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An essential role for endocytosis of rhodopsin through interaction of visual arrestin with the AP-2 adaptor

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Summary

Previously, we have identified a class of retinal degeneration mutants in *Drosophila* in which the normally transient interaction between arrestin2 (Arr2) and rhodopsin is stabilized and the complexes are rapidly internalized into the cell body by receptor-mediated endocytosis. The accumulation of protein complexes in the cytoplasm eventually results in photoreceptor cell death. We now show that the endocytic adapter protein AP-2 is essential for rhodopsin endocytosis through an Arr2–AP-2β interaction, and mutations in Arr2 that disrupt its interaction with the β subunit of AP-2 prevent endocytosis-induced retinal degeneration. We further demonstrate that if the interaction between Arr2 and AP-2 is blocked, this also results in retinal degeneration in an otherwise wild-type background. This indicates that the Arr2–AP-2 interaction is necessary for the pathology observed in a number of *Drosophila* visual system mutants, and suggests that regular rhodopsin turnover in wild-type photoreceptor cells by Arr2-mediated endocytosis is essential for photoreceptor cell maintenance.

Key words: Rhodopsin, Arrestin, Retinitis pigmentosa, Endocytosis, AP-2

Introduction

Inherited retinal diseases are heterogeneous in both their pathology and genetic origin. These diseases are characterized by the premature and progressive loss of rod and cone photoreceptor cells. A large number of loci have been implicated in causing retinal disorders (Rivolta et al., 2002; Saleem and Walter, 2002). Owing to the heterogeneous nature of retinal diseases, there have been many molecular mechanisms implicated. The disruption of genes that encode proteins involved in the phototransduction cascade, metabolism of the retinal chromophore of rhodopsin, and the structural support of photoreceptors have been shown to be responsible for the observed pathology.

The *Drosophila* visual system has proved to be an excellent model system for the study of retinal degeneration (Hardie and Raghu, 2001; Montell, 1999; Zuker, 1996). The phototransduction cascade in *Drosophila* is initiated by a photon of light activating the major light-sensitive receptor, rhodopsin (Rh1). Once activated, Rh1 catalyzes the exchange of GDP for GTP on the G_{α} subunit ($G_{\alpha q}$) of the heterotrimeric G protein to which it is coupled. The $G_{\alpha q}$ subunit then dissociates from the inhibitory βγ subunits and is able to activate retinal-specific phospholipase C, eventually leading to the opening of the cation-specific channels Trp and Trpl. The influx of cations through Trp and Trpl leads to the depolarization of the photoreceptor cell and neurotransmitter release.

The regulation of the visual transduction cascade is crucial for temporal resolution as well as adaptation to varying intensities of light. One key aspect of regulation is the inactivation of rhodopsin. In vertebrates, this inactivation is achieved by two proteins, rhodopsin kinase and arrestin (Bennett and Sitaramayya, 1988; Kühn and Wilden, 1987). Rhodopsin kinase phosphorylates the C-terminal tail of rhodopsin, which partially decouples it from the pathway. Arrestin is then able to bind the phosphorylated form of rhodopsin and completely inactivate it, presumably by competing for a common binding site with G_{α}. In addition to their role in inactivating receptors, arrestins have also been implicated in receptor internalization. This is best characterized by the non-visual system arrestins (β-arrestins), which trigger the internalization of these receptors by recruiting clathrin to the plasma membrane via a 'clathrin box' in the extreme C terminus (Ferguson et al., 1996; Goodman, Jr et al., 1996). Recently, an interaction between β-arrestin and the AP-2 adaptor complex has also been demonstrated to be critical for receptor endocytosis (Laporte et al., 2000). Visual arrestins have traditionally been discounted as serving the role of recruiting rhodopsin to clathrin-coated pits largely because they do not contain a clathrin-binding domain. However, recent work in *Drosophila* has implicated visual arrestins in rhodopsin endocytosis. The minor visual arrestin (Arr1) has been demonstrated to be essential for light-induced Rh1 internalization (Satoh and Ready, 2005), and the more abundant Arr2 has been shown to be involved in rhodopsin endocytosis under certain pathological situations (Alloway et al., 2000; Kiselev et al., 2000; Lee et al., 2003). Moreover, it has been demonstrated that Arr2 can interact with clathrin in vitro (Kiselev et al., 2000).

Previously, we and others have identified a novel retinal

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degeneration pathway in *Drosophila* photoreceptors that is characterized by persistent complexes between arrestin and rhodopsin that form at the rhabdomeric membrane (Alloway et al., 2000; Kiselev et al., 2000). These stable complexes are toxic because of the extensive endocytosis of the receptor into the photoreceptor cell body (Alloway et al., 2000; Orem and Dolph, 2002b). Although the photoreceptor cells undergo what appears to be programmed cell death, it has been demonstrated that death does not occur by classic developmental apoptosis (Hsu et al., 2004) and may therefore involve an alternative cell death pathway. Here we report that an AP-2 interaction motif found in the C terminus of Arr2 is essential for endocytosis-mediated photoreceptor cell death. Furthermore, we show that AP-2-mediated internalization of Rh1 in wild-type photoreceptors is essential for the maintenance of normal retinal morphology.

