Lin et al., 1997) and that Arr2 is phosphorylated at a similar position upon light activation (Matsumoto et al., 1994) raises the possibility that the Arr2-clathrin interaction is also regulated by phosphorylation. To test this, we used the coimmunoprecipitation assay to study the interaction of clathrin cages with purified phosphorylated Arr2. To stoichiometrically phosphorylate Arr2 at its single physiological site (Ser-366), we first partially purified hexahistidine-tagged Arr2 (His_6-Arr2) from *E. coli* and incubated this protein in *Drosophila* head homogenates either with or without excess ATP and inhibitors of phosphatases (Figure 6B, lanes 4 and 2, respectively). Electrospray mass spectrometry of His_6-Arr2 repurified from both samples (Figure 6B, lanes 5 and 3) demonstrates the nearly quantitative addition of a single phosphate (mass difference = 81 Da) per molecule in the presence of ATP to form p-Arr2. A similar experiment with His_6-Arr2 carrying a mutation at Ser-366 (S366A) shows no modification (data not shown), consistent with specific phosphorylation at position 366. To demonstrate reversibility of the effect of phosphorylation of Arr2, we dephosphorylated p-Arr2 by incubation of this protein once again in *Drosophila* head membrane homogenates in the absence of added ATP to produce dephosphorylated Arr2 (dp-Arr2). Quantitative removal of phosphate from p-Arr2 was again confirmed by electrospray mass

spectrometry (data not shown). To compare the interaction of Arr2, p-Arr2, and dp-Arr2 with clathrin, we coimmunoprecipitated Arr2 and clathrin as described above (Figure 6A). These data and their quantitation (Figure 6C) show that like β-arrestin1, Arr2 interacts with clathrin in the unphosphorylated state, and the interaction is reversibly controlled by phosphorylation at the single carboxy-terminal site.

Genetic Evidence for Regulation of Photoreceptor Apoptosis by Arr2 Phosphorylation

If the interaction of Arr2 with clathrin is a necessary step in triggering photoreceptor apoptosis in *rdgC*^{306} and is regulated by phosphorylation in vivo, then phosphorylation-deficient Arr2 mutants should show enhancement of degeneration in *rdgC*^{306} due to the enhanced clathrin interaction. To test this prediction, we made flies carrying genetic combinations of *rdgC*^{306} and an Arr2 allele (*arr2*^{1}) containing a 45 amino acid carboxy-terminal truncation that eliminates the Arr2 phosphorylation site (Dolph et al., 1993). As with previous experiments, these studies were carried out in a *dgq*^{1} genetic background to suppress photoreceptor degeneration through the visual signaling defects of arrestin mutations. Light-stimulated *dgq*^{1}*;arr2*^{1} mutants show normal ommatidial structure (Figure 7A), but *dgq*^{1}*;arr2*^{1} *rdgC*^{306} mutants (Figure 7B) show rapid degeneration of photoreceptor cells, demonstrating dramatic enhancement of the *rdgC*^{306} phenotype (compare Figures 7B and 3C).

To assess the time course of photoreceptor degeneration in *rdgC*^{306} and the influence of *arr2* mutations in this process, we screened populations of flies carrying various combinations of mutant alleles using the deep pseudopupil (DPP) assay. The DPP is a pattern of dark spots seen on the surface of the compound eye that results from the optical superposition of several photoreceptor rhabdomeres from neighboring ommatidia (Figure 7C); the DPP intensity is directly related to the quantity of rhodopsin in photoreceptor cells and to the proper relative organization of the ommatidial units. Since both of these are affected by photoreceptor degeneration, the intensity of the DPP is a good measure of this process. For example, the light-dependent apoptotic process in *rdgC*^{306} results in nearly complete loss of the DPP (Figure 7D). Figure 7E shows a graph of the fraction of flies with DPPs following continuous exposure to room light for all genotypes tested. Consistent with the suggestion of a prolonged commitment phase for apoptosis in *dgq*^{1}*;rdgC*^{306} flies, these animals show normal DPPs for several days and then undergo a dramatic loss of photoreceptor cells between days 4 and 6. Loss of Arr2 function results in suppression of this phenotype (*dgq*^{1}*;arr2*^{3} *rdgC*^{306}), and deletion of the phosphorylation domain of Arr2 (*dgq*^{1}*;arr2*^{1} *rdgC*^{306}) shows significant enhancement of the *rdgC*^{306} phenotype.

