Visual Arrestin Interaction with Rhodopsin

SEQUENTIAL MULTISITE BINDING ENSURES STRICT SELECTIVITY TOWARD LIGHT-ACTIVATED PHOSPHORYLATED RHODOPSIN*

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Visual arrestin plays an important role in regulating light responsiveness via its ability to specifically bind to the phosphorylated and light-activated form of rhodopsin. Previously, we utilized an *in vitro* translation system to express and characterize the full-length (404 amino acids) and two truncated forms of visual arrestin. Here we have extended these studies to include a total of 33 different truncation and deletion mutants of arrestin, ranging from 69 to 391 amino acids in length. Mutants were produced by cutting within the open reading frame of the bovine arrestin cDNA with selective restriction enzymes followed by *in vitro* translation of the transcribed truncated mRNAs. Mutant arrestin binding to dark, light-activated, dark phosphorylated, and light-activated phosphorylated rhodopsin as well as to opsin, phosphoopsin, and truncated rhodopsin was then extensively characterized. In addition, the sensitivity of arrestin/rhodopsin interactions to conditions of increasing ionic strength was measured. These studies suggest the localization of multiple functional domains within the arrestin molecule that include: 1) a "phosphorylation recognition" domain, which interacts with the phosphorylated carboxyl terminus of rhodopsin, was localized predominately between residues 158–185; 2) an "activation recognition" domain, which interacts with those portions of the rhodopsin molecule that change conformation upon light activation, was found to consist of at least three regions within the first 191 residues of the arrestin molecule; 3) a hydrophobic interaction domain, localized between residues 191 and 365, appears to be mobilized upon binding of arrestin to activated phosphorylated rhodopsin; 4) a regulatory domain, localized in the COOH-terminal region of arrestin (residues 365–391), was found to play a role in controlling the conformational change in arrestin necessary for mobilization of the hydrophobic interaction domain; and 5) The NH₂ terminus of arrestin (residues 2–16) was found to be important for interacting with the regulatory COOH-terminal region as well as maintaining the conformation of the NH₂-terminal half of arrestin. A mechanism which ensures strict arrestin binding selectivity toward phosphorylated light-activated rhodopsin is proposed.

The ability of an organism to regulate the intensity of a response in the presence of a continuous stimulus plays an important role in cell function. This phenomenon, often termed desensitization, is well recognized in biological regulatory and sensory systems (1). One of the best models for studying the mechanisms involved in desensitization has been phototransduction in retinal rod cells (2). Visual transduction in photoreceptor cells results from a series of chemical reactions that translate a light signal into a hyperpolarization of the plasma membrane of the cell. The highly regulated reactions in this cascade are responsible for initiating, amplifying, and quenching the light-induced electrical response. Three distinct proteins modulate these various reactions via their specific photon-activated binding to the cytoplasmic surface of stimulated receptors. These include the guanine nucleotide regulatory protein (G protein)[1] transducin, a protein kinase termed rhodopsin kinase, and the regulatory protein arrestin (also termed S-antigen or 48K protein). Transducin, the retinal-specific G protein, is involved in amplifying the light signal via its interaction with metarhodopsin II. Conversely, rhodopsin kinase and arrestin are involved in rapid inactivation of the phototransduction cascade. Following light activation, rhodopsin kinase phosphorylates metarhodopsin II at multiple sites on its carboxyl-terminal domain (3, 4). This phosphorylation not only reduces the ability of rhodopsin to interact with transducin but also promotes the association of arrestin. Arrestin binding to phosphorylated metarhodopsin II completely blocks further activation of the cGMP phosphodiesterase cascade (2, 5). To recycle this system, arrestin is released and the deactivated photoreceptor is then dephosphorylated by protein phosphatase 2A and regenerated with 11-*cis*-retinal (6–8).

Retinal arrestin was initially identified as a major protein which redistributed (along with rhodopsin kinase) from the cytoplasm to the disc membrane following light activation of rod outer segments (9). In 1984, Kuhn and co-workers (10) demonstrated that the binding of arrestin to photoreceptor membranes was significantly enhanced by the phosphorylation of rhodopsin. While phosphorylated rhodopsin has a reduced ability to interact with transducin and consequently stimulate cGMP phosphodiesterase, the binding of arrestin to rhodopsin suppresses phosphodiesterase activation by ~98% (2). In contrast, arrestin does not quench non-phosphorylated rhodopsin activation of phosphodiesterase. Reti-