Results

The AP-2 adaptor complex is required for the *norpA*-mediated endocytosis of rhodopsin-arrestin complexes

We have previously shown that mutations in the eye-specific phospholipase C gene (*norpA*) cause persistent complexes of rhodopsin and Arr2 (Alloway et al., 2000), and that these complexes are rapidly removed from the rhabdomere resulting in approximately 80% of the Rh1 being internalized into the cell body (Orem and Dolph, 2002a). Arrestin disassociates from Rh1 once in the cytoplasm and the rhodopsin accumulates as cytoplasmic aggregates. This massive endocytosis eventually results in photoreceptor apoptosis (Orem and Dolph, 2002b). Recently, a physical interaction between AP-2 and β-arrestin has been demonstrated to be important in the endocytosis of the β-adrenergic receptor (Laporte et al., 2000). To study the possibility that Arr2 mediates the endocytosis of rhodopsin-arrestin complexes through the AP-2 adaptor, we analyzed flies containing a mutation in the *α-adaptin* gene. This mutation disrupts the α subunit of the heterotetrameric AP-2 adaptor complex, thus reducing AP-2 function (Forjanic et al., 1997; Gonzalez-Gaitan and Jackle, 1997). The P element insertion in the *α-adaptin* gene results in embryonic lethality, and therefore, all analysis was performed in somatic mosaic clones in the adult eye. In wild-type flies, after 24 hours of light treatment the localization of Rh1 and Arr2 is largely rhabdomeric (Fig. 1A-C). In *norpA* flies, persistent complex formation promotes the internalization of Rh1 and Arr2 resulting in at least 80% of the rhodopsin and most of the arrestin being removed from the rhabdomere (Fig. 1D-F). This results in an apparent decrease in Rh1 levels that may be due to the aggregation of rhodopsin in the cytoplasm or the degradation of Rh1 in the proteosome or lysosome. In contrast, the *norpA* α-*adaptin* double mutant displays a wild-type phenotype, with the majority of the rhodopsin and arrestin remaining in the rhabdomere (Fig. 1G-I). Thus the AP-2 adaptor complex plays a critical role in the *norpA*-mediated endocytosis of Arr2-rhodopsin complexes.

To investigate the role of the AP-2 adaptor on *norpA*-mediated retinal degeneration we examined the retinal morphology of light-treated flies. Wild-type retinas have a highly organized structure consisting of rows of ommatidia, with each ommatidium containing seven photoreceptor cells in any one plane of section. The photoreceptor cells are distinguished by the rhabdomeres, which are the dark staining areas that project into the intraommatidial space (Fig. 2A). This is in sharp contrast to *norpA* mutants, which undergo massive retinal degeneration (Fig. 2B). These mutants exhibit the loss of ommatidial organization, and most noticeably the shrinking or disappearance of most of the rhabdomeres. The *α-adaptin* mutant retinas show some signs of retinal degeneration with a few large holes in the sections (Fig. 2C), but the degeneration is much less severe than in *norpA* flies. Previously it has been shown that dynamin loss-of-function alleles exhibit significant degeneration (Acharya et al., 2003; Alloway et al., 2000). However, the *α-adaptin*^{06694} allele is not a null (Gonzalez-Gaitan and Jackle, 1997), and therefore, we would not anticipate the same degree of retinal degeneration as would be observed if

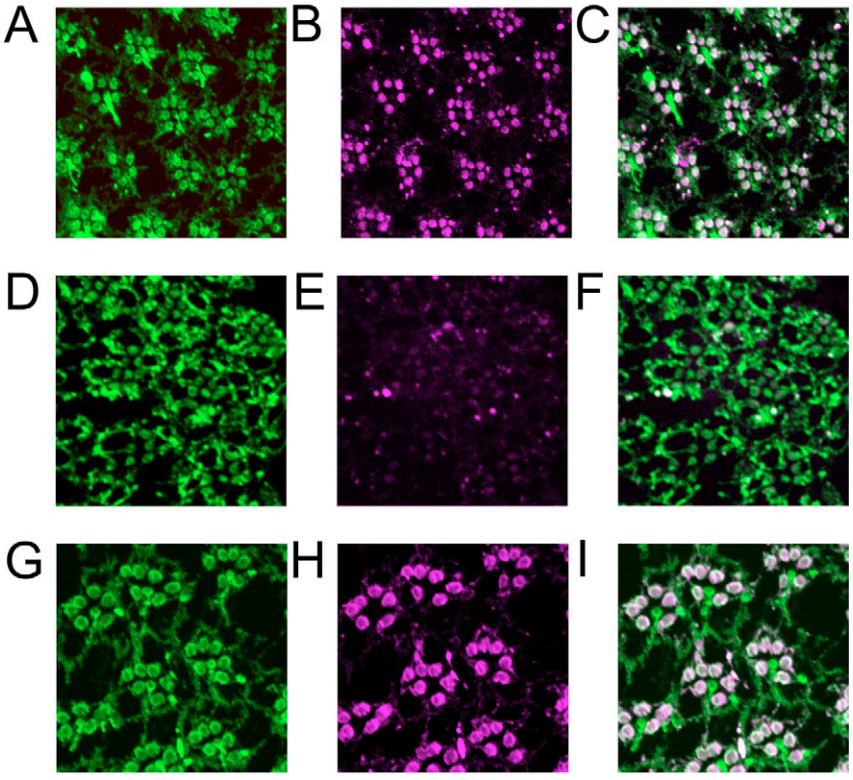

Fig. 1. The AP-2 adaptor complex is necessary for the *norpA*-induced endocytosis of stable rhodopsin-arrestin complexes. All panels show cross sections (0.75 μm) from a frozen *Drosophila* retina stained with both arrestin and rhodopsin antibodies followed by fluorescein- and rhodamine-labeled secondary antibodies respectively. (A-C) Wild-type, (D-F) *norpA* and (G-I) *norpA;α-adaptin*. The flies were dark-reared and then treated with constant room light for 24 hours prior to fixation. Dissected eyes were fixed and sectioned as described in Materials and Methods. A, D and G were stained with arrestin-specific antibodies, B, E and H were stained with rhodopsin-specific antibodies, C, F and I are merged images.

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endocytosis were completely blocked. Interestingly, in *norpA α-adaptin* double mutants, the *norpA*-mediated retinal degeneration is rescued and the photoreceptors exhibit wild-type morphology (Fig. 2D). Therefore, *norpA*-mediated retinal degeneration is rescued by loss-of-function alleles of α-*adaptin*. These data demonstrate that the AP-2 adaptor complex is essential for the endocytosis of Arr2-rhodopsin complexes, and is required for the retinal degeneration observed in *norpA* mutants.