The enhancement of photoreceptor degeneration in *rdgC*^{306} by *arr2*^{1} is particularly intriguing since *arr2*^{1} mutants display completely wild-type photoreceptor physiology (Dolph et al., 1993), suggesting normal rate of quenching of metarhodopsin activity. Thus, like for β-arrestin1 (Lin et al., 1997), Arr2 phosphorylation appears to primarily regulate its interaction with clathrin rather than regulate interaction with the receptor. To demonstrate this, we measured difference spectra (blue light illuminated minus dark) of rhodopsin-containing membranes extracted from *Drosophila* heads in the presence of phosphorylated Arr2 or dephosphorylated Arr2 (Figure 7F). Both Arr2 species result in the production of an identical quantity of 580 nm–absorbing stabilized metarhodopsin, consistent with the model that both phosphorylated and dephosphorylated forms of Arr2 bind equally to metarhodopsin. These results are consistent with studies showing that mutation of the Arr2 phosphorylation site does not prevent light-dependent binding to rhodopsin-containing membranes (Alloway and Dolph, 1999). Taken together, these data support the model that Arr2 is a phosphorylation-dependent adaptor protein for clathrin interaction in vivo and that photoreceptor apoptosis in *rdgC*^{306} proceeds through clathrin-dependent internalization of M-p.

Endocytosis Is Necessary for Photoreceptor Apoptosis in *rdgC*^{306}

As a rigorous test for the requirement of internalization of Arr2-M-p complexes for photoreceptor apoptosis in *rdgC*^{306}, we reasoned that *Drosophila* mutants known to disrupt the process of clathrin-mediated endocytosis should act as suppressors of *rdgC*^{306}. The best characterized of this class of mutant is the temperature-sensitive paralytic mutant of the dynamin GTPase *shibere* (*shi*^{ts1}). *shi*^{ts1} flies display reversible rapid paralysis upon temperature shifts from permissive (18°C) to restrictive (29°C) conditions (Grigliatti et al., 1973). This phenotype is due to loss of synaptic function caused by defective reuptake of neurotransmitter, which in turn is due to failure of clathrin-mediated internalization (van der Bliek and Meyerowitz, 1991). The dynamin family is conserved throughout evolution and in many diverse cell types, suggesting that it is an integral component of the general clathrin-dependent endocytic pathway (McClure and Robinson, 1996). In *Drosophila* photoreceptor cells, *shi*^{ts1} causes ultrastructural features at the rhabdomeric membrane typical of defective endocytosis even at temperatures that fail to produce complete paralysis (Sapp et al., 1991), suggesting that Shibere is a mediator of endocytosis of photoreceptor membranes. Since studies of photoreceptor degeneration require observation of flies over a several days, we titrated incubation temperature in *shi*^{ts1} mutants to find conditions that cause defective endocytosis in photoreceptor cells without inducing complete paralysis (data not shown). Thus, if photoreceptor apoptosis in *rdgC*^{306} requires clathrin-dependent endocytosis then *shi*^{ts1} should act as a partial suppressor of the *rdgC*^{306} phenotype at such temperature conditions.

To first verify that endocytosis of rhabdomeric membranes is defective in photoreceptors homozygous for the *shi*^{ts1} allele, we examined the process of light-dependent internalization of Arr2 in *rdgC*^{306} or *shi*^{ts1}*;;rdgC*^{306} flies grown at 23°C. As expected, both *rdgC*^{306} and *shi*^{ts1}*;;rdgC*^{306} photoreceptors show cytosolic localization of Arr2 in animals maintained in the dark for 2 days (Figures 8A and 8B). A 10 min exposure to blue light followed by 2 days of dark incubation shows an intracellular distribution of Arr2 in *rdgC*^{306} mutants but shows a largely rhabdomeric distribution in *shi*^{ts1}*;;rdgC*^{306}; these