[1] The abbreviations used are: G protein, guanine nucleotide-binding regulatory protein; ARR, arrestin; βARK, β-adrenergic receptor kinase; ORF, open reading frame; RRL, rabbit reticulocyte lysate; Rh, dark rhodopsin; Rh*, light-activated rhodopsin; P-Rh, phosphorylated rhodopsin; P-Rh*, phosphorylated light-activated rhodopsin; ^{329}G-Rho, truncated rhodopsin; ROS, rod outer segment membranes; dpm, disintegrations/minute; rpm, revolutions/minute.

nal arrestin was initially purified and characterized from bovine retinas (11) and has been shown to specifically interact with phosphorylated metarhodopsin II with a K_d of 50 nM (12). While arrestin interaction with rhodopsin has been reported to be enhanced by ATP and GTP (13, 14), recent studies have provided no evidence for the direct binding of these nucleotides to purified arrestin (15). Cloning of a bovine retinal arrestin cDNA reveals that arrestin has 404 amino acids (45.3 kDa) with several short stretches of amino acid homology with the α subunit of transducin (16, 17, 18). Recent studies on purified trypsinized arrestin suggest that the carboxyl-terminal domain of arrestin is involved in recognition of the activated form of rhodopsin (19). Overall, these studies demonstrate that while rhodopsin phosphorylation is critical for quenching, it is arrestin binding which effectively disrupts the ability of rhodopsin to activate transducin.

In vitro translation has been a useful system for the functional expression (20–23), mutagenesis (24, 25), post-translational modification (26, 27), and high specific activity radiolabeling (28) of a variety of proteins. The combination of specific properties of the in vitro transcription and translation systems also provides a unique ability to produce mutant proteins truncated from the COOH terminus without the need for introducing any mutations into the cDNA itself. Expression of the radiolabeled protein enables characterization of the protein without tedious and often troublesome purification. These advantages coupled with a relatively short preparation time render this expression system most suitable for the first stage of structure-function studies which is deletion mutagenesis. Recently, we have utilized in vitro translation to express visual arrestin and characterize its binding to rhodopsin (28). These studies demonstrated that in vitro translated arrestin is fully functional in terms of its ability to specifically bind to the light-activated phosphorylated form of rhodopsin. Two truncated arrestins were also in vitro translated and characterized, enabling us to localize functionally important domains in the amino-terminal half and carboxyl terminus of arrestin. In the present work we have produced and characterized a total of 33 arrestin mutations using the in vitro translation system. These studies have enabled us to more precisely localize the functional domains on the arrestin molecule which are involved in the exquisite specificity of arrestin/rhodopsin interaction. A model is proposed for the sequential multisite binding which ensures strict selectivity of arrestin binding to the light-activated phosphorylated form of rhodopsin.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP, [^{35}S]dATP, [^{3}H]leucine (179.6 Ci/mmol), and [^{14}C]leucine (0.316 Ci/mmol) were purchased from New England Nuclear while RNasin was from Promega. The restriction enzymes *Bal*I, *Ban*I, *Ppu*MI, and *Sfa*NI were purchased from New England Biolabs while all other restriction enzymes were from Boehringer Mannheim. Sepharose 2B, Sephadex G-25, and all other chemicals were from Sigma. Rabbit reticulocyte lysate (RRL) was either purchased from Promega or prepared as previously described (29). SP6 RNA polymerase was isolated from an overproducing *Escherichia coli* strain HB101/pTISP6 (30) while 11-*cis*-retinal was generously supplied by Dr. R. K. Crouch, National Institutes of Health. Other reagents were from sources previously described (28).

Plasmid Constructions—A bovine visual arrestin cDNA was generously donated by Dr. T. Shinohara (17). The coding region was excised with *Hpa*I and *Hind*III and subcloned in the vector pG2S6-I which contains an SP6 promoter and an "idealized" 5′-nontranslated region (24). This construction resulted in the insertion of 18 base pairs, beginning with the starting ATG codon from the vector, which preceded the arrestin open reading frame (ORF). These additional bases encode the sequence Met-Asn-Thr-Ala-Ala-Ser (this protein is referred to as ARR (-6–404)). In order to remove these additional

codons, and to create on optimal context for the starting ATG (31), oligonucleotide-directed mutagenesis was performed. In this construction an additional GCC (Ala) codon was inserted between the normal first and second arrestin codons. This protein is referred to as ARR (1–404). In order to create an NH$_2$-terminal deletion, the arrestin cDNA in pBluescript was partially digested with *Bgl*II, blunted with mung bean nuclease, and the resulting fragment, starting with codon 17, was excised with *Hind*III. This cDNA fragment was subcloned into pG2S6-I digested with *Nco*I (this end was subsequently blunted with Klenow and therefore contains a starting ATG codon) and *Hind*III. The resulting construct lacks amino acid codons 2–16, and the encoded protein is referred to as ARR Δ(2–16). The sequences of all constructs were confirmed by DNA sequencing.