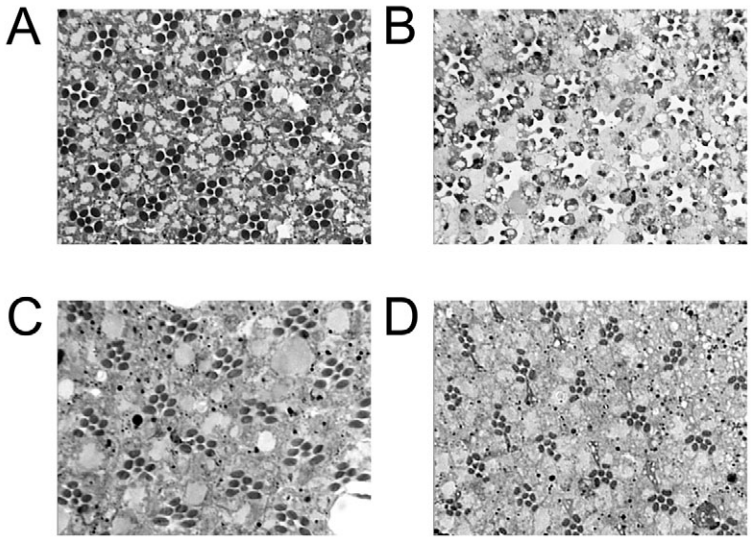

Fig. 2. The AP-2 adaptor complex is necessary for *norpA*-mediated retinal degeneration. All panels show cross sections (0.5 μm) of retinas from (A) wild-type, (B) *norpA*, (C) *α-adaptin* and (D) *norpA;α-adaptin* flies. The flies were dark-reared and then exposed to 5 days of constant room light prior to fixation. Tissue was fixed and embedded as described in Materials and Methods.

Single point mutations in the AP-2 binding domain of arrestin do not disrupt its ability to bind and regulate rhodopsin

Since it is clear that the AP-2 adaptor complex is necessary for *norpA*-mediated retinal degeneration, we were interested in investigating an interaction between Arr2 and AP-2. It has long been known that, unlike the β-arrestins, visual arrestins do not contain a clathrin-interaction domain. However, upon closer examination of the amino acid alignments, it was clear that both the β-arrestins and the visual arrestins contain a consensus sequence for interaction with AP-2 (Fig. 3A). This suggests that visual arrestin and *Drosophila* Arr2 may serve to recruit arrestin-rhodopsin complexes to clathrin-coated pits, through an AP-2 interaction. To investigate the possibility that arrestin interacts with AP-2, we constructed two point mutations in the Arr2 C terminus, $arr2^{K391A}$ and $arr2^{R393A}$ and introduced these mutations into an $arr2^{null}$ background. These two basic residues have previously been shown to be essential for AP-2–β-arrestin interaction (Laporte et al., 2000). The construction of these transgenic flies enabled us to directly test the interaction between Arr2 and AP-2. The 'ear' domain from the β-subunit was expressed in vitro fused in frame with the maltose-binding protein. Arr2 from wild-type flies bound to the β-adaptin fusion protein whereas lysates from $arr2^{K391A}$ and $arr2^{R393A}$ failed to show any β-adaptin binding (Fig. 3B). This demonstrates that *Drosophila* Arr2, either directly or indirectly, binds to the β-subunit of AP-2, and, moreover, the single point mutations completely eliminate the interaction.

Prior to an examination of the role of these amino acids in endocytosis, it was imperative to demonstrate that these mutant proteins are able to regulate rhodopsin in a manner similar to wild-type arrestins. For example, these mutations could disrupt the ability of arrestin to interact with rhodopsin, or it is possible that the variant arrestins will constitutively bind rhodopsin. In order to investigate the role of these mutations on Arr2 function, they were analyzed by biochemical, ultrastructural and electrophysiological methodologies.

To examine the interaction between the mutant arrestins and rhodopsin, we took advantage of a biochemical assay that measures the binding of the two proteins in response to light (Alloway and Dolph, 1999). This assay relies on the fact that the major *Drosophila* opsin, Rh1, can be photoconverted between the inactive and active forms by different wavelengths of light. When Rh1 is converted to the M form by a photon of blue (480 nm) light, the regulatory protein arrestin binds rhodopsin and, therefore, is localized to the membrane fraction. Alternatively, when the M form is converted to the inactive R form by absorbing a photon of orange (580 nm) light, Arr2 is released from rhodopsin and subsequently is found in the soluble fraction. In wild-type flies, all of the Arr2 binds to Rh1 upon blue light stimulation and ~80% is released after orange light treatment (Fig. 3C). Similarly, when either $arr2^{K391A}$ or $arr2^{R393A}$ are exposed to blue light virtually all of the Arr2 is found in the pellet fraction, demonstrating that the amino acid substitutions have not disrupted the ability of arrestin to bind rhodopsin. Moreover, both mutant arrestin proteins are able to release from rhodopsin after orange light stimulus ($arr2^{K391A}$ ~80% release and arr^{R393A} ~50% release). These data demonstrate that the mutations in the AP-2 binding domain do not significantly compromise the ability of Arr2 to bind and release from rhodopsin.

