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Mechanism of Phosphorylation-Recognition by Visual Arrestin and the Transition of Arrestin into a High Affinity Binding State

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SUMMARY

Arrestin plays an important role in quenching phototransduction via its ability to interact specifically with the phosphorylated light-activated form of the visual receptor rhodopsin (P-Rh*). Previous studies have demonstrated that Arg175 in bovine arrestin is directly involved in the phosphorylation-dependent binding of arrestin to rhodopsin and seems to function as a phosphorylation-sensitive trigger. In this study, we further probed the molecular mechanism of phosphorylation recognition by substituting 19 different amino acids for Arg175. We also assessed the effects of mutagenesis of several other highly conserved residues within the phosphorylation-recognition region (Val170, Leu172, Leu173, Ile174, Val177, and Gln178). The binding of all of these mutants to P-Rh*, light-activated rhodopsin, and truncated rhodopsin, which lacks the carboxyl-terminal phosphorylation sites, was then characterized. Overall, our results suggest that arrestin interaction with the phosphorylated carboxyl-terminal domain of rhodopsin activates two relatively independent changes in arrestin: (a) mobilization of additional binding sites and (b) increased affinity of the phosphorylation-recognition region for the rhodopsin carboxyl-terminal domain. Together, these two mechanisms ensure the exquisite selectivity of arrestin toward P-Rh*. Mutagenesis of residues that play a major role in binding site mobilization and phosphorylation-recognition enabled us to create "constitutively active" (phosphorylation-independent) arrestin mutants that have high affinity for both P-Rh* and light-activated rhodopsin. The introduction of a negative charge in position 175 was particularly effective in this respect. A detailed molecular model of phosphorylation-recognition is proposed.

The visual amplification cascade has long served as an archetype G protein-coupled receptor signaling system. Signal transduction in rod cells is initiated by the photon-induced isomerization of 11-*cis*-retinal covalently attached to Lys296 of rhodopsin. Rhodopsin is thus converted into metarhodopsin II, the active form of the molecule that interacts with the visual G protein transducin. This leads to the activation of transducin, which in turn activates a cGMP phosphodiesterase, resulting in the closing of cGMP-gated sodium channels and hyperpolarization of the rod cell. A major mechanism for quenching the visual transduction cascade is initiated by the rapid light-dependent phosphorylation of rhodopsin by the enzyme rhodopsin kinase, followed by the highly selective binding of the protein arrestin to P-Rh* (1). In this manner, rhodopsin/transducin interactions are blocked and visual signaling is quenched effectively (2, 3).

In an effort to characterize arrestin interaction with rhodopsin, we previously developed a direct binding assay to assess arrestin binding to various forms of rhodopsin (4). Binding studies with wild-type, mutant, and chimeric arrestins have enabled the identification of several functional regions within arrestin (4–6). The amino-terminal half of arrestin (residues 1–191) was found to contain both an activation-recognition domain, which interacts with the regions of rhodopsin that change conformation upon activation, and a phosphorylation-recognition domain, which interacts with the phosphorylated carboxyl terminus of rhodopsin (see Fig. 1) (4–6). These two regions serve as the primary binding sites mediating arrestin/rhodopsin interaction. However, when both of these domains are engaged in interaction with rhodopsin (i.e., when arrestin binds to P-Rh*), arrestin undergoes a conformational rearrangement (7) that results in mobilization of secondary binding sites located within the carboxyl-terminal half of arrestin (5) (see Fig. 1). We hypothesized that this transition is triggered by the simultaneous engagement of both primary binding sites (double-trigger mechanism) and seems to be controlled by intramolecular interactions within arrestin (5, 6, 8–10).

Recently, we attempted to identify the residues involved in interaction with the phosphorylated carboxyl terminus of

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ABBREVIATIONS: Rh*, light-activated rhodopsin; βARK, β-adrenergic receptor kinase; Rh, dark rhodopsin; P-Rh, phosphorylated rhodopsin; P-Rh*, phosphorylated light-activated rhodopsin; PCR, polymerase chain reaction; bp, base pair; 329G-Rh*, truncated rhodopsin.

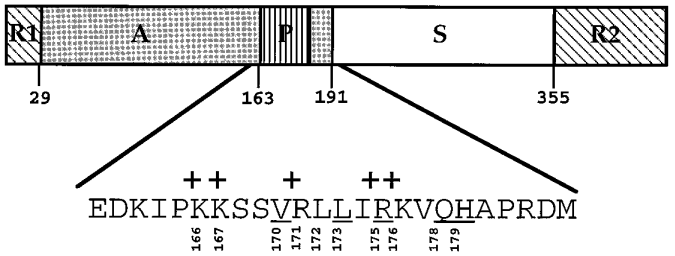

Fig. 1. Functional regions of bovine visual arrestin and the sequence of its phosphorylation-recognition region. Major functional regions are designated as follows: *R1*, basic amino-terminal regulatory region; *R2*, acidic carboxyl-terminal regulatory region; *A*, activation-recognition region; *P*, phosphorylation-recognition region; *S*, region that contains secondary (hydrophobic) binding sites (5, 6, 10). The number of residues approximately corresponding to the borders between functional regions are shown below the schematic. +, Residues involved in the interaction with phosphates on P-Rh; *underlined*, residues that are detrimental to Rh* binding.

rhodopsin by introducing point mutations into a basic region in visual arrestin (residues 163–189) (11). These studies demonstrated that Arg171, Arg175, and Lys176 in bovine arrestin play a primary role in phosphate interaction, whereas Lys166 and Lys167 probably play a minor role in phosphate interaction. In addition, these studies suggested that Arg175 functions as a phosphorylation-sensitive trigger because charge neutralization enabled an R175N mutant arrestin to bind to Rh* as well as wild-type arrestin binds to P-Rh*.

In the present study, we provide a detailed analysis of the critical role of Arg175 in phosphorylation-recognition by characterizing a series of mutant arrestins in which Arg175 has been mutagenized to a different amino acid. The roles of six highly conserved neutral residues in the phosphorylation-recognition domain also are examined by site-directed mutagenesis. Importantly, these studies have led to the generation of a number of "constitutively active" (phosphorylation-independent) arrestin mutants that represent new tools in the assessment of arrestin function. Based on these extensive structure/function analyses, we propose a detailed model of phosphorylation-recognition by visual arrestin.

Experimental Procedures

Materials. [γ-^{32}P]ATP, [^{35}S]dATP, and [^{3}H]leucine were purchased from Dupont-New England Nuclear (Boston, MA). All restriction enzymes were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN), Promega (Madison, WI), or New England Biolabs (Beverly, MA). Sepharose 2B, Sephadex G25, and all other chemicals were from Sigma Chemical (St. Louis, MO). Rabbit reticulocyte lysate and SP6 RNA polymerase were prepared as described previously (4). 11-*cis*-Retinal was supplied generously by Dr. R. K. Crouch (Medical University of South Carolina, Charleston, SC), and other reagents were from sources mentioned previously (4–6).