Plasmid Linearizations for Transcription—The plasmids for in vitro transcription were linearized using either *Hind*III (which cuts 120 base pairs downstream from the arrestin stop-codon) to produce mRNAs encoding full-length proteins or different restriction enzymes which cut within the coding region to produce truncated mRNAs (Fig. 1). When unique restriction sites were used, the digestion was carried to completion. When restriction enzymes that cut at multiple sites were used, the plasmids were initially linearized with *Hind*III and then further digested for various times with the enzyme in order to obtain a ratio of different size products.

In Vitro Transcription—Transcription was carried out at 37 °C for 2 h as described (32) in the presence of 120 mM potassium HEPES, pH 7.5, 16 mM MgCl$_2$, 2 mM spermidine, 40 mM dithiothreitol, 3 mM of each NTP, 200 units/ml of RNasin, 2.5 units/ml of inorganic pyrophosphatase, 1500 units/ml of SP6 RNA polymerase, and 30 μg/ml of template DNA. The mRNA (3–3.5 mg/ml) was precipitated by addition of 0.4 volume of 9 M LiCl and incubation for 10 min on ice and then pelleted by centrifugation for 10 min at 3,000 $\times$ g at 4 °C. The pellet was resuspended by vortexing in 1 ml of ice-cold 2.5 M LiCl, recentrifuged for 5 min at 4 °C, and then washed with 1 ml of 70% ethanol at room temperature. The final pellet was drained, immediately dissolved in diethylpyrocarbonate-treated water, and reprecipitated by addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and 3.3 volumes of ethanol. The mRNA was stored as a suspension at −80 °C.

In Vitro Translation—Translations using RRL were carried out essentially as described (29) in 120 mM potassium acetate, 30 mM creatine phosphate, 160 μg/ml creatine kinase, 200 units/ml RNasin, 0.1 μg/ml pepstatin, 0.1 μg/ml leupeptin, 0.1 mg/ml soybean trypsin inhibitor, 5 mM cAMP, 50 μM of 19 unlabeled amino acids, and 20–50 μM of [^{14}C]leucine (14,000–35,000 dpm/μl). The translation mix was 70% RRL and was found to contain sufficient concentrations of ATP, GTP, magnesium, and spermine. The RRL also contained 5.9 μM endogenous leucine (as determined by the isotope dilution method). When [^{3}H]leucine was used 800,000–1,000,000 dpm/μl were added along with cold leucine to obtain a final concentration of 20–25 μM (including the endogenous leucine). This resulted in a specific activity for leucine of 15 Ci/mmol.

Uncapped mRNAs containing an idealized 5′-nontranslated region (24) were added at a concentration of 100–150 μg/ml in the RRL mix. Aliquots of the mRNA suspension were centrifuged at room temperature (3,000 $\times$ g, 5 min) and the mRNA pellets were washed with 0.5 ml of 70% ethanol, dried, and dissolved in diethylpyrocarbonate-treated water just before addition to the translation mix. Translations were carried out at 22 °C for 2 h. The number of leucine residues in either full-length or truncated arrestins were taken into account in calculating the specific activities of the corresponding proteins. Following translation, 1 mM ATP and 1 mM GTP were added, and the samples were incubated for 10 min at 37 °C ("run-off"). Then EDTA (10 mM) and RNase A (20 μg/ml) were added, and the samples were incubated at 25 °C for 10 min. Samples were cooled on ice, and aggregated proteins were pelleted by centrifugation in a TLA 100.1 rotor (Beckman) at 100,000 rpm for 60 min at 4 °C. The supernatants, which contained about 95% of the synthesized protein, were then used for functional assays either directly or after gel filtration on a Sephadex G-25 column equilibrated with 20 mM Tris-Cl, pH 7.5, 2 mM EDTA (buffer A). Protein synthesis was determined using the amount of [^{3}H] or [^{14}C]leucine incorporated into a hot trichloroacetic acid-insoluble fraction or into the respective band after resolution by gel electrophoresis as described (5, 28). These values agreed within 10% of each other. The number of leucine residues in the respective arrestin along with the known specific activity of the leucine were then used to calculate the specific activities of corresponding proteins. The radioactivity in the arrestin band along with the specific activity

were used to determine the yields, which ranged from 50 to 200 pmol/ml of translation mix.