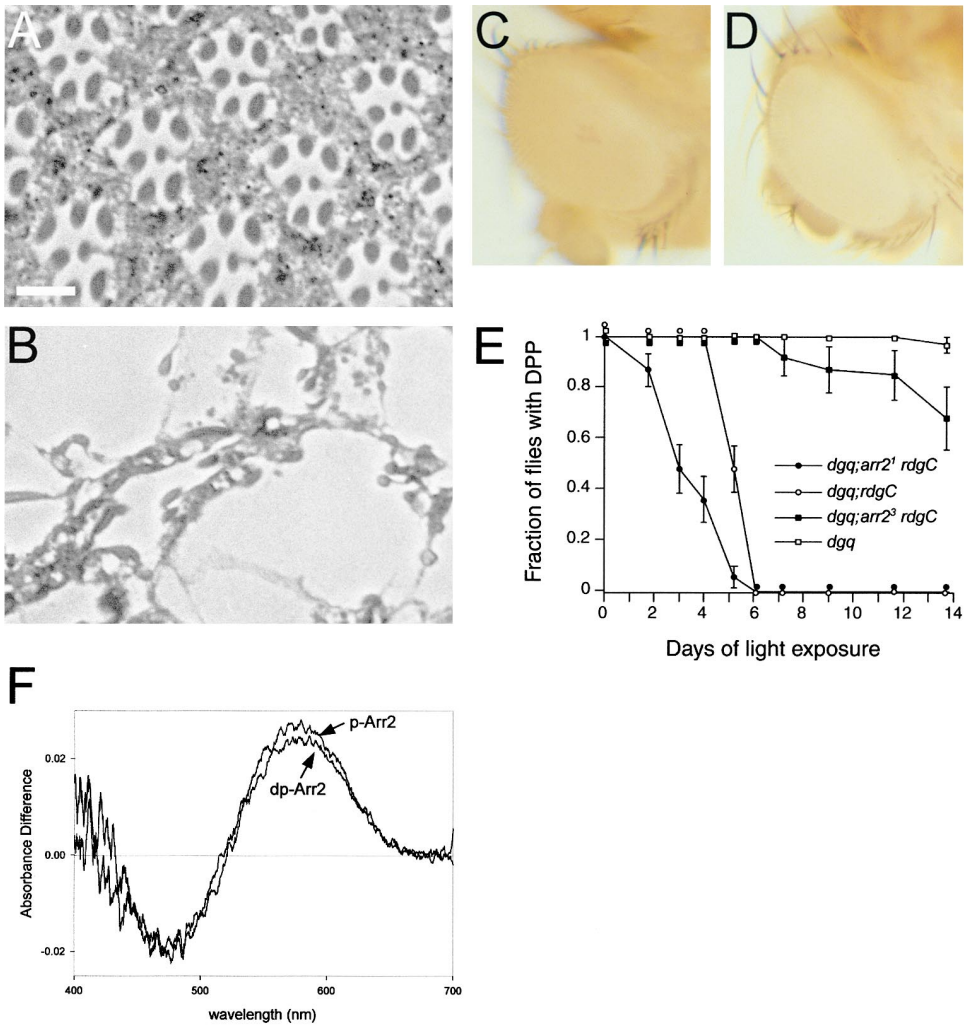

Figure 7. Arrestin2 Truncation Mutants Lacking the Phosphorylation Domain Enhance Degeneration in *rdgC*^{306} Mutant

(A and B) Cross sections of eyes (1 μm thick) in *dgq*^{1};*arr2*^{1} (A) and *dgq*^{1};*arr2*^{1} *rdgC*^{306} (B) 6 days after light exposure, showing enhancement of the *rdgC*^{306} degeneration phenotype (compare with Figure 3C).

(C and D) The deep pseudopupil (DPP) in *rdgC*^{306} flies maintained in the dark for 6 days (C). The DPP is a pattern of dark spots on the surface of the compound eye whose intenisty is strongly sensitive to degenerative changes in the eye that disrupt the normal ommatidial structure. *rdgC*^{306} flies grown in the light for 6 days have a complete lack of DPP (D).