Plasmid construction and site-directed mutagenesis. A bovine visual arrestin cDNA (12) was generously supplied by Dr. T. Shinohara (National Institutes of Health, Bethesda, MD). An arrestin construct containing the wild-type amino terminus was constructed as described (5, 9) in the plasmid pARR. This pGEM2-based plasmid contains the bovine wild-type arrestin open reading frame with an "idealized" 5′-untranslated region (5) under control of the SP6 promoter. The previously generated mutant ARR-R175N and plasmid pARR-VSP (11), which has silent mutations creating the unique restriction sites *Bcl*I (codons 173–175), *Sph*I (codons 178–180), and *Sac*II (codons 181–182), were used in these studies. Mutagenesis of residue 175 was performed by PCR using *Taq* DNA polymerase and pARR-VSP as a template. Three degenerate antisense primers with the general sequence 5′-cat atc ccg cgg cgc atg ctg tac ctt xxx gat cag cag acg-3′ [where xxx (anticodon 175) was either g(a/c/g/t)(a/c/g/t) (I), c(a/c/t)(a/c/g/t) (II), or t(a/g/t)t (III)] were used with a common-sense primer 5′-ag agc ctg atc aag aag ctg-3′ (codons 105–111). The amplified 239-bp fragments were purified by electrophoresis on low melting agarose, digested with *Sal*I and *Sac*II, and subcloned into *Sal*I/*Sac*II digested pARR-VSP. Screening of multiple PCR products identified clones encoding all possible amino acid replacements for R175 (codons shown in parentheses): Q(cag), M(atg), E(gag), L(ttg), G(ggg), W(tgg) (primer I); S(tcc), A(gcc), V(gtc), F(ttc), Y(tac), P(ccc), H(cac), C(tgc), D(gac) (primer II); and T(aca), I(ata), K(aaa) (primer III).

To create the point mutants V170A, L172A, L173A, I174A, V177A, and Q178A, pairs of mutant oligonucleotides corresponding to the *Sac*I-*Bcl*I (codons 168–174) and *Bcl*I-*Sph*I (codons 173–180) regions were synthesized, annealed, phosphorylated, and subcloned into *Sac*I/*Sph*I-digested pVSP (11), which contains the same silent mutations found in pARR-VSP. Subsequently, 320-bp *Sal*I-*Xho*I fragments of each mutation were subcloned into *Sal*I/*Xho*I-digested pARR and resequenced.

The arrestin mutants (I) R175N/H179Q, (II) V170A/L173A, (III) V170A/L173A/Q178A, (IV) V170A/L173A/Q178A/H179Q, (V) V170A/R175N/H179Q, (VI) V170A/R175E, (VII) V170A/L173A/R175E, and (VIII) V170A/L173A/R175E/H179Q were created using PCR amplification of pARR-VSP (for R175N/H179Q) or pV170A (for all others) template DNA using the appropriate mutant antisense primer (corresponding to codons 171–184) and a common-sense primer (corresponding to codons 105–111). The amplified 239-bp fragments were purified (Wizard PCR Preps; Promega), digested with *Sal*I and *Sac*II, and subcloned into *Sal*I/*Sac*II digested pARR-VSP. The complete sequences of the PCR-generated portions of all of the above constructs were confirmed by dideoxy sequencing in both directions.

***In vitro* transcription and translation.** Plasmids were linearized with *Hin*dIII before *in vitro* transcription using SP6 polymerase to obtain full-length mRNAs. For truncated mRNAs, plasmids were linearized with *Sty*I or *Stu*I to obtain mRNAs encoding ARR(1–365) or ARR(1–191) species as described previously (4, 5). *In vitro* transcription and translation were performed as described previously (4, 5). All arrestin proteins were labeled by incorporation of [^{3}H]leucine with specific activity 20–40 Ci/mmol, resulting in specific activities of full-length and truncated arrestin proteins of 400–600 Ci/mmol (890–1300 dpm/fmol). All *in vitro* translated arrestin mutants used in this study migrated as single, labeled protein bands of the expected mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, mutants with either bulky hydrophobic residues (methionine, leucine, valine, phenylalanine, isoleucine, and, to a lesser extent, tryptophan and tyrosine) or cysteine in position 175 all demonstrated a slightly faster mobility than wild-type arrestin. Because this altered mobility also was observed in the corresponding truncated arrestin(1–365) and arrestin(1–191) forms of these mutants, it cannot be attributed to premature termination or proteolysis and most likely reflects an increased sodium dodecyl sulfate-binding capability of these proteins (data not shown).

Rhodopsin preparations. Urea-treated rod outer segment membranes were prepared, phosphorylated with βARK, and regenerated with 11-*cis*-retinal as described (5). The stoichiometry of phosphorylation for the rhodopsin preparations used in these studies was 5.2 mol phosphate/mol rhodopsin. The binding of wild-type and several of the mutant arrestins also was studied at a rhodopsin phosphorylation level of 2.2 mol phosphate/mol rhodopsin (data not shown). No significant differences in arrestin binding to rhodopsin preparations with phosphorylation levels from 2.2 to 6.8 mol/mol were observed (data not shown; see Refs. 5, 9–11). Recent studies have demonstrated that rhodopsin kinase, βARK1 and 2, and GRK5 preferentially phosphorylate Ser338 and Ser343 on rhodopsin

(Ser338~Ser343 for rhodopsin kinase; Ser338>Ser343 for GRK5; Ser343>Ser338 for βARK1 and 2) (13). This suggests that at phosphorylation levels of 2 mol/mol or higher, both Ser338 and Ser343 are phosphorylated regardless of the kinase used. 329G-Rh*, in which the portion of the carboxyl terminus containing all of the rhodopsin kinase phosphorylation sites was removed proteolytically, was prepared as described (14).

Arrestin binding to rhodopsin. The basis of the arrestin/receptor binding assay involves assessing a change in the mobility of arrestin on a gel filtration column upon binding to the receptor. To study binding to rhodopsin, the *in vitro* translated tritiated arrestins were incubated in 50 mM Tris·HCl, pH 7.5, 0.5 mM $MgCl_2$, 1.5 mM dithiothreitol, 50 mM potassium acetate with 7.5 pmol of the various functional forms of rhodopsin in a final volume of 50 μl for 5 min at 37° either in the dark or in room light. The samples were cooled immediately on ice and, under dim red light, were loaded onto 2 ml Sepharose 2B columns equilibrated with 20 mM Tris·HCl, pH 7.5, 2 mM EDTA. Bound arrestin eluted with the rod outer segments in the void volume (between 0.5 and 1.1 ml). In all experiments, the nonspecific binding, determined in the presence of 0.3 μg of control liposomes, was subtracted. Nonspecific binding was ≤10% of the total binding for the full-length arrestins and ≤25% for the truncated arrestins. This low nonspecific binding amounted to less than 0.5% of the total arrestin present in any particular assay.

Data analysis. Correlation analysis of the binding of arrestins with 20 different residues in position 175 to different functional forms of rhodopsin (P-Rh*, Rh*, and 329G-Rh*) was performed on SuperANOVA-Macintosh (Abacus Concepts, Berkeley, CA).