Arrestin function can also be analyzed electrophysiologically with an electroretinogram (ERG). An ERG measures the sum of the light-induced electrical activity of the photoreceptor cells and their downstream neurons. The electroretinogram of wild-type flies shows a stereotypical trace where the photoreceptors rapidly depolarize in response to light, and rapidly repolarize once the light stimulus has terminated (Fig. 3E). The rate-limiting step in the termination of the ERG is determined by the binding of Arr2 to rhodopsin and, therefore, provides a good measure of arrestin function (Ranganathan and Stevens, 1995). The $arr2^{null}$ flies elicit an ERG that is significantly different from wild-type. When flashed with blue activating light $arr2^{null}$ mutant photoreceptor cells rapidly depolarize and remain depolarized during the light stimulus just as in wild type, but once the light stimulus is terminated, the photoreceptor cells repolarize very slowly, taking about four times longer to terminate the response. This is because the activated rhodopsin in $arr2^{null}$ flies remains coupled to the signal transduction pathway until it is decoupled by the much less abundant arrestin, Arr1 (Dolph et al., 1993). The $arr2^{K391A}$ and the $arr2^{R393A}$ flies both display a largely

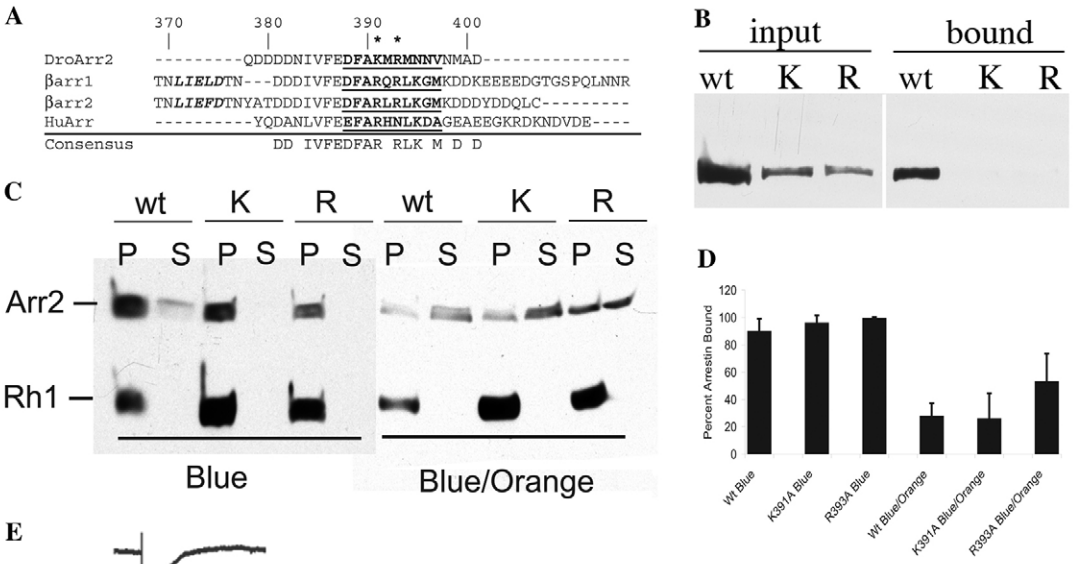

Fig. 3. Point mutations in the AP-2 binding domain of Arr2 do not inhibit the binding of arrestin to rhodopsin or the ability of the mutant arrestins to regulate the phototransduction cascade. (A) An alignment of β and visual arrestins. The 'clathrin box' found in βarr1 and 2 are in bold and italicized. The AP-2 binding domain is in bold and underlined. The two residues that were mutated for this study are marked with an asterisk. (B) Head lysates from wild-type, $arr2^{K391A}$ and $arr2^{R393A}$ flies were generated and incubated with a maltose binding protein–AP-2β Ear domain fusion protein. Lanes 1-3 show represent head equivalents from the lysate. Lanes 4-6 represent Arr2 protein bound to the AP-2β Ear domain. wt, wild-type; K, $arr2^{K391A}$; R, $arr2^{R393A}$. Overexposure of the blot labeled 'bound' failed to show any significant binding of the K or R point mutants (data not shown) (C) Western blot of fractionated fly head lysates. Dark-reared flies were treated with either 2 seconds of blue light or 2 seconds of blue light followed by 4 seconds of orange light. wt, wild-type; K, $arr2^{K391A}$; R, $arr2^{R393A}$. Pellet (P) and supernatant (S) fractions were subjected to SDS-PAGE and western blot analysis. (D) Quantification of the percentage of arrestin bound to rhodopsin-containing membranes. The data shown are the averages of five independent experiments. For the blue light treatment the following percentages of arrestin were bound, wild type 89±9%, $arr2^{K391A}$ 95±5% and $arr2^{R393A}$ 99% ±1%. For the blue/orange light treatment the following percentages of arrestin were bound, wild-type 27±10%, $arr2^{K391A}$ 25±19% and $arr2^{R393A}$ 52±20%. Data are means ± s.d. (E) Electroretinogram analysis of wild-type, $arr2^{5}$, $arr2^{K391A}$ and $arr2^{R393A}$ dark reared newly eclosed flies. The flies were treated with 1 second of 480 nm (blue) light and the total electrical response of the eye was measured.

wild-type electroretinogram, which indicates that the variant arrestins are able to bind and inactivate rhodopsin. The $arr2^{K391A}$ mutant has a trace that is almost identical to that of wild type. The $arr2^{R393A}$ mutant exhibits repolarization kinetics that are much faster than $arr2^{null}$ alleles, but the return to baseline is slower than tat of wild type. However, it is clear that both mutants are able to bind and inactivate photoactivated rhodopsin.

A third method to measure the efficacy of the arrestin variants is to examine retinal morphology after exposure to 5 days of constant light. The wild-type retina retains its highly organized structure after 5 days of constant light and shows very little sign of degeneration (Fig. 4A). However, the retina from $arr2^{null}$ flies shows severe degeneration due to the defect in rhodopsin inactivation. Large intracellular holes have developed and the ommatidial organization is completely lost (Fig. 4B). This clearly contrasts with both the $arr2^{K391A}$ and the $arr2^{R393A}$ mutants. These *arr2* alleles show some degeneration, but it is significantly decreased compared to that of the $arr2^{null}$ allele. (Fig. 4C,D, respectively). Taken together these data indicate that the mutations in the C-terminal AP-2-binding domain of Arr2 do not significantly impair the ability of Arr2 to regulate rhodopsin.