(E) A graph showing the fraction of flies with DPPs as a function of days of light exposure. *rdgC*^{306} flies show DPP loss between 4 and 6 days. In contrast, *dgq*^{1};*arr2*^{3} *rdgC*^{306} flies show substantial suppression of DPP loss, and *dgq*^{1};*arr2*^{1} *rdgC*^{306} show enhancement.

(F) Difference spectra (blue illuminated minus dark) of rhodopsin-containing membranes isolated from arrestin null mutants supplemented with either phosphorylated or dephosphorylated recombinant Arr2. Both Arr2 isoforms show similar ability to bind metarhodopsin. Scale bar, 5 μm.

data show that *shi*^{ts1};;*rdgC*^{306} flies are indeed defective in the clathrin-dependent internalization pathway and provide additional support to the model that Arr2 is internalized through this process. To examine the consequence of defective clathrin-dependent internalization for photoreceptor apoptosis, we conducted DPP analysis of light-dependent photoreceptor degeneration in *rdgC*^{306} or *shi*^{ts1};;*rdgC*^{306} animals. *rdgC*^{306} (Figure 8E) or *shi*^{ts1};;*rdgC*^{306} (Figure 8F) flies maintained without stimulation in the dark for 4 days show equivalently strong DPPs. Siblings exposed to 10 min of blue light followed by 4 days of dark incubation show elimination of DPP in *rdgC*^{306} (Figure 8G) and partial, but significant, suppression of DPP loss in *shi*^{ts1};;*rdgC*^{306} (Figure 8H), consistent with requirement of Shibere for photoreceptor degeneration. Overall, these results indicate that clathrin-mediated internalization is a necessary process for photoreceptor apoptosis.

Discussion

In this work, we use a combination of genetic and biochemical methods to reveal a novel light-dependent but G protein–independent signaling pathway regulating photoreceptor cell viability in *Drosophila*. We show that in addition to its established function in inactivation of metarhodopsin, Arr2 acts as a clathrin adaptor protein to mediate the internalization of Arr2-M complexes. The

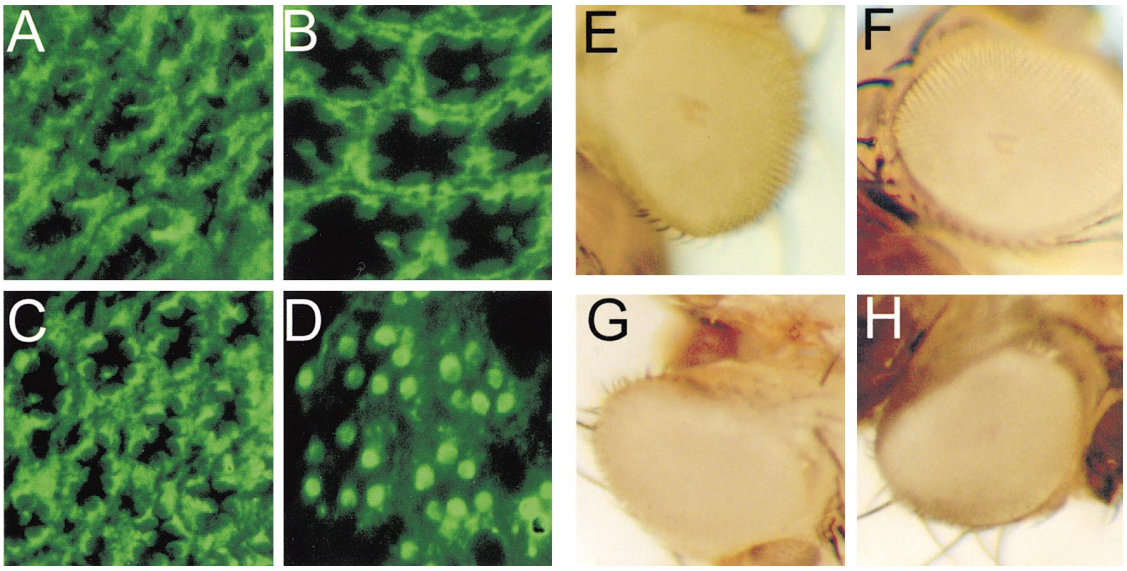

Figure 8. Loss-of-Function Mutation in the Shibere Dynamin GTPase Partially Suppresses Light-Dependent Degeneration in *rdgC*^{306}