Results and Discussion

The role of Arg175 as a phosphorylation-sensitive trigger in arrestin. Previous mutagenesis studies have demonstrated that Arg175 is one of the three major residues involved in arrestin interaction with the phosphorylated carboxyl-terminal domain of rhodopsin (11). Arg175 also seems to be involved in an intramolecular interaction that controls the transition of arrestin into a high affinity binding state (11), thus making this residue a likely candidate to serve as a phosphorylation-sensitive switch in arrestin. To probe the nature of the interactions involving Arg175, as well as the mechanism of this putative intramolecular switch, we created a series of arrestin mutants containing all possible amino acid substitutions in position 175. These mutants were then expressed by *in vitro* translation and their binding to P-Rh*, Rh*, and 329G-Rh* was compared.

The binding of these mutants to P-Rh*, which presumably reflects the ability of the mutant proteins to interact with the phosphorylated carboxyl terminus and to assume a high affinity binding conformation, does not follow any uniform pattern as far as the size and chemical nature of the residue in position 175 is concerned (Fig. 2A). The binding is highest (>30 fmol) when histidine, phenylalanine, tyrosine, glutamine, asparagine, cysteine, threonine, or aspartate occupy this position; intermediate (22–28 fmol) with valine, alanine, methionine, tryptophan, serine, or glutamate; similar to the wild-type level (18 fmol) with glycine, isoleucine, leucine, or proline; and lowest (11 fmol) with lysine. Interestingly, all substitutions except the most conservative R175K either do not change arrestin binding or, in some cases, increase binding up to 2-fold (e.g., tyrosine and threonine).

Arrestin binding to Rh* probably reflects predominantly its propensity to assume a high affinity binding conformation due to engagement of the activation-recognition site alone.

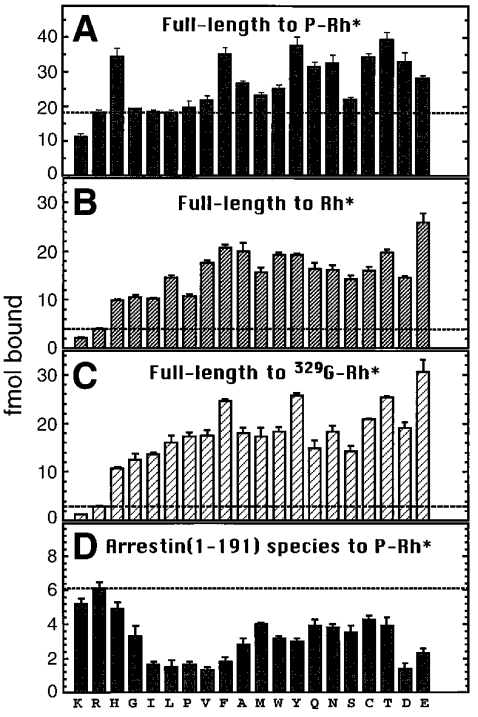

Fig. 2. Effects of mutation of residue 175 on full-length arrestin binding to P-Rh* (A), Rh* (B), and 329G-Rh* (C), and on truncated arrestin(1–191) to P-Rh* (D). The various functional forms of rhodopsin (150 nM) were incubated with the indicated arrestin mutants (2 nM, specific activities 897 to 963 dpm/fmol for full length, 365 to 417 dpm/fmol for arrestin(1–191)) at 37° for 5 min in a total volume of 50 μl. Samples were then cooled on ice, and bound and free arrestin was separated by Sepharose 2B chromatography as described in Experimental Procedures. *Dotted line*, the level of wild-type arrestin binding, which was: full-length arrestin to P-Rh*, 18.1 ± 0.9 fmol; to Rh*, 3.9 ± 0.2 fmol; and to 329G-Rh*, 2.9 ± 0.2; arrestin(1–191) to P-Rh*, 6.1 ± 0.4 fmol. The binding of all arrestin proteins was activation-dependent (data not shown). The binding to the corresponding forms of rhodopsin in the dark was 5- to 10-fold (P-Rh), 11- to 37-fold (Rh), and 12- to 44-fold (329G-Rh*) lower than under illumination for full-length proteins. For all arrestin(1–191) forms, including that with wild-type sequence, the binding to dark P-Rh was about 1.5-fold lower than to P-Rh*, because this binding predominantly reflects interaction with the phosphorylated rhodopsin carboxyl terminus (4–6, 11). Complete selectivity profiles of full-length and truncated forms of wild-type, arrestin(R175E), and arrestin(R175K) mutants are presented in Fig. 3. Mean ± standard deviation from two to three experiments each performed in duplicate are shown.

This binding again is inhibited by only one mutation, R175K, whereas all other substitutions result in a significant stimulation of binding: 5- to 7-fold with phenylalanine, alanine, tryptophan, tyrosine, threonine, or glutamate; >4-fold with valine, methionine, glutamine, asparagine, or cysteine; and >2.5-fold with histidine, glycine, isoleucine, leucine, proline, serine, or aspartate (Fig. 2B).

Because previous studies have suggested that arrestin can interact weakly with the unphosphorylated rhodopsin carboxyl terminus (5, 6), we also studied binding of the various mutants to 329G-Rh*, a truncated form of rhodopsin that lacks the carboxyl-terminal sites of phosphorylation (Fig. 2C). For these studies, we expected the binding to reflect only the ability of arrestin to undergo transition into a high affinity binding state upon engagement of the activation-recognition site, because interaction with the rhodopsin carboxyl terminus is precluded. Thus, if an intramolecular interaction

of Arg175 is involved in maintaining arrestin in a low affinity binding conformation, we would predict that amino acid substitutions that result in a weaker intramolecular interaction would lead to higher arrestin binding. Indeed, the presence of a positive charge in position 175 seems to keep arrestin in a low affinity state (the binding to 329G-Rh* follows the pattern Lys<Arg<His), whereas a negative charge in position 175 promotes its transition into a high affinity binding state (R175E has the highest binding to 329G-Rh*). However, the order of potency of other mutants suggests that additional characteristics of residue 175 are also important. Phenylalanine, tyrosine, cysteine, or threonine in this position seem more detrimental for the intramolecular interaction (and therefore favorable for the binding) than aspartate, whereas glycine, isoleucine, leucine, serine, and glutamine partially support the intramolecular interaction, yielding arrestins with a more modest ability to bind to 329G-Rh*.

The order of potency of the various mutants toward 329G-Rh* also gives some insight into the chemical nature of the putative intramolecular interaction involving Arg175. Both arginine and lysine, with positive charges on long side chains, seem to keep arrestin in a low affinity binding state. In contrast, histidine promotes 4- to 5-fold increased binding, suggesting that the negatively charged residue presumably involved in interaction with Arg175 is not readily accessible to this side chain. Moreover, the relatively low binding levels of the R175S and R175Q mutant arrestins suggest that these residues might be able to participate (probably via hydrogen bonding) in the intramolecular interaction. The ~2-fold difference between R175S and R175T binding to 329G-Rh*, as well as less dramatic but significant differences between R175Q and R175N binding, suggest that the exact positioning of the interacting group on the side chain is very important for this interaction. The low to moderate binding of all but one arrestin mutant containing a bulky hydrophobic residue (most notably R175I and R175L) suggests that hydrophobic interactions also may be involved.