The AP-2 binding domain of arrestin is necessary for the endocytosis of arrestin-rhodopsin complexes in a *norpA* background

Having established that the Arr2 variants serve to regulate rhodopsin in a manner similar to that of wild-type Arr2, we investigated the role of the AP-2 binding domain in *norpA*-mediated retinal degeneration. As previously described, when *norpA* mutants are exposed to 24 hours of constant room light, at least 80% of the rhodopsin is removed from the rhabdomere by endocytosis. Additionally, the majority of Arr2 is also found in the cytoplasm (Fig. 5A). This is in sharp contrast to the almost wholly rhabdomeric localization of

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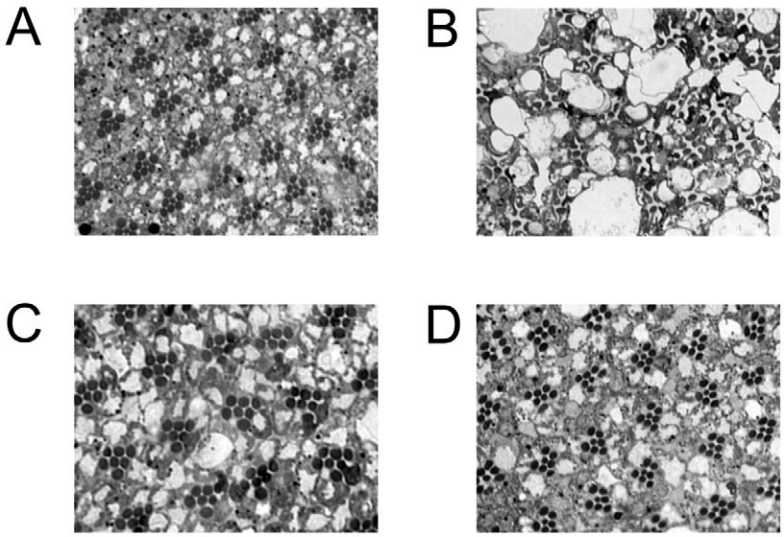

Fig. 4. Point mutations in the AP-2 binding domain of *arrestin* do not cause rapid light-dependent degeneration. All panels are cross sections (0.5 μm) of *Drosophila* retinas from (A) wild-type, (B) $arr2^{null}$, (C) $arr2^{K391A}$ and (D) $arr2^{R393A}$ flies. Dark reared flies were treated with 5 days of constant room light then fixed and sectioned as described in Materials and Methods.

both rhodopsin and Arr2 seen in wild-type flies treated with 24 hours of room light (Fig. 1A). Interestingly, both the $arr2^{K391A}$ and the $arr2^{R393A}$ mutations block the *norpA*-induced endocytosis of rhodopsin-arrestin complexes (Fig. 5F,I). Interestingly there are some cytoplasmic puncta immunoreactive for both arrestin and rhodopsin in the $arr2^{K391A}$ and the $arr2^{R393A}$ background. This may represent leaky endocytosis or internalization of Arr2 and Rh1 by an alternative mechanism. Moreover, another interesting phenomenon exhibited by the $arr2^{R393A}$ allele is the lack of Arr2 staining in the R7 cell. We see this routinely and presently we are unable to provide an explanation for the missing Arr2 in this cell type. These data demonstrate that simply by changing one amino acid that is crucial for the arrestin–AP-2 interaction it is possible to block the *norpA*-induced endocytosis of rhodopsin-Arr2 complexes.

To examine the role of the Arr2–AP-2 interaction in *norpA*-mediated degeneration, we examined the retinal morphology of the Arr2 point mutants in a *norpA* background. After 5 days of light, the wild-type flies exhibit a highly ordered array of ommatidia with no evident degeneration (Fig. 6A). This is in marked contrast to *norpA* flies, which have much smaller rhabdomeres (Fig. 6B). Strikingly, both the $arr2^{K391A}$ and the $arr2^{R393A}$ mutations completely rescue the *norpA*-mediated retinal degeneration (Fig. 6C and D, respectively). Taken together these data add to our knowledge of endocytosis-mediated retinal degeneration. The apoptotic pathway is initiated by the formation of stable arrestin-rhodopsin complexes. These complexes are then recruited to clathrin-coated pits by the interaction of the C terminus of arrestin with AP-2. The massive internalization of rhodopsin finally results in apoptosis of the photoreceptor cells.