All panels show experiments in either dark maintained flies (A, B, E, and F) or in flies stimulated with a 10 min exposure of blue light followed by 2 (C and D) or 4 (G and H) days in the dark. Arr2 immunolocalization in 0.5 μm eye sections from *rdgC*^{306} (A and C) or *shi*^{ts1}*;;rdgC*^{306} (B and D) flies shows that loss of Shibere function correlates with reduced ability to internalize Arr2 from rhabdomeric membranes (compare [C] and [D]). DPP analysis of *rdgC*^{306} (E and G) or *shi*^{ts1}*;;rdgC*^{306} double mutants (F and H). These data show that light eliminates the DPP in *rdgC*^{306} mutants and that loss of Shibere function partially suppresses the *rdgC*^{306} phenotype.

analysis of *rdgC*^{306} mutants (that are unable to dephosphorylate metarhodopsin and display light-dependent retinal degeneration) shows that the Arr2-dependent internalization process is necessary for photoreceptor cell apoptosis and that the triggering condition for apoptosis is the long-term accumulation of Arr2-M-p complexes in an internal compartment. These findings are in strong agreement with the work of Alloway et al. (2000 [this issue of *Neuron*]), who also show that formation and internalization of stable Arr2-M complexes are associated with photoreceptor apoptosis in *Drosophila*. This study shows that mutations in several phototransduction genes show Arr2-dependent retinal degeneration, suggesting that the Arr2-dependent internalization pathway is likely to be a common mechanism for triggering apoptotic photoreceptor cell death.

How does this pathway function in wild-type photoreceptor cells and how is apoptosis suppressed under normal physiological conditions? We show that two phosphorylation events control this process: (1) the phosphorylation of M promotes apoptosis through an unknown mechanism downstream of the internalization process and the RdgC phosphatase counteracts this process by efficient dephosphorylation of M, and (2) the phosphorylation of Arr2 suppresses apoptosis by disrupting clathrin interaction and preventing internalization of the receptor complexes. The net flux of the proapoptotic Arr2-M-p complexes through the internalization pathway is therefore determined by the quantity of metarhodopsin created upon light exposure, the activity of the RdgC phosphatase, and the phosphorylation status of Arr2. Interestingly, both the activity of the RdgC phosphatase and the phosphorylation of Arr2 are upregulated by the light-dependent increase in intracellular calcium that occurs during visual signaling in *Drosophila* photoreceptors (Byk et al., 1993; Matsumoto et al., 1994). Thus, phototransduction appears to promote the survival of photoreceptor cells by reducing the accumulation of internalized Arr2-M-p complexes.

These results help clarify a number of unexplained and mysterious observations in the study of the photoreceptor signaling in *Drosophila*. For example, mutations that eliminate the phosphorylation domain of rhodopsin (Vinos et al., 1997) or of arrestin (Dolph et al., 1993) have no effect on the sensitivity or kinetics of light transduction. Nevertheless, these phosphorylation events occur rapidly upon light activation (Matsumoto et al., 1994) and require the investment of substantial metabolic resources since rhodopsin and arrestin are among the most abundant of photoreceptor proteins. We suggest that these phosphorylation events are dedicated regulatory processes that apply primarily to the control of cell viability. The large commitment of metabolic energy in these steps presumably highlights the concept that like all cells, photoreceptors need to suppress the latent apoptotic machinery for survival (Raff et al., 1994).