These data suggest that Arg175 may play an important role in supporting the conformation of arrestin and, therefore, may be crucial in folding. To ascertain that the differences in binding levels reflect affinity differences of the Arg175 mutants rather than the proportion of the protein that folds correctly, we determined the percentage of functional arrestin in the *in vitro* translated wild-type, R175E, and R175K preparations. To this end, we measured binding of the different arrestins (present at 100 fmol/assay) as a function of P-Rh* concentration (varied from 0.0375 to 4.8 μg/assay). In each case, >85% of the arrestin added was bound to P-Rh* at the highest concentration tested. However, the rhodopsin concentration necessary to bind 50% of the added arrestin was different (1.2, 0.6, and 2.4 μg/assay for wild-type, R175E, and R175K arrestin, respectively). These data demonstrate that >85% of the wild-type and mutant arrestins fold correctly and that the observed differences in binding at relatively low rhodopsin concentrations (e.g., the 0.3 μg/assay we routinely use) probably reflects different affinities of the wild-type and mutant arrestins for rhodopsin.

To more directly assess the role of residue 175 in the interaction with the phosphorylated carboxyl terminus of rhodopsin, we also produced truncated arrestin(1–191) versions of these mutants and studied their binding to P-Rh* (Fig. 2D). Truncated arrestin(1–191) has been shown previously to retain both activation- and phosphorylation-recognition sites while lacking the secondary binding site (4–6). As a result, this truncated form of arrestin does not display the complicated sequential multisite binding characteristics of full-length arrestin (4–6), thus simplifying interpretation of differences in binding levels. The data shown in Fig. 2D support the hypothesis that residue 175 interacts with a phosphate because the binding is highest when a positively charged residue is in this position (Arg>Lys~His). Neutral residues capable of hydrogen bonding (methionine, glutamine, asparagine, serine, cysteine, threonine) yield intermediate binding levels, whereas the presence of negatively charged (aspartate, glutamine) or bulky hydrophobic (isoleucine, leucine, proline, valine, phenylalanine) residues impair binding. Residues with small side chains (glycine, alanine) and bulky residues capable of hydrogen bonding (tryptophan, tyrosine) yield binding levels intermediate between the second and third groups (Fig. 2D).

Although Arg175 mutations do not affect arrestin(1–191) binding to Rh* (Ref. 11 and data not shown), the binding of these mutants to P-Rh* varies >4-fold (1.3 to 6.1 fmol) (Fig. 2D). This supports our previous conclusion that residue 175 plays an important role in phosphate interaction (11). However, full-length arrestin binding to 329G-Rh* varies >20-fold (1.3 to 30.6 fmol) (Fig. 2C), strongly suggesting that Arg175 also plays a key role in controlling the transition of arrestin into a high affinity binding state. In an effort to distinguish the relative contribution of Arg175 to phosphate-recognition versus high affinity binding, we compared all of our Arg175 mutant binding data by regression analysis. We found no correlation between full-length and arrestin(1–191) binding to P-Rh* ($r = 0.015$, $F(1,18) = 0.04$, $p = 0.951$). In contrast, comparison of full-length Arg175 mutant arrestins binding to P-Rh* and 329G-Rh* revealed a significant correlation ($r = 0.682$, $F(1,18) = 15.693$, $p = 0.0009$), whereas full-length mutant arrestin binding to Rh* and 329G-Rh* had the highest correlation ($r = 0.926$, $F(1,18) = 108.093$, $p = 0.0001$). This suggests that the role of residue 175 in triggering the transition of arrestin into a high affinity binding state is far more important for P-Rh* binding than its direct interaction with phosphates on the rhodopsin carboxyl terminus. Indeed, the observation that a synthetic phosphopeptide comprising the fully phosphorylated carboxyl-terminal region of bovine rhodopsin induces arrestin binding to Rh* and 329G-Rh* supports this notion (15).

One possible mechanism whereby Arg175 could serve as a phosphorylation-sensitive trigger would involve its interaction with a negatively charged residue within the carboxyl-terminal half of arrestin. This constraining intramolecular interaction would serve to maintain arrestin in a low-affinity binding state. When arrestin interacts with phosphorylated rhodopsin, the positively charged residues in the phosphorylation-recognition region (primarily Arg171, Arg175, and Lys176) come in contact with phosphoserines on rhodopsin. This interaction neutralizes the positive charge of Arg175, thus disrupting the intramolecular interaction, releasing the constraint, and allowing the transition of arrestin into a high affinity binding state.

In an effort to better localize potential residue(s) on arrestin involved in intramolecular interaction with Arg175, we compared the binding of wild-type, R175E, and R175K ar-

restins that were truncated at residue 365, thus removing the highly negatively charged carboxyl terminus of arrestin. This truncated form of arrestin is very similar to a recently identified splice variant of arrestin (termed p44) that has the carboxyl-terminal 35 residues replaced by a single alanine (16, 17). Binding of the R175E mutant to P-Rh* was reduced ~25%, compared with that of wild-type and R175K arrestin(1–365) (Fig. 3). Interestingly, the binding of both R175E and R175K arrestins to Rh* was modestly reduced compared with that of wild-type arrestin(1–365) (Fig. 3). These results are in striking contrast to the 7-fold increased binding of full-length R175E arrestin and the 2-fold decreased binding of R175K compared with wild-type arrestin (Fig. 3). Thus, the deletion of 39 residues from the carboxyl terminus eliminates both the strong stimulatory effect of R175E mutation and the strong inhibitory effect of R175K mutation on the interaction with both Rh* and P-Rh* (Fig. 3). These results suggest that the residue(s) involved in the intramolecular interaction with Arg175 most likely lies within the carboxyl-terminal 39 amino acids of arrestin. It is interesting to note that the effects of R175E and R175K mutation in arrestin(1–365) qualitatively resemble the effects of these mutations in arrestin(1–191) (i.e., virtually no effect of R175K and an inhibitory effect of R175E; see Fig. 3, compare B with C). This is in sharp contrast to the effects of R175E and R175K in the full-length protein, in which potent stimulatory effects of R175E and inhibitory effects of R175K mutation are observed (see Fig. 3, compare A with B and C).