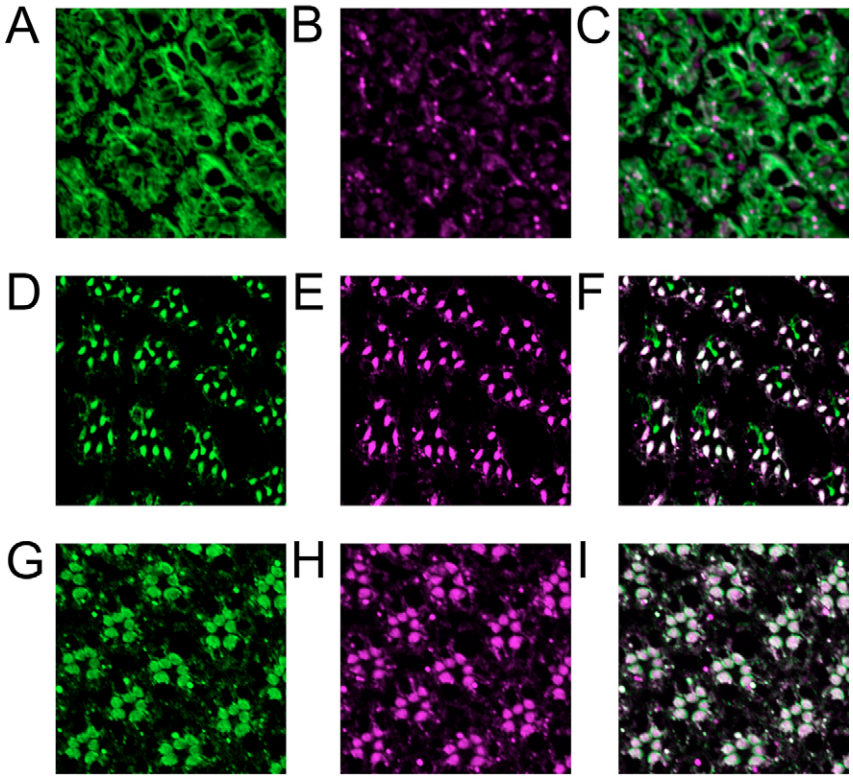

Fig. 5. An intact AP-2 binding domain in arrestin is necessary for *norpA*-induced endocytosis of persistent rhodopsin-arrestin complexes. Cross sections (0.75 μm) from a frozen *Drosophila* retina were stained with both arrestin and rhodopsin antibodies followed by fluorescein and rhodamine-labeled secondary antibodies, respectively. (A-C) *norpA*, (D-F) *norpA;* $arr2^{K391A}$ and (G-I) *norpA;* $arr2^{R393A}$. The flies were dark-reared and then treated with constant room light for 24 hours prior to fixation. Dissected eyes were fixed and sectioned as described in Materials and Methods. A, D and G were stained with arrestin-specific antibodies, B, E and H were stained with rhodopsin-specific antibodies, C, F, and I are merged images.

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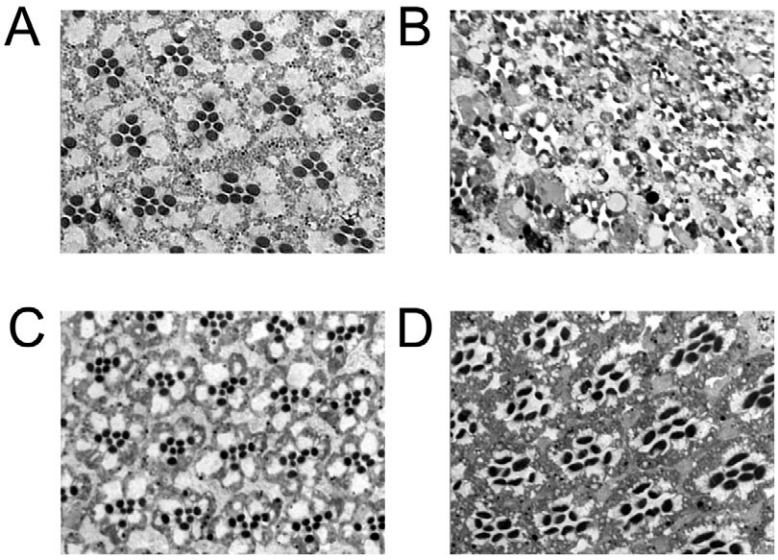

Fig. 6. The interaction between arrestin and AP-2 is required for *norpA*-mediated retinal degeneration. All panels show cross sections (0.5 μm) of *Drosophila* retinas from (A) wild-type, (B) *norpA*, (C) *norpA; arr2*^{K391A} and (D) *norpA; arr2*^{R393A}. Dark-reared flies were treated with 5 days of constant room light then fixed and sectioned as described in Materials and Methods.

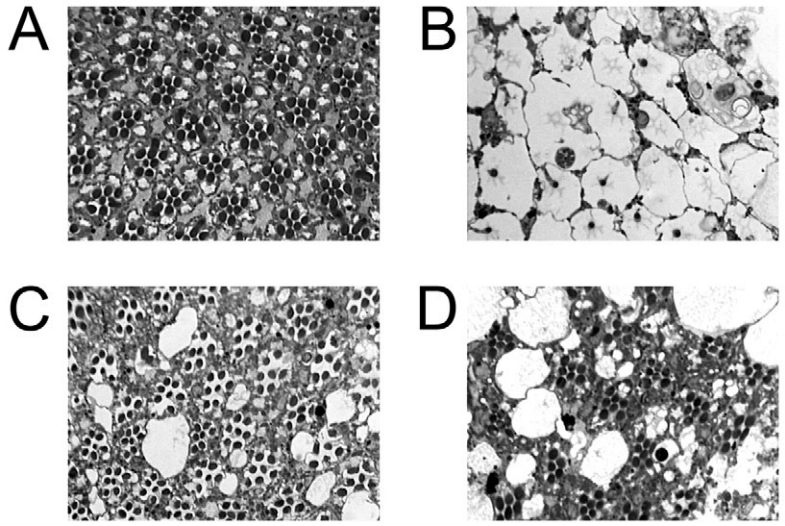

Fig. 7. Mutations in the AP-2 binding domain of arrestin cause slow retinal degeneration. All panels show cross sections (0.5 μm) of *Drosophila* retinas from (A) wild-type, (B) *arr2*^{5}, (C) *arr2*^{K391A} and (D) *arr2*^{R393A} flies. Dark-reared flies were treated for 30 days of 12 hours light/12 hours dark, then fixed and embedded as described in Materials and Methods.

Arrestin-mediated internalization of rhodopsin is essential for photoreceptor cell maintenance

These data suggest that Arr2 plays two roles in wild-type photoreceptors. First, it binds to and uncouples activated rhodopsin from the phototransduction pathway, and second, it suggests that Arr2 also promotes the internalization of rhodopsin through an interaction with AP-2. Therefore, we reasoned that mutating this highly conserved domain should disrupt normal photoreceptor physiology. In order to test this idea further, wild-type, *arr2*^{null}, *arr2*^{K391A} and *arr2*^{R393A} flies were treated with a 12-hour light-dark cycle for 30 days. A cross-section of wild-type photoreceptors after a 30-day treatment shows little, if any, degeneration with a highly ordered array of ommatidia (Fig. 7A). In contrast, the *arr2*^{null} ommatidia show severe and almost complete retinal degeneration, with all except the R7 rhabdomere completely missing (Fig. 7B). Although, the *arr2*^{K391A} and *arr2*^{R393A} mutants exhibited retinal morphology that was largely intact after 5 days of light treatment (Fig. 4C,D), longer treatments under physiological light conditions produce moderate degeneration (Fig. 7C,D). This degeneration is characterized by large intracellular holes and darkened cytoplasm. These data suggest that the internalization of rhodopsin through Arr2-mediated endocytosis is essential to maintain photoreceptor integrity. This defines a new retinal degeneration mechanism in which defective internalization and degradation of rhodopsin results in photoreceptor cell degeneration. Since human visual arrestin also possesses an identical AP-2 interaction motif, it is likely that rod photoreceptors also have an essential function of internalizing rhodopsin through arrestin-mediated endocytosis.