Rhodopsin Preparations—Urea-treated rod outer segment (ROS) membranes were prepared as described (33). Briefly, 50 frozen bovine retinas (Hormel) were suspended in 50 ml of 34% (w/v) sucrose, 65 mM NaCl, 2 mM MgCl$_2$, 10 mM Tris acetate buffer, pH 7.4, shaken vigorously, and centrifuged at 2,000 × g for 5 min. The supernatant was diluted with 2 volumes of 10 mM Tris acetate buffer, pH 7.4, and centrifuged as above. The crude ROS pellets were resuspended in 30 ml of 0.77 M sucrose, 1 mM MgCl$_2$, 10 mM Tris acetate buffer, pH 7.4, and further purified on a discontinuous sucrose gradient. The interface between 0.84 and 1.0 M sucrose was collected, diluted 2-fold with buffer, and pelleted at 48,000 × g for 20 min. The ROS were then suspended in 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5.0 M urea (1 ml/retina), sonicated on ice (4 min), diluted with 2 volumes of 50 mM Tris-HCl, pH 7.4, and centrifuged at 100,000 × g for 45 min. The pellet was washed three times with Tris buffer, resuspended in 50 mM Tris-HCl, pH 7.4, sonicated on ice, snap-frozen in liquid nitrogen, and stored at −80 °C wrapped in foil. All operations were carried out under dim red light at 4 °C. The concentration of rhodopsin was measured by absorbance at 498 nm using an extinction coefficient of 40,600.

A truncated form of rhodopsin with 19 amino acid residues proteolytically removed from the COOH terminus ([329]G-Rho) was made as previously described (34). Briefly, urea-treated ROS were incubated with endopeptidase Asp-N in a ratio of 750:1 (w/w, rhodopsin/Endo Asp-N) in 10 mM Tris-HCl, pH 7.5, in the dark at 22 °C for 16 h. The reaction was quenched by addition of 1 mM dithiothreitol and 1 mM EDTA, and the ROS membranes were recovered by centrifugation (40,000 × g for 10 min). The ROS were resuspended in 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5.0 M urea, sonicated, pelleted by centrifugation, washed three times in 50 mM Tris-HCl, pH 7.4, and finally resuspended in 50 mM Tris-HCl, pH 7.4. Complete digestion to [329]G-Rho was validated by the presense of a single protein band of 38 kDa on a 12% SDS-polyacrylamide gel that was not phosphorylated by the β-adrenergic receptor kinase (βARK) (35).

Rhodopsin was phosphorylated using βARK purified from Sf9 cells infected with a recombinant baculovirus.[2] βARK appears to phosphorylate light-activated rhodopsin at the same sites as rhodopsin kinase (36). 250–300 μg of rhodopsin was incubated with 5–30 μg of βARK, 2.0 mM ATP, 6 mM MgCl$_2$ in 1 ml of buffer A at 30 °C for 60 min with illumination. The reaction was stopped by dilution with ice-cold buffer A followed by centrifugation at 50,000 rpm in a TLS 55 rotor for 30 min. The pellet was washed two times with 2.5 ml of buffer A, thoroughly resuspended in 1 ml of the same buffer, and sonicated on ice for 1 min. The opsin was then regenerated by addition of a 3-fold molar excess of 11-*cis*-retinal, incubation in the dark at 37 °C for 40 min, followed by the addition of an equal portion of 11-*cis*-retinal and 2 h of incubation in the dark at 37 °C. The phosphorylated rhodopsin was aliquoted under dim red light and stored at −80 °C wrapped in foil. The efficiency of regeneration (determined by absorption at 498 nm) was 98 ± 3%. To measure the stoichiometry of phosphorylation, 1–2 μCi of [γ-^{32}P]ATP was added to a 20-μl aliquot of the initial reaction mix. This sample was incubated under the same conditions except that it was stopped by the addition of 5 μl of sodium dodecyl sulfate (SDS) sample buffer followed by gel electrophoresis. The gel was dried, autoradiographed, and the rhodopsin band was excised and counted in 5 ml of scintillation fluid. It was assumed that all of the receptors were accessible to the kinase during the phosphorylation.