Thus, the introduction of a negative charge within the arrestin phosphorylation-recognition region promotes its high affinity binding to Rh*. Interestingly, there are one or

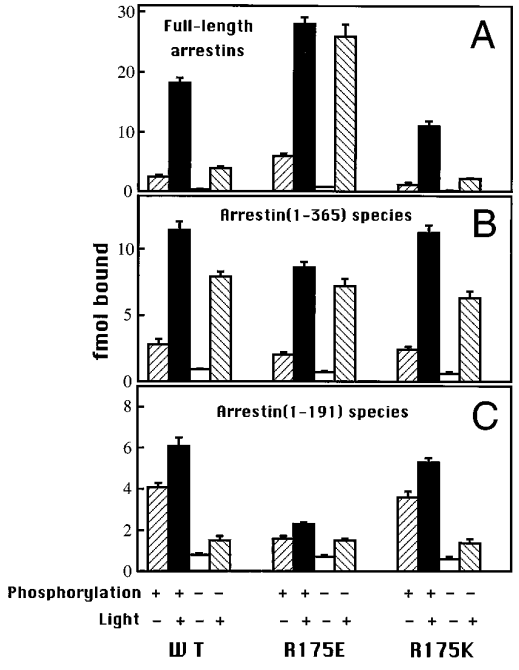

Fig. 3. Effects of mutations R175E and R175K on selectivity of full-length and truncated arrestin binding to rhodopsin. The indicated functional forms of rhodopsin (150 nM) were incubated with the wild-type (WT) or mutant full-length (A), truncated (1–365) (B), or (1–191) (C) arrestins (2 nM) at 37° for 5 min in a final volume of 50 μl. Samples were then treated as described in Experimental Procedures. Mean ± standard deviation from two experiments each performed in duplicate are shown.

two serine residues within the phosphorylation-recognition regions of all arrestins sequenced thus far (e.g., S168 and S169 in visual arrestin) (11). Conceivably, the phosphorylation of any of these serines *in vivo* potentially may serve as a mechanism for switching arrestin-receptor interaction from phosphorylation-dependent to phosphorylation-independent. This potential mechanism of arrestin regulation probably does not occur with the *Drosophila melanogaster* visual arrestins, because phosphorylation occurs at residue Ser366 (18–22). However, we believe that this hypothesis deserves experimental evaluation in the case of vertebrate arrestins, even though the phosphorylation of these proteins has not been reported to date.

It is worth noting that interpretation of results from any mutagenesis study is complicated by the possibility of global folding or conformational perturbances of the molecule due to mutation. Several lines of evidence suggest that this is not the case for any of the R175 mutants we have tested. In our experience, there are several indicators of folding problems in arrestin mutants (4–6, 9–11). First, the yield of such mutants from the rabbit reticulocyte lysate *in vitro* translation system typically are much lower than that of wild-type arrestin. This is due to the ability of heat-shock proteases, which are abundant in the lysate, to effectively degrade misfolded proteins. The yields of all mutants was 80 to 110% of the wild-type arrestin. Second, the undegraded portions of misfolded proteins often aggregate and therefore pellet after high-speed centrifugation. We found that >90% of wild-type arrestin and all R175 mutants remain in the supernatant after centrifugation at 356,000 × *g* for 60 min, routinely performed after *in vitro* translation (4–6). Finally, misfolded arrestins demonstrate similar binding to all functional forms of rhodopsin and opsin and very high nonspecific binding to liposomes. In contrast, all of the Arg175 mutants demonstrated specific light-dependent binding to phosphorylated, unphosphorylated, and 329G-Rh* and a very low level of nonspecific binding similar to that observed for wild-type arrestin. Therefore, we believe that mutation of Arg175 does not significantly change the overall conformation of the arrestin molecule but rather promotes its transition into a high affinity rhodopsin-binding state triggered by its interaction with any form of light-activated rhodopsin.

Functional role of neutral residues in the phosphorylation-recognition region. Within the core of the arrestin phosphorylation-recognition region, there are six neutral residues that are highly conserved throughout the arrestin family (Fig. 1): Val170, Leu172, Leu173, Ile174, Val177, and Gln178. To probe the functional role of these residues, we created six point mutants in which alanine was substituted for each of these bulky residues and then analyzed the binding of these mutants to various functional forms of rhodopsin (Fig. 4).

Only V170A was found to significantly increase arrestin binding to P-Rh* and P-Rh while three mutations increase the binding to Rh* (the order of potency being V170A>L173A>Q178A) and L173A modestly increases binding to Rh. In contrast, mutation L172A decreases arrestin binding to all functional forms of rhodopsin. Mutations I174A and V177A have no effect on P-Rh* binding while decreasing binding to P-Rh and Rh. These data suggest that several of the neutral residues within the phosphorylation-recognition region may have a role in rhodopsin interaction.

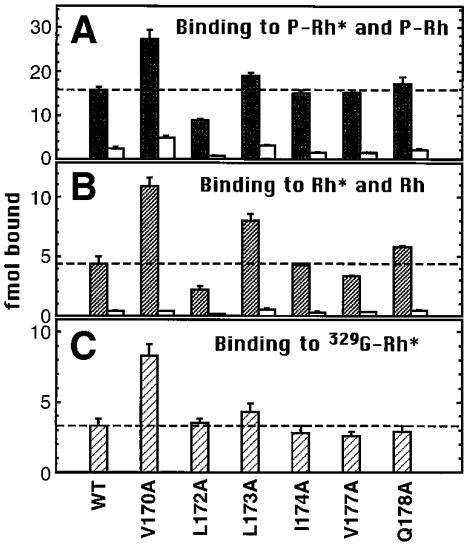

Fig. 4. Effect of mutagenesis of neutral residues in the phosphorylation-recognition region on arrestin binding to light-activated (*shaded bars*) or dark (*open bars*) P-Rh (A), Rh (B), and 329G-Rh* (C). Rhodopsin (150 nM) was incubated with the indicated arrestin mutants (2 nM, specific activities 875 to 914 dpm/fmol) at 37° for 5 min in a final volume of 50 μl. Samples were then treated as described in Experimental Procedures. *Dotted line*, level of wild-type arrestin binding, which was: to P-Rh*, 15.8 ± 0.6 fmol; to dark P-Rh, 2.4 ± 0.2 fmol; to Rh*, 4.4 ± 0.6 fmol; to Rh, 0.40 ± 0.06 fmol; and to 329G-Rh*, 3.3 ± 0.5 fmol. Mean ± standard deviation from two experiments each performed in duplicate are shown.