How can internalized Arr2-M-p complexes trigger apoptosis? Several studies support for the idea that internalized, arrestin-bound GPCRs can trigger additional signaling events (Lefkowitz, 1998). For example, β-arrestin1 recruits c-Src to form three-protein complexes with activated β2 adrenergic receptors, and mediates the internalization of the entire complex through direct interaction with clathrin (Luttrell et al., 1999a). Upon internalization, the complex triggers stimulation of mitogenic signaling through activation of the Ras-MAP kinase pathway (Luttrell et al., 1999a). Cell survival has been shown to be promoted by this pathway through both suppression of the apoptotic machinery and transcriptional activation of prosurvival factors (Bonni et al.,

1999), and β-arrestin- and receptor internalization-dependent activation of MAP kinases is reported to be the basis for the antiapoptotic effects of substance P (DeFea et al., 2000). Thus GPCRs can trigger a second round of signaling through arrestin-mediated internalization and may generally promote the survival of cells through activation of mitogenic signaling. One possibility is that Arr2-mediated internalization of dephosphorylated metarhodopsin in *Drosophila* photoreceptors plays a similar role in triggering a prosurvival signaling pathway and that phosphorylated M-Arr2 complexes may trigger the apoptotic machinery by disruption of this process.

The conservation of arrestin-mediated internalization between *Drosophila* rhodopsin and mammalian β receptors highlights the similarity in functional mechanisms throughout the GPCR superfamily. The stimulus-dependent phosphorylation at the carboxyl terminus is no exception; this property is shared by most GPCRs and suggests that RdgC-like receptor phosphatases may also be found in other signaling systems. Two mammalian homologs of RdgC, PPEF-1 and PPEF-2, have been cloned that show tissue distributions consistent with a role in several sensory signaling processes (Montini et al., 1997; Sherman et al., 1997; Huang and Honkanen, 1998). Indeed, PPEF-2 is found exclusively in the retinal rod photoreceptors and in the pineal gland (Sherman et al., 1997), although the role of this protein in regulating function of vertebrate rhodopsin is not yet established. However, retinal degeneration in transgenic mice expressing a constitutively active form of rhodopsin (K296E) does not result from excessive stimulation of the visual signaling cascade; instead the mutant rhodopsin is found in a constitutively phosphorylated form, tightly bound to arrestin molecules (Li et al., 1995; Rim and Oprian, 1995). The corresponding mutation in human rhodopsin is associated with one form of retinitis pigmentosa (Keen et al., 1991), suggesting that stable phosphorylated GPCR-arrestin complexes may also be proapoptotic in humans.

An interesting issue of the apoptotic mechanism in *rdgC*^{306} photoreceptors is the long but apparently reliable time delay in the commitment to cell death. For example, DPP analysis of a population of *rdgC*^{306} flies exposed to light show no individuals with retinal degeneration until the fourth day, but then nearly all animals show complete degeneration over the next 2 days (Figure 7E). Such fidelity in the timing of apoptosis in many independent animals is reminiscent of many human age-dependent macular degenerations in which affected individuals show a similar age of onset of symptoms, and argues that the timing of degeneration must be a well-regulated feature of the commitment process and not a simple, randomly distributed event. Since formation and internalization of the Arr2-M-p complexes proceeds with a time course of hours, and commitment to apoptosis requires days, we conclude that the mechanism controlling the decision for cell death must reside in the multivesicular bodies (MVB) that represent the stable pool of internalized membranes in *Drosophila* photoreceptors (Stark and Sapp, 1987). The detailed characterization of commitment to apoptosis in *rdgC*^{306} photoreceptors should provide insight into understanding the factors controlling programmed timing of apoptosis.

Experimental Procedures

Phosphorylation of Rhodopsin In Vivo

rdgC^{306} flies were fed standard fly food supplemented with 250 μCi of γ−[^{32}P]ATP (ICN Biochemicals) for 2 days in the dark and illuminated with either blue light (440 nm) or blue light followed immediately by orange light (600 nm). Fly heads (five) were collected under dim red illumination and were manually disrupted in 50 μl of 2× sample buffer (60 mM Tris-HCl [pH 6.8], 20% SDS, 0.0004% bromophenol blue, 10% β-mercaptoethanol, 20% glycerol). 10 μl samples were subjected to SDS-PAGE, transferred to nitrocellulose, and analyzed by autoradiography or Western blot analysis using anti-Rh1 antibody (1:10,000 dilution). For all experiments, flies were illuminated using a 100 W Xenon arc lamp source filtered through appropriate interference filters (10 nm spectral bandwidth) (Oriel Corp).