Arrestin Binding Assays—Two experimental designs were employed to measure arrestin binding to phosphorylated, phosphorylated light-activated, dark and light-activated rhodopsin. The *in vitro* translated arrestins (0.2–1 pmol) were incubated in 30 mM potassium HEPES, 2 mM MgCl$_2$, 150 mM potassium acetate, pH 7.5 (buffer B) with 0.3–0.6 μg (7.5–15 pmol) of the various rhodopsins in a volume of 50 μl for 5 min at 37 °C either in the dark or with illumination (room light). The samples were immediately cooled on ice and under dim red light were diluted with 200 μl of buffer B and loaded on a 0.2 ml cushion of 0.2 M sucrose in buffer B. The samples were then centrifuged in a TLA 100.1 rotor at 100,000 rpm at 2 °C for 30 min. After carefully removing the supernatants, the pellets were dissolved in 15 μl of SDS sample buffer and separated on a 10% or 15% SDS-polyacrylamide gel (37). The gels were stained with Coomassie Blue G-250 and then soaked in 20% 2,5-diphenyloxazole in glacial acetic

acid for 10 min. The fluorochrome was precipitated by washing the gel with two to three changes of water, and the gels were then dried. Fluorographs were exposed at −80 °C for 2–8 days using Fuji x-ray film, and the labeled protein bands were then excised and counted in a liquid scintillation counter. Alternatively, following the initial incubation the samples were cooled and loaded onto a 2-ml Sepharose 2B column equilibrated with buffer A. Bound arrestin eluted with the ROS in the void volume (between 0.5–1.1 ml). Nonspecific binding (*i.e.* the binding to 0.3 μg of liposomes) was subtracted and in all cases was <20% of the total binding. The kinetics of arrestin-rhodopsin complex dissociation were measured by incubating a 10-fold concentrated sample (in respect to rhodopsin and arrestin) at 37 °C followed by dilution with ice-cold buffer B. The samples were kept on ice and at the appropriate time were loaded onto a 2-ml Sepharose 2B column equilibrated with buffer A to separate bound and free arrestins.

RESULTS

Truncation Mutagenesis of Visual Arrestin—Previously, we used *in vitro* translation to produce and characterize two arrestin mutants using unique restriction sites found within the open reading frame of the arrestin cDNA (28). These studies demonstated that both the COOH-terminal region and NH$_2$-terminal half of the arrestin molecule are important for rhodopsin binding. In an effort to more precisely localize functional domains within the visual arrestin molecule, we have produced a variety of additional truncated arrestins using restriction sites found within the arrestin ORF. Restriction enzymes that cut uniquely within the arrestin ORF were used to generate mRNAs that encode ARR(1–365), ARR(1–355), ARR(1–191), and ARR(1–145) with or without an additional 6 amino acids at the amino terminus. Another strategy for generating mutant arrestins involved initial linearization of the plasmid with *Hind*III (a *Hind*III site is located ~120 bases downstream from the arrestin stop codon), followed by a partial digestion with restriction enzymes that have two to four sites within the arrestin ORF (Fig. 1). *In vitro* transcription of the resulting plasmids generated a mixture of truncated and full-length mRNAs, the position of truncation being determined by the position of the cut within the transcribed DNA strand (38). Translation of these mRNAs using a rabbit reticulocyte lysate gave rise to a mixture of truncated arrestins with the last amino acid being determined by the last full codon present in the mRNA (Fig. 2A). The relative amount of each truncated protein was roughly proportional to the percentage of the initial partially digested DNA in the mixture (Fig. 2A, *lanes 1–9*). While we were not able to determine the actual COOH-terminal residue of the truncated arrestins, the proteins all migrated on a polyacrylamide gel as sharp discrete bands with the expected electrophoretic mobility (Fig. 2A).

We next tested the ability of the various truncated arrestins to bind to phosphorylated (P-Rh), phosphorylated light-activated (P-Rh*), dark (Rh), and light-activated rhodopsin (Rh*). For these studies the various truncated arrestins were incubated with the different forms of rhodopsin for 5 min, and bound and free arrestins were then separated by centrifugation through a 0.2 M sucrose cushion. The pellets, which contained bound arrestins, were electrophoresed, and the labeled arrestins were visualized by fluorography. Representative data from three of the arrestin mutant binding studies are shown in Fig. 2B. In these experiments a high molar excess (~10-fold) of rhodopsin was used to enable the mixture of truncated arrestins to bind independently to rhodopsin. This was confirmed by comparing the binding of several of the individual mutants with a mixture of these same mutants (data not shown). Following electrophoresis the appropriate arrestin bands were excised and counted. This approach enabled us to produce and characterize the binding of 33 differ-