Discussion

In this study our results demonstrate that *Drosophila* Arr2 plays a role as a mediator of rhodopsin endocytosis by interacting with the AP-2 adaptor complex. We have shown that the AP-2 adaptor complex is required for the endocytosis of rhodopsin during certain pathological conditions and that the disruption of this complex rescues *norpA*-mediated retinal degeneration. In addition, we have shown that in flies with a single point mutation in the AP-2 binding domain of Arr2, *norpA*-induced endocytosis of stable rhodopsin-arrestin complexes and the subsequent retinal pathology is blocked. Furthermore, internalization of rhodopsin by an Arr2–AP-2 interaction has been shown to be essential for photoreceptor cell viability.

In previous work it has been demonstrated that certain Arr2 variants bind tightly to rhodopsin (Alloway and Dolph, 1999). This results in the recruitment of the endocytic machinery and cell death via excessive rhodopsin endocytosis. In this study we have also found an Arr2 variant (*arr2*^{R393A}) that binds more tightly to rhodopsin than wild-type Arr2. However, this mutant does not trigger extensive retinal degeneration. This is further evidence for the essential role of the Arr2–AP-2 interaction in receptor internalization. In this Arr2 background complexes are formed between Arr2 and Rh1, but since Arr2 cannot interact with AP-2 they are not internalized and no photoreceptor cell death is induced.

Our data point to an essential role for the endocytosis of rhodopsin through Arr2 in the maintenance of photoreceptor

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cells. Previous work has implicated an essential role for *Drosophila* arrestins in endocytosis; however, these studies used mutations that either blocked all endocytosis in the photoreceptor cell or utilized loss-of-function mutants that could have other effects on the photoreceptor. Photoreceptor cell degeneration can be induced by inhibiting dynamin function with a dominant negative mutation (Acharya et al., 2003) or by blocking AP-2 function (this study). However, it is possible that a global inhibition of endocytosis may halt the internalization of compounds essential for cell viability. Therefore, cell death may be unrelated to defects in the phototransduction cascade or the internalization of rhodopsin. In addition, retinal degeneration in Arr1 mutants may be due to the defect in endocytosis but pleiotropic affects associated with the loss of Arr1 may also contribute to the aberrant retinal morphology. By using Arr2 variants that are unable to internalize rhodopsin, we have selectively blocked the endocytosis of one protein (rhodopsin) while leaving general endocytosis intact. Therefore, the retinal degeneration observed in this study (Fig. 7) is due solely to defects in rhodopsin internalization through its interaction with Arr2.

One interesting question concerns the purpose of the essential role for rhodopsin-Arr2 endocytosis. One possibility is that this may be a mechanism to remove damaged rhodopsin from the cell. If rhodopsin is photochemically damaged in such a way that it becomes constitutively active, it would be deleterious to the cell, and would need to be removed. Presumably constitutively active rhodopsin would form a stable complex with Arr2 and be targeted for endocytosis through the interaction of arrestin with the AP-2 adaptor complex. This would provide a surveillance mechanism for the cell, whereby defective rhodopsin molecules are quickly and efficiently removed. Second, it may be an adaptive mechanism. In high light conditions the amount of activated rhodopsin may exceed the ability of arrestin to quickly decouple the metarhodopsin from the phototransduction pathway. This would lead to a loss of visual temporal resolution and be detrimental to cell viability. However, under high light conditions at any given time a higher percentage of the cellular arrestin will be bound to rhodopsin and increase the likelihood that the Arr2 bound to rhodopsin will interact with AP-2 and drive internalization of rhodopsin. This would serve to lower the concentration of rhodopsin available to activate the phototransduction cascade and thereby reduce sensitivity under conditions of intense illumination.

Materials and Methods

Drosophila stocks

All *Drosophila melanogaster* stocks were in a white (*w*) background to eliminate screening pigments in the eye. The white mutant (w^{1118}) is a spontaneous mutation that has been extensively characterized (Lindsley and Zimm, 1992). The $norpA^{EE5}$ mutation was induced by ethyl methanesulfonate (EMS). The $arr2^{5}$ allele has a stop codon at amino acid 20 and is referred to as $arr2^{null}$ throughout the text (Dolph et al., 1993) The *α-adaptin* (α-$adaptin^{06694}$) mutant is a P-element insertion in the first exon (Gonzalez-Gaitan and Jackle, 1997). The stocks used to produce the eye-specific somatic mosaic clones were modified from previously described stocks (Newsome et al., 2000).

Arrestin 2 constructs

The AP-2-binding domain mutations in *arr2* were constructed using standard PCR techniques. The nucleic acid substitutions were induced in a genomic arrestin clone. Therefore the final clones were driven by the endogenous promoter and contained all genomic introns and exons. The mutant constructs were inserted into modified version of the Y.E.S. vector (Patton et al., 1992) and injected into *yw* fly embryos. The modified version of the Y.E.S. vector was a generous gift from Steven Britt (University of Colorado Health Sciences Center). The y^{+} transformants were then selected and the *arr2* mutant constructs were crossed into an $arr2^{null}$ background.