Transmission Electron Microscopy

Fly heads were collected under dim red illumination, fixed for 4 hr in 0.1 M sodium cacodylate (pH 7) containing 2.0% glutaraldehyde, and 2% formaldehyde at 4°C followed by 2 hr in 1% OsO_4 in the same buffer, rinsed with water, and stained for 2 hr in aqueous uranyl acetate. Heads were then dehydrated through an ethanol series (70%, 95%, 100%, 5 min each) followed by three changes of propylene oxide (15 min each) and embedded in Spurr's resin (EM Sciences). Transverse sections (50–100 nm thick) were poststained with uranyl acetate and lead citrate and imaged using a JEOL 1200 EX electron microscope.

Photoreceptor Degeneration and Light Microscopy

Flies were exposed to white light (30 W white lamp, attenuated 1:100 at 15 cm) for 6 days. Fly heads were cut, fixed, and embedded in resin as described (Smith et al., 1991). Sections (1 μm thick) were stained with methylene blue and borax before analysis.

Arrestin2 Membrane Binding Assays

Head homogenates from 100 flies were prepared as previously described (Kiselev and Subramaniam, 1997) under dim red light, illuminated by blue light (440 nm) for 2 min at room temperature, and rhodopsin containing membranes were isolated by centrifugation at 45,000 × g for 1 hr at 4°C. Membranes were resuspended in 10 mM MOPS (pH 7.0) containing 0.25 M sucrose, 0.12 M KCl, 5 mM $MgCl_2$, 1 mM DTT, PMSF (1 mM), leupeptin (10 μg/ml), and pepstatin (2 μg/ml) and were divided and treated as follows: sample 1 was kept in the dark for 20 min; sample 2 was illuminated with orange light (600 nm) for 1 min and then kept in the dark for 19 min; and sample 3 was illuminated with orange light for 1 min, kept in the dark for 19 min, and again illuminated with blue light for 1 min. The resulting samples were then centrifuged at 150,000 × g for 20 min. at 4°C and supernatants (2 μl) were analyzed by Western blot probed with an anti-Arr2 antibody (1:1000).

Difference Spectra

Head homogenates from flies were prepared as described above and divided into two cuvettes placed into sample and reference positions in a double beam spectrophotometer (Perkin-Elmer λ18). After baseline correction, one sample was illuminated for 1 min at the wavelength indicated, and the difference spectrum was measured against the nonilluminated sample. For experiments involving recombinant Arr2, homogenates were prepared from arrestin null mutants (*arr1*^{1};*arr2*^{3}) to avoid M stabilization from endogenous arrestins and were incubated with a three-molar excess of either phosphorylated or dephosphorylated Arr2 prior to collection of difference spectra.

Immunofluorescence Analysis

Dark-reared flies were illuminated as indicated, and the heads collected under dim red illumination. Heads were fixed for 4 hr in 0.1 M PBS (pH 7.0) containing 4% formaldehyde at 4°C, cryoprotected in PBS containing 2.3 M sucrose, and frozen in liquid nitrogen. 0.5 μm sections were cut at −120°C. Sections were washed for 30 min with PBS plus 0.1% saponin at room temperature and then incubated for 1 hr with anti-Arr2 antibodies (1:100) in 0.1 M PBS plus 0.1% saponin. Sections were washed four times in PBS and

incubated for 1 hr with fluorescein-conjugated goat antibodies to rabbit IgG (1:8) (Amersham Pharmacia) in PBS plus 0.1% saponin. Sections were washed four times in PBS, allowed to dry, and covered with 90% glycerol containing p-phenylenediamine (Sigma) as an antibleaching agent. Sections were imaged using an inverted fluorescent microscope (Olympus IX70) and photographed using a digital camera (Princeton Instruments). Images were acquired using Axon Imaging Workbench (Axon Instruments).