FIG. 1. **Restriction map of the bovine visual arrestin cDNA (ORF is *shaded*) with the relative positions of all restriction sites used to produce the various truncated mutants.** Sites used for production of the individual mutants are shown in *boldface* under the cDNA. The *Hind*III site is located 120 base pairs downstream from the arrestin stop codon. The exact positions of all restriction sites are shown in Table I.
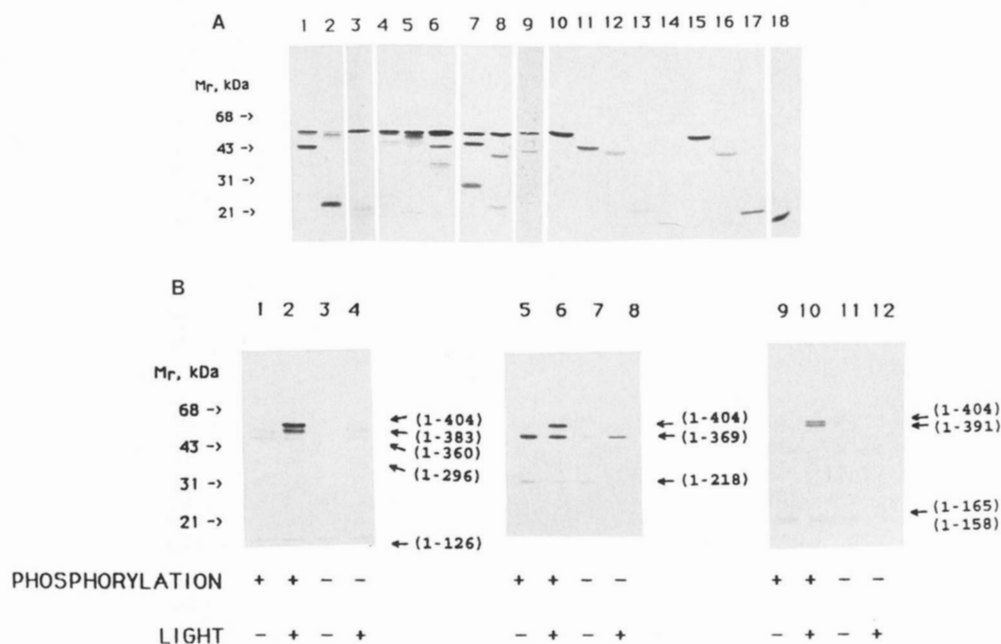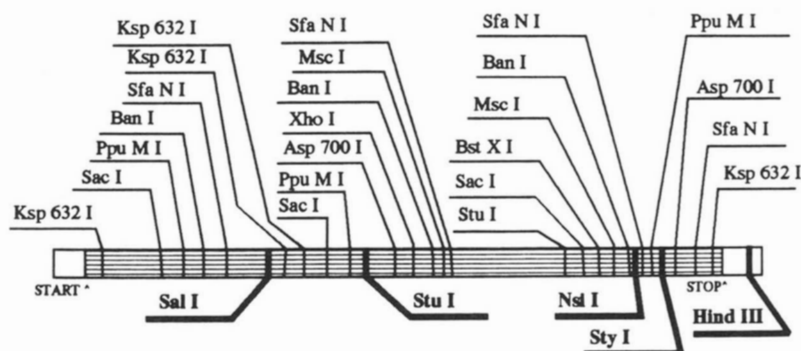