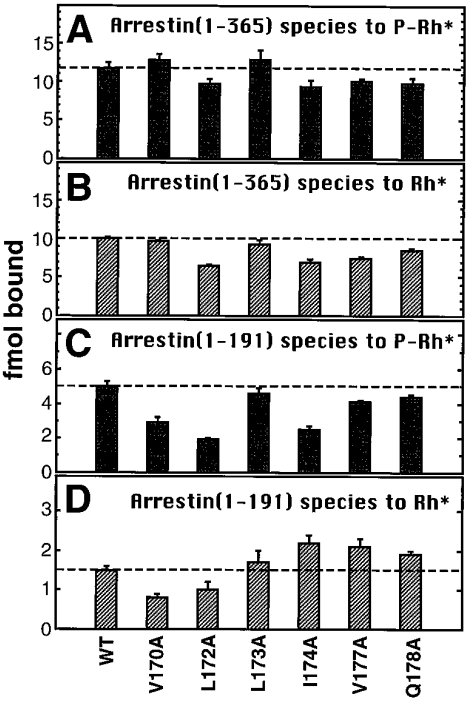

Fig. 5. Effect of mutagenesis of neutral residues in phosphorylation-recognition region on truncated arrestin(1–365) (A, B) and arrestin(1–191) (C, D) binding to P-Rh* (A, C) and Rh* (B, D). The various functional forms of rhodopsin (150 nM) were incubated with the respective arrestins (2 nM) at 37° for 5 min. Samples were then treated as described in Experimental Procedures. *Dotted line*, level of wild-type arrestin binding, which was as follows: arrestin(1–365) to P-Rh*, 11.7 ± 0.8 fmol; arrestin(1–365) to Rh*, 10.0 ± 0.2 fmol; arrestin(1–191) to P-Rh*, 5.0 ± 0.3 fmol; arrestin(1–191) to Rh*, 1.5 ± 0.1 fmol. The binding of all arrestin proteins was activation-dependent (see Fig. 3 and legend of Fig. 2). Mean ± standard deviation from two experiments each performed in duplicate are shown.

To assess whether this role involves direct interaction with the rhodopsin carboxyl terminus, we tested the effects of these mutations on arrestin binding to 329G-Rh* (Fig. 4). Comparison of binding to 329G-Rh* versus Rh* reveals that the stimulatory effect of the V170A mutation is retained fully, whereas the effect of L173A and Q178A is reduced substantially. In addition, the potent inhibitory effect of the Leu172 mutation is lost completely.

To test whether any of these neutral residues are involved in an intramolecular interaction with the carboxyl terminus of arrestin (5, 8, 11), we also characterized binding of the truncated arrestin(1–365) versions of these mutants (Fig. 5). All stimulatory effects of mutations V170A, L173A, and Q178A are eliminated completely by the truncation of arrestin, whereas the inhibitory effect of L172A and V177A on Rh* binding is retained. This suggests that interaction with the arrestin carboxyl terminus may play a role in the stimulatory effects observed with the full-length proteins.

To study the direct role of these residues in P-Rh* and Rh* binding, we also characterized the binding of the respective arrestin(1–191) proteins (Fig. 5). In this case, V170A and L172A mutations substantially inhibit binding, suggesting that these residues play a direct role in rhodopsin interaction. L173A has no appreciable effect, although the other three mutations decrease binding to P-Rh* while increasing the binding to Rh* (Fig. 5).

Mutagenesis of Arg175 and His179, which increases arrestin binding to unphosphorylated rhodopsin (Fig. 2, see also Ref. 11), facilitates the transition of arrestin into a high affinity binding state by mobilizing secondary hydrophobic binding sites for Rh* interaction. This is accompanied by a dramatic decrease in salt sensitivity of arrestin-Rh* interaction. To determine whether the V170A and L173A mutations exert their effect via a similar mechanism, we tested the salt sensitivity of Rh* binding for the V170A and L173A mutants as well as for the double mutant. No significant changes in the salt sensitivity of these mutants were observed when compared with that exhibited by wild-type arrestin (data not shown). This suggests that these mutations probably increase arrestin binding to Rh*, not via an enhanced mobilization of secondary hydrophobic binding sites, but via removal of the hindrance for Rh* interaction and an increase in the affinity of the phosphorylation-recognition site for unphosphorylated rhodopsin. Such a mechanism also explains the relatively moderate effects of these mutations (compare Figs. 2, 4, and 5). Conceivably, Val170 and Leu173 (as well as Gln178) normally hinder the interaction with Rh*. However, the binding of Arg171, Arg175, and Lys176 to phosphates on rhodopsin probably would change the overall conformation of the phosphorylation-recognition region, and potentially move Leu173 and Gln178 out of the way and Val170 into a position favorable for interaction. The hindrance of arrestin interaction with Rh* by Val170 does not seem to depend on the presence of the rhodopsin carboxyl terminus since the same stimulation by the V170A mutation is observed for arrestin binding to Rh* and 329G-Rh*. Thus, Val170 interacts with a domain of rhodopsin other than the carboxyl-terminal 19 amino acids. Conversely, the L173A and Q178A mutations lose their stimulatory effect with 329G-Rh*, suggesting that Leu173 and Gln178 prevent the interaction of other arrestin

residues with the portion of unphosphorylated rhodopsin carboxyl terminus that is removed in 329G-Rh*. As a result, the binding of these mutants to 329G-Rh* is only ~50% of that observed for Rh*.

Leu172 seems to take part in direct interaction with rhodopsin, because the L172A mutation decreases binding of full-length and truncated arrestins to all forms of rhodopsin. This interaction may occur with the rhodopsin carboxyl terminus, because the L172A mutation fails to inhibit arrestin binding to 329G-Rh*. Ile174 and Val177 may play a supportive role in arrestin interaction with phosphorylated rhodopsin because full-length arrestin binding to P-Rh and arrestin(1–191) binding to P-Rh* are decreased by the I174A and V177A mutations. These residues also seem to slightly hinder the interaction with Rh*. Because the Q178A mutation stimulates both full-length and truncated arrestin binding to Rh*, Gln178 may play a role in impeding binding to Rh*. This effect is dependent on the presence of the rhodopsin carboxyl terminus because no stimulatory effect of Q178A mutation on 329G-Rh* binding is apparent.

Mechanisms involved in reducing phosphorylation sensitivity of visual arrestin. To further explore the mechanisms involved in the increased ability of various arrestin mutants to bind to Rh*, we studied the effects of several combinations of mutations. These mutations either facilitate the transition of arrestin into a high affinity binding state by mobilization of the secondary binding site (e.g., R175N, R175E, and H179Q) or increase arrestin binding to Rh* by removing the direct hindrance of the primary binding site interaction (e.g., V170A, L173A, Q178A). To this end, we constructed eight combination mutants and analyzed their binding to P-Rh*, Rh*, and 329G-Rh* (Fig. 6).

All proteins with mutations solely or predominantly from the second group (i.e., removing the direct hindrance of the primary binding site interaction) (II, III, IV) demonstrate substantially higher binding to Rh* (3.7- to 4.7-fold) compared with 329G-Rh* (2.2- to 3.3-fold) as well as an ~2-fold increased binding to P-Rh*. The addition of a potent mutation in position 175 (R175N or R175E) overcomes this difference between Rh* and 329G-Rh* producing a pattern characteristic for R175N and R175E point mutants.