Isolation of Clathrin Cages and Purification of Recombinant of Arr2

Clathrin coat proteins were isolated from bovine brain exactly as described in Kibbey et al. (1998). Arr2 cDNA was subcloned into PET28 (Novagen) as an N-terminal His_6-tagged construct. His_6-Arr2 was expressed by overnight IPTG induction (100 μM) at 17°C in BL21(DE3) cells grown to an OD_{600} of 1.2 in Terrific Broth (TB). Cells were harvested and resuspended in buffer A (10 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 10 mM imidazole, 0.1% Tween-20, 1 mM PMSF, leupeptin [10 μg/ml], and pepstatin [2 μg/ml]), sonicated, and centrifuged at 50,000 × g for 1 hr at 4°C. The supernatant was incubated with Ni-NTA (Qiagen) resin overnight at 4°C, washed with 25 bed volumes of buffer A followed by a 25 bed volume wash with buffer A lacking Tween-20. His_6-Arr2 was eluted in buffer A containing 0.25 M imidazole, digested with thrombin (Sigma) (for removal of the His_6-tag) during dialysis for 12 hr against buffer B (50 mM Tris-HCl, 0.15 M NaCl, 2.5 mM $CaCl_2$, 5 mM $MgCl_2$, 10% glycerol, and 10 mM β-mercaptoethanol). Uncut His_6-Arr2 was removed by reincubation with Ni-NTA resin. Purified Arr2 was stored at 4°C and used immediately for all experiments.

Phosphorylation and Dephosphorylation of Arr2

Total head homogenate from 25 grams of dark-reared *arr1[1];arr2[3]* flies was isolated as described above. The soluble and membranes fractions were separated by centrifugation twice at 250,000 × g for 30 min at 4°C. Recombinant His_6-Arr2 (750 μg) was quantitatively phosphorylated by mixing with the soluble fraction from head homogenate supplemented with 5 mM ATP and 2 μM of okadaic acid (Calbiochem), incubated for 1 min, and immediately purified using Ni-NTA affinity chromatography. To obtain dephosphorylated Arr2 (dp-Arr2), His_6-tagged p-Arr2 was mixed with the soluble fraction from *arr1[1];arr2[3]* head homogenate and incubated for 40 min at room temperature. Dephosphorylated Arr2 (dp-Arr2) was again purified by Ni-NTA affinity chromatography.

Coimmunoprecipitation of Clathrin/Arrestin2 Complexes

Clathrin cages were incubated at 4°C for 30 min in 150 μl of 10 mM MOPS (pH 7.0) containing 0.25 M sucrose, 0.12 M KCl, 5 mM $MgCl_2$, and 0.4% CHAPS with or without one of the following: Arr2, pArr2, or dpArr2 at a 1:15 clathrin:arrestin ratio. Anti-Arr2 antibodies (25 μg) were added, and the mixtures were incubated an additional 30 min at 4°C. Samples were mixed with 5 μl of Protein A-Sepharose (6 mg/ml) (Sigma), incubated 15 min at 4°C, and centrifuged at 500 × g for 1 min to pellet any clathrin-Arr2 complexes. Supernatants and pellets were diluted 5-fold in 2× sample buffer and analyzed by Western blot using monoclonal anti-clathrin heavy chain antibodies (1:1000) (Transduction Laboratories). Approximately 2 μg of clathrin was used as a positive control.

Fly Stocks

The fly stocks used were *w[1118]*, *w[1118];;P[ninaE[Δ356] ry[+]] ry ninaE[117] e[s] (Rh1Δ356)*, *w[1118];;rdgC[306] cu red*, *w;arr1[1];arr2[3]*, *w[1118];dgq[1] cn*, *dgq[1];arr2[1]*, *dgq[1];rdgC[306] cu red*, and *dgq[1];arr2[3] rdgC[306]*. *shi[ts1]* flies were kindly provided by Dr. Helmut Kramer. *shi[ts1]*, *cn bw*, and *w;;rdgC[306] cu red* flies were crossed using standard *Drosophila* genetics to generate *shi[ts1];cn bw;rdgC[306] cu red*.

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