FIG. 2. *A, in vitro* translation products for the various truncated arrestion mRNAs. The visual arrestin cDNA was linearized with *Hind*III and then partially digested with either *Msc*I (*lane 1*), *Ksp*632I (*lane 2*), *Ban*I (*lane 3*), *Ppu*MI (*lane 4*), *Sfa*NI (*lane 5*), *Ban*I (*lane 6*), *Asp*700I (*lane 7*), *Stu*I (*lane 8*), or *Bst*XI (*lane 9*). The plasmids were then transcribed, and the resulting mixture of truncated mRNAs was translated using the rabbit reticulocyte system and [¹⁴C]leucine. Alternatively, individual full-length and truncated mRNAs, encoding arrestins (-6–404) (*lane 10*), (-6–365) (*lane 11*), (-6–355) (*lane 12*), (-6–191) (*lane 13*), (-6–145) (*lane 14*), (1–404) (*lane 15*), (1–355) (*lane 16*), (1–191) (*lane 17*), and (1–145) (*lane 18*) were generated using the unique restriction sites shown in Fig. 1. The plasmids were transcribed and translated using RRL and [³H]leucine. Two μl of each respective translation mix was then electrophoresed on a 10% SDS-polyacrylamide gel which was then fixed and autoradiographed. *B,* functional binding of several truncated arrestins to various forms of rhodopsin. Two μl of the different translation mixes containing the following ¹⁴C-labeled arrestins were used: (1–404)+(1–383)+(1–360)+(1–296)+(1–126) (*Sfa*NI, *lanes 1–4*); (1–404)+(1–369)+(1–218) (*Asp*700I, *lanes 5–8*); (1–404)+(1–391)+(1–165)+(1–158)+(1–26) (*Ksp*632I, *lanes 9–12*). The respective translation mixes were incubated with the indicated functional forms of rhodopsin for 5 min at 37 °C, cooled on ice, and then diluted with 0.2 ml of ice-cold buffer B. The samples were then loaded on a 0.2-ml cushion of 0.2 M sucrose in buffer B and the membranes, containing the bound arrestins, were sedimented by centrifugation at 100,000 rpm for 35 min. The pellets were dissolved in 20 μl of SDS sample buffer and electrophoresed, fixed, and autoradiographed as described above.

ent mutant arrestins, ranging from ARR(1–69) to ARR(1–391) (Table I). The binding of several of the individually produced truncated arrestins to different functional forms of rhodopsin and opsin was also characterized in more detail (Fig. 3). For these studies bound and free arrestins were resolved by chromatography on Sepharose 2B columns.

When both the affinity and selectivity of truncated arrestins for the different forms of rhodopsin are taken into account, these mutants fall into at least six different groups (Table I, Fig. 3). The full-length arrestins ARR(-6–404) and ARR(1–404) bind very selectively to P-Rh*. However, a low but measurable level of binding to Rh* and P-Rh can also be detected while the binding to Rh is negligible (Table I, Fig. 3). The binding of the full-length arrestins was also very dependent on the phosphorylation level of the receptor since binding to P-Rh* with 2 mol P$_i$/mol receptor was ~5-fold

higher then to P-Rh* with 0.6 mol P$_i$/mol receptor (Fig. 3). Both full-length arrestins also bound substantially to phosphoopsin (Fig. 3). The longest mutant arrestin tested ARR(1–391) has properties very similar to those of full-length arrestin. Thus, removal of the COOH-terminal 13 amino acids of arrestin has a minimal effect on its binding properties.

A second class of mutants ranging from ARR(-6–355) to ARR(1–383) have partially reduced binding to P-Rh*. However, these mutants also bind substantially to both Rh* and dark P-Rh as well as weakly to dark Rh (Table I, Fig. 3). This apparent loss of selectivity (*i.e.* increased binding to Rh* and P-Rh) increases with decreasing mutant size in this group. For example, while ARR(1–383) is still somewhat selective for binding to P-Rh*, the other mutants in this group bind to Rh* and P-Rh nearly as well as to P-Rh*. Two of the mutants in this group, ARR(-6–365) and ARR(-6–355), also bind read-

TABLE I

Full-length and truncated arrestin binding selectivity to different forms of rhodopsin

Arrestin[a]	Restriction enzyme	Restriction site[b]	Binding to rhodopsin (Bound to free ratio)[c]			
			P-Rh	P-Rh*	Rh	Rh*
1–404	HindIII	1727	0.04	0.86	0.03	0.04
–6–404	HindIII	1727	0.05	1.17	0.01	0.05
1–391	Ksp632I	1503	0.05	0.62	0.01	0.08
1–383	SfaNI	1480	0.14	0.50	0.05	0.12
1–369	Asp700I	1437	0.17	0.44	0.07	0.28
–6–365	StyI	1428	0.42	0.87	0.09	0.57
1–362	PpuMI	1414	0.37	0.63	0.15	0.28
1–360	SfaNI	1408	0.34	0.39	0.15	0.34
–6–355	NsiI	1401	0.27	0.36	0.18	0.28
Δ(2–16)	HindIII	1727	0.05	0.31	0.08	0.29
Δ(2–16)-365	StyI	1428	0.06	0.57	0.10	0.22
1–355	NsiI	1401	0.12	0.12	0.02	0.05
1–351	BanI	1381	0.15	0.15	0.10	0.11
1–347	MscI	1373	0.09	0.07	0.05	0.04
1–344	BstXI	1369	0.06	0.07	0.05	0.04
1–340	SacI	1356	0.03	0.07	0.06	0.08
1–336	StuI	1341	0.03	0.03	0.03	0.02
1–296	SfaNI	1215	0.12	0.09	0.10	0.07
1–284	MscI	1184	0.12	0.12	0.10	0.07
1–281	BanI	1171	0.14	0.12	0.13	0.11
1–251	XhoI	1081	0.15	0.11	0.12	0.11
1–218	Asp700I	987	0.16	0.09	0.10	0.12
Δ(2–16)-191	StuI	905	0.01	0.02	0.02	0.02
1–191	StuI	905	0.38	0.51	0.06	0.11
–6–191	StuI	905	0.34	0.61	0.14	0.19
1–185	PpuMI	884	0.63	0.60	0.10	0.10
1–167	SacI	836	0.18	0.19	0.10	0.11
1–165	Ksp632I	824				
			0.30	0.25	0.21	0.17
1–158	Ksp632I	804				
1–145	SalI	764	0.39	0.24	0.38	0.24
–6–145	SalI	764	0.41	0.20	0.44	0.24
1–126	SfaNI	711	0.37	0.50	0.37	0.42
1–98	BanI	623	0.69	0.30	0.51	0.19
1–82	PpuMI	574	0.60	0.27	0.44	0.17
1–69	SacI	483	0.37	0.38	0.38	0.29