The multiple mutants also enable an assessment of the effects of individual mutations in different settings. For example, four proteins differ by V170A mutation (wild-type to V170A; L173A to II; I to V; and R175E to VI). This mutation increases the binding to all forms of activated rhodopsin, the effect being greater when the binding of the first protein is relatively low (first two cases). Three proteins differ by L173A mutation (wild-type to L173A; V170A to II; and VI to VII), which yields moderate increases in binding in the first two cases and no effect on P-Rh* and Rh* binding and a slight decrease of 329G-Rh* binding in VI-to-VII (i.e., when the binding of the first protein is already high). Similar tendencies are observed for Q178A mutation (compare wild-type to Q178A and II to IV), and H179Q mutation (compare the gradual decline of its effects in wild-type-to-H179Q, III-to-IV, R175N-to-I, and VII-to-VIII pairs of proteins concomitant with the increase of binding of the first protein in each pair). It seems that there is a "ceiling effect"; i.e., there is a certain maximum level of binding (approximately 44 fmol for P-Rh*, 30 for Rh*, and 40 for 329G-Rh* in our assay conditions, even though 100 fmol of arrestin is present) for fully activated arrestin, and therefore, potentially activating mutations may have no effect on the protein that is already fully activated by other mutations. Interestingly, the maximum levels of mutant arrestin binding to 329G-Rh* and P-Rh* are similar, whereas maximum binding to Rh* is reduced. Mutant VI (V170A, R175E), combining the most potent activating mutations from both groups, is the most potent "constitutively active" arrestin, being capable of binding better to Rh* than wild-type arrestin binds to P-Rh*.

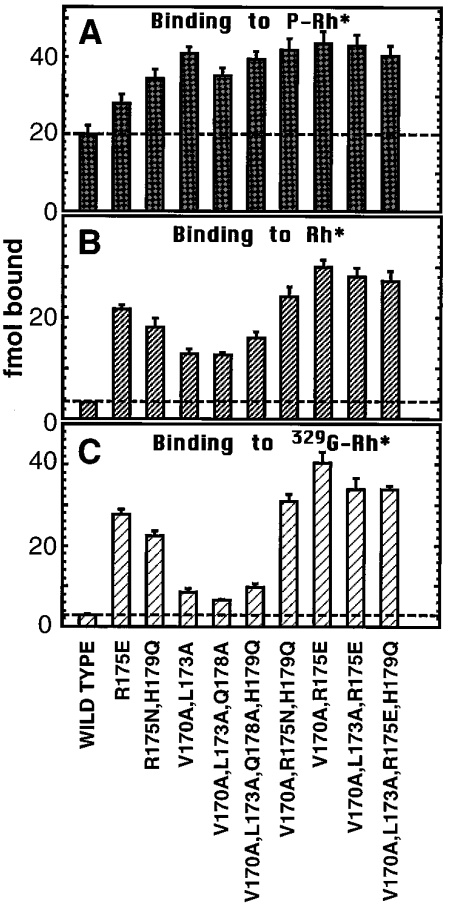

Fig. 6. Effect of multiple mutations in the phosphorylation-recognition region on arrestin binding to P-Rh* (A), Rh* (B), and 329G-Rh* (C). The indicated functional forms of rhodopsin (150 nM) were incubated with the respective arrestins (2 nM) at 37° for 5 min. The dotted line shows the level of wild-type arrestin binding, which was as follows: to P-Rh*, 20.1 ± 2.1 fmol; to Rh*, 3.4 ± 0.1 fmol; and to 329G-Rh*, 2.9 ± 0.2 fmol. The binding of all arrestin mutants was activation-dependent (data not shown). The binding to corresponding forms of rhodopsin in the dark was always 7- to 10-fold (P-Rh), 12- to 32-fold (Rh), and 14- to 41-fold (329G-Rh*) lower than under illumination (data not shown). Mean ± standard deviation from three experiments each performed in duplicate are shown.

Molecular mechanism of phosphorylation-recognition. Considered collectively, these data (Figs. 2 through 6, see also Ref. 11) enable us to propose a detailed model of the molecular mechanisms involved in phosphorylation-recognition and triggering the transition of arrestin into a high affinity binding state.

There are two relatively stable functional, and most likely conformational (7), states of arrestin. One is the "low-affinity" basal state of arrestin in which the activation-recognition and phosphorylation-recognition sites are both exposed, al-

though not in a conformation favorable for interaction (6), whereas the secondary binding site is not exposed. This conformation does not seem to change significantly when arrestin interacts with either Rh* or P-Rh (7). It should be noted that in truncated arrestin(1–365), both primary binding sites seem to be in a favorable conformation for binding and the secondary site seems to be mobilized readily, whereas in arrestin(1–191) the primary binding sites are also in a favorable conformation for interaction (5, 6), although the secondary binding site is absent.

In the basal state, residues Lys166, Lys167, Arg171, Lys176, and possibly Arg175 (within the phosphorylation-recognition region) are available for interaction with phosphates on the rhodopsin carboxyl terminus. Leu172 also is exposed and ready to interact with rhodopsin regardless of its phosphorylation state (Leu172 seems to interact with residues within the 19-residue carboxyl-terminal domain of rhodopsin, possibly with one of the hydrophobic residues in this region, Val337 or Val345). At the same time, Arg175 and His179 are involved in intramolecular interactions with the carboxyl-terminal domain of arrestin that help to keep arrestin in the low affinity state. Residues Val170, Leu173, and Gln178 are positioned to hinder arrestin interaction with unphosphorylated rhodopsin.

When arrestin binds to phosphorylated rhodopsin, the interaction of the positively charged arrestin residues with the phosphoserines on rhodopsin changes the overall conformation of the phosphorylation-recognition region. Phosphate interaction also neutralizes the positive charge of Arg175. These events disrupt the constraining intramolecular interactions in which Arg175 and His179 are involved, enabling arrestin to mobilize its secondary binding site (provided that the rhodopsin is also light-activated, thereby releasing other constraints). This interaction simultaneously moves Leu173 and Gln178 out of the way and places Val170, Ile174, and Val177 into a position favorable for interaction with an unidentified region of rhodopsin.

Such a mechanism implies that the phosphorylation-recognition region is exposed and very flexible. Indeed, sequence analysis of this region of arrestin shows a high surface probability and flexibility and a low probability of α-helix or β-sheet structure. In addition, recent studies have demonstrated that antibodies raised against a peptide corresponding to arrestin residues Val170 to Arg182 bind readily to arrestin but not to arrestin complexed to P-Rh* (23). Moreover, the peptide is able to compete with arrestin for binding to P-Rh* when used at high concentrations (0.4–3.2 mM) (23).

The finding that the most potent arrestin mutants bind comparably to P-Rh* and 329G-Rh* and substantially lower to Rh* suggests that arrestin interaction with the phosphorylated rhodopsin carboxyl terminus may promote a conformational rearrangement in rhodopsin to unmask additional sites on rhodopsin for arrestin binding. Conceivably, similar conformational changes may also unmask additional phosphorylation sites on rhodopsin (e.g., blowfly arrestin-rhodopsin interaction), leading to the notion that arrestin binding stimulates rhodopsin phosphorylation (24, 25).

The findings reported in this communication suggest three major directions for future research. First, arrestin mutants capable of phosphorylation-independent high affinity binding to rhodopsin should be tested further for their ability to quench transducin activation by Rh* and P-Rh*. Second, similar phosphorylation-independent mutants of nonvisual arrestins should be constructed and functionally characterized. Third, the neutralization of individual negative charges in the carboxyl-terminal region (residues 366–404) of visual arrestin by site-directed mutagenesis and the comparison of the effects of these mutations on the binding characteristics of wild-type arrestin and arrestin-R175E mutant will help identify the residue(s) with which Arg175 interacts.