AP-2β interaction assay

The Ear domain of AP-2β was cloned in frame with the maltose binding protein. Genomic DNA was amplified by the polymerase chain reaction using the following primers: GGGAATTCAAGGGCAAGGGACTCGAAATACAAG and ACTGAG-TGACAAGCAGCTCCGATT. The 900 bp fragment was cloned into the pMAL-c2X vector using *Eco*RI and *Sal*I sites. The resulting fusion protein was induced and purified using amylose resin by standard techniques. A head lysate from 20 flies was incubated with pMAL-βEAR fusion protein bound to amylose beads for 30 minutes at 4°C. The beads were washed three times with column buffer and the samples were boiled for 5 minutes. Western bots were performed using Arr2 polyclonal antisera.

Somatic mosaics

Somatic mosaic clones in the adult eye were made as previously described (Newsome et al., 2000) except that the FLP recombinase was provided by a recombinant chromosome containing both the *norpA* mutation and the *ey*FLP transgene. The *α-adaptin* P element mutation was recombined onto a FRT42 chromosome by standard genetic methods. Eye-specific mosaics were made by crossing *norpA eyFLP;* FRT42 *cl2R11* females with FRT42 *α-adaptin* males. The presence of the clR211 recessive cell lethal allele results in a large portion of the eye being homozygous for the *α-adaptin* mutation in both males and females. In the resulting progeny, the males were used to analyze *norpA; α-adaptin* double mutants, whereas the females were heterozygous for the *norpA* mutation and therefore used to study the effect of the *α-adaptin* mutation in an otherwise wild-type background.

Histology

Heads from light-treated flies were removed, bisected and placed in ice cold 2% glutaraldehyde until all samples were dissected. An equal volume of 2% osmium tetroxide was added to each sample and then incubated at 4°C for 30 minutes on a rotary shaker. The glutaraldehyde/osmium tetroxide mix was removed and replaced with 2% osmium tetroxide, and the samples incubated at 4°C for 1 hour. Samples were dehydrated with increasing concentrations of ice-cold ethanol. Dehydration was followed by two 10-minute incubations at room temperature with propylene oxide. Samples were then incubated overnight with rotation in 50% Durcupan resin/50% propylene oxide (Fluka). The Durcupan propylene oxide mix was removed and replaced with 100% Durcupan and the incubation continued for an additional 4 hours. The samples were cured in molds at 80°C overnight, and 0.5 μm sections were cut with a Sorvall MT-1 ultra-microtome (Sorvall, a Kendro company, Ashville, NC). Tissue was observed with a Zeiss Axiophot (Carl Zeiss MicroImaging) microscope using a 100×/NA 1.3 oil immersion objective. Digital images were collected using an Optronics DEI 750 camera and MetaVue (Universal Imaging) image capturing software. Images were processed using Adobe Photoshop for Windows.

Immunological staining of cross sections of *Drosophila* photoreceptors

Newly eclosed dark-reared flies were either kept in the dark or exposed to 24 hours of constant room light. The eyes were removed and fixed with 4% paraformaldehyde in PBS for 1 hour. Fixed tissue was rinsed once with PBS and then infused with 40% sucrose in PBS overnight at 4°C. The eyes were then frozen and cut into 0.75 μm thick sections using a Sorvall MT5000 ultra microtome with a RMC CR2000 cryo attachment (RMC Products, Tucson AZ). Sections were cut with a sample temperature of –23°C and a knife temperature of –50°C. The sections were treated with a blocking solution of PBS + 1% BSA (PBS-B) for 15 minutes at room temperature and then incubated with antibodies against Arr2 and Rh1 at 4°C overnight. Antibodies were diluted 1:100 in PBS-B. FITC- and TRITC-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used at 1:100 for 4 hours at room temperature. Stained sections were observed with a Leica TCS SP confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany) using a 100×/NA 1.4 oil immersion objective. Rhodopsin (TRITC) is displayed as magenta to be red/green color blind compatible. The co-localization of FITC (green) and TRITC appears white. The anti-Arr2 antibody was a generous gift from Rama Rangananthan (University of Texas, Southwestern). The Rh1 antibody (4C5) developed by David Blest was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. Images were processed using Adobe Photoshop 7 for Windows.

Biochemistry

Arr2 bind and release assays were performed as previously described (Alloway and Dolph, 1999). For the binding assay, five heads were dissected and floated in 30 μl of PBS at room temperature. The heads were then dark adapted for 10 minutes followed by exposure to 2 seconds of blue (480 nm) light. The heads were homogenized in the dark and centrifuged for 5 minutes at 13,000 ***g***. The supernatant

fraction was removed to a fresh tube under very dim red light. Both the pellet and supernatant fractions were then placed on ice until SDS/PAGE and western blot analysis was performed. The release assay followed the same procedure except that the 2 seconds of blue light was followed immediately by 4 seconds of orange (580 nm) light. Blue and orange illumination were produced with a 300-W xenon/mercury lamp (Oriel, Stamford, CT) and 480 nm or 580 nm band pass filters (Oriel), respectively. To quantitate the western blots, the blots were scanned using a UMAX scanner (UMAX Data Systems) and the band density was analyzed using Lab Works (UVP Bioimaging Systems Inc.).

Electroretinogram analysis

Electroretinograms were performed on newly eclosed dark-reared adult flies immobilized on a solid support. The glass electrodes were filled with 0.7% NaCl. The recording electrode was placed onto the eye and the reference electrode was inserted into the thorax. Electrical signals were amplified through a DAM50 amplifier (World Precision Instruments, Sarasota, FL), digitized with a MacAdios ADPO (GW Instruments, Somerville, MA), and viewed with SUPERSCOPE software (GW Instruments). Stimulating light was generated with a 300 W xenon/mercury lamp (Oriel) and attenuated with neutral density filters. Blue and orange light was generated by filtering through 480 nm and 580 nm band-pass filters (Oriel), respectively. All light pulses were 1 second in duration and at 1/100 maximum intensity.

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