[a] The last residue of each truncated arrestin was determined by assuming that the last base in the mRNA is determined by the position of the cut in the transcribed DNA strand (38). Thus, the last amino acid residue in the resulting protein would be encoded by the last full codon in the mRNA.

[b] The restriction site positions are from Ref. 17 where the open reading frame is from base pair 332 to 1543.

[c] Binding to rhodopsin was determined by pelleting the rhodopsin membranes containing the bound arrestins followed by electrophoresis, fluorography and cutting and counting the respective radiolabeled protein bands (see "Experimental Procedures"). The amount of the respective arrestins initially added to each assay was determined by electrophoresis of the translation mix and cutting and counting of the respective arrestin. Due to variations in specific activities and molar quantities of the different truncated proteins produced during translation, bound to free ratios are presented since they better reflect the affinity and selectivity of the mutants tested. In all cases values represent the mean from at least two independent experiments, variations never exceeded 20% of the respective values.

ily to phosphoopsin, even with a low level of phosphorylation (Fig. 3).

Another interesting group of mutants includes ARRΔ(2–16) and ARR(Δ(2–16)-365) which lack residues 2–16 at the NH₂ terminus of arrestin. This deletion leads to a significant reduction in binding to P-Rh* as compared to full-length arrestin (Table I). In addition, ARRΔ(2–16) also lacks the ability to discriminate between the phosphorylated and non-
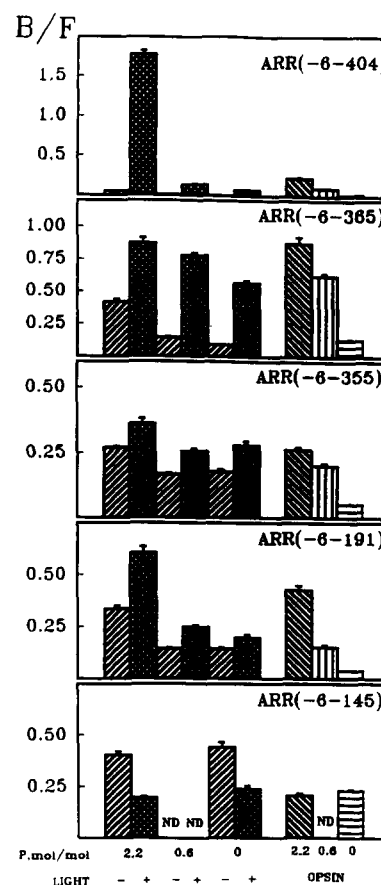

FIG. 3. **Direct binding studies of several truncated arrestins to different forms of rhodopsin.** The indicated full-length or truncated [³H]arrestins were incubated with 0.3 μg (7.5 pmol) of the different functional forms of rhodopsin or opsin (containing 0, 0.6 or 2.2 mol Pᵢ/mol receptor) for 5 min at 37 °C. The membranes containing bound arrestins were then separated from unbound arrestin by Sepharose 2B chromatography as described under "Experimental Procedures." Blank values (radioactivity in collected fractions present upon incubation of the individual arrestin with 0.3 μg of liposomes) were substracted and in all cases were <20% of the total binding. The actual quantities of arrestins added varied in the range of 85–138 fmol/assay while specific activities