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References

1. Kuhn, H. Light-regulated binding of rhodopsin kinase and other proteins to cattle photoreceptor membranes. *Biochemistry* **17:**4389–4395 (1978).
2. Bennett, N., and A. Sitaramayya. Inactivation of photoexcited rhodopsin in retinal rods: the roles of rhodopsin kinase and 48-kDa protein (arrestin). *Biochemistry* **27:**1710–1715 (1988).
3. Wilden, U., S. W. Hall, and H. Kuhn. Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein (arrestin). *Proc. Natl. Acad. Sci. USA* **83:**1174–1178 (1986).
4. Gurevich, V. V., and J. L. Benovic. Cell-free expression of visual arrestin: truncation mutagenesis identifies multiple domains involved in rhodopsin interaction. *J. Biol. Chem.* **267:**21919–21923 (1992).
5. Gurevich, V. V., and J. L. Benovic. Visual arrestin interaction with rhodopsin: sequential multisite binding ensures strict selectivity toward light-activated phosphorylated rhodopsin. *J. Biol. Chem.* **268:**11628–11638 (1993).
6. Gurevich, V. V., C.-Y. Chen, C. M. Kim, and J. L. Benovic. Visual arrestin binding to rhodopsin: intramolecular interaction between the basic N-terminus and acidic C-terminus of arrestin may regulate binding selectivity. *J. Biol. Chem.* **269:**8721–8727 (1994).
7. Schleicher, A., Kuhn, H., and Hofmann, K. P. Kinetics, binding constant, and activation energy of the 49-kDa protein-rhodopsin complex by extra-metarhodopsin II. *Biochemistry* **28:**1770–1775 (1989).
8. Krupnick, J. G., V. V. Gurevich, T. Schepers, H. E. Hamm, and J. L. Benovic. Arrestin-rhodopsin interaction: multi-site binding delineated by peptide inhibition. *J. Biol. Chem.* **269:**3226–3232 (1994).
9. Gurevich, V. V., R. M. Richardson, C. M. Kim, M. M. Hosey, and J. L. Benovic. Binding of wild type and chimeric arrestins to the m2 muscarinic cholinergic receptor. *J. Biol. Chem.* **268:**16879–16882 (1993).
10. Gurevich, V. V., S. B. Dion, J. J. Onorato, J. Ptasienski, C. M. Kim, R. Sterne-Marr, M. M. Hosey, and J. L. Benovic. Arrestin interactions with G-protein-coupled receptors: direct binding studies of wild type and mutant arrestins with rhodopsin, β_2-adrenergic, and m2 muscarinic cholinergic receptors. *J. Biol. Chem.* **270:**720–731 (1995).
11. Gurevich, V. V., and J. L. Benovic. Visual arrestin binding to rhodopsin: diverse functional roles of positively charged residues within the phosphorylation-recognition region of arrestin. *J. Biol. Chem.* **270:**6010–6016 (1995).
12. Shinohara, T., B. Dietzschold, C. M. Craft, G. Wistow, G., J. J. Early, L. A. Donoso, J. Horwitz, and R. Tao. Primary and secondary structure of bovine retinal S-antigen (48-kDa protein). *Proc. Natl. Acad. Sci. USA* **84:**6975–6979 (1987).
13. Palczewski, K., H. Ohguro, R. T. Premont, and J. Inglese. Rhodopsin kinase autophosphorylation: characterization of site-specific mutations. *J. Biol. Chem.* **270:**15294–15298 (1995).
14. Palczewski, K., J. Buczylko, M. W. Kaplan, A. S. Polans, and J. W. Crabb. Mechanism of rhodopsin kinase activation. *J. Biol. Chem.* **266:**12949–12955 (1991).
15. Puig, J., A. Arendt, F. L. Tomson, G. Abdulaeva, R. Miller, P. A. Hargrave, and J. H. McDowell. Synthetic phosphopeptide from rhodopsin sequence induces retinal arrestin binding to photoactivated unphosphorylated rhodopsin. *FEBS Lett.* **362:**185–188 (1995).
16. Palczewski, K., J. Buczylko, H. Ohguro, R. S. Annan, S. A. Carr, J. W. Crabb, M. W. Kaplan, R. S. Johnson, and K. A. Walsh. Characterization of a truncated form of arrestin isolated from bovine rod outer segments. *Protein Sci.* **3:**314–324 (1994).
17. Smith, W. C., A. H. Milam, D. Dugger, A. Arendt, P. A. Hargrave, and K. Palczewski. A splice variant of arrestin: molecular cloning and localization in bovine retina. *J. Biol. Chem.* **269:**15407–15410 (1994).
18. Matsumoto, U., and W. L. Pak. Light-induced phosphorylation of retina-

specific polypeptides of *Drosophila in vivo*. *Science (Washington D. C.)* **223:**184–186 (1984).
19. Byk, T., M. Bar-Yaacov, Y. N. Doza, B. Minke, Z. Selinger. Regulatory arrestin cycle secures the fidelity and maintenance of the fly photoreceptor cell. *Proc. Natl. Acad. Sci. USA* **90:**1907–1911 (1993).
20. LeVine, H., D. P. Smith, M. Whitney, D. M. Malicki, P. J. Dolph, G. F. H. Smith, W. Burkhart, and C. S. Zuker. Isolation of a novel visual-system-specific arrestin: an *in vivo* substrate for light-regulated phosphorylation. *Mech. Dev.* **33:**19–26 (1991).
21. Yamada, T., Y. Takeuchi, N. Komori, H. Kobayashi, Y. Sakai, Y. Hotta, and H. Matsumoto. A 49-kilodalton phosphoprotein in the drosophila photoreceptor is an arrestin homolog. *Science (Washington D. C.)* **248:**483–486 (1990).
22. Matsumoto, H., B. T. Kurien, Y. Takagi, E. S. Kahn, T. Kinumi, N. Komori, T. Yamada, F. Hayashi, K. Isono, W. L. Pak, K. W. Jackson, and S. L. Tobin. Phosrestin I undergoes the earliest light-induced phosphorylation by calcium/calmodulin-dependent protein kinase in drosophila photoreceptors. *Neuron* **12:**997–1010 (1994).
23. Kieselbach, T., K.-D. Irrgang, and H. Ruppel. A segment corresponding to amino acids Val170-Arg182 of bovine arrestin is capable of binding to phosphorylated rhodopsin. *Eur. J. Biochem.* **226:**87–97 (1994).
24. Bentrop, I., A. Plangger, and R. Paulsen. An arrestin homolog of blowfly photoreceptors stimulates visual-pigment phosphorylation by activating a membrane-associated protein kinase. *Eur. J. Biochem.* **216:**67–73 (1993).
25. Plangger, A., D. Malicki, M. Whitney, and R. Paulsen. Mechanism of arrestin 2 function in rhabdomeric photoreceptors. *J. Biol. Chem.* **269:** 26969–26975 (1994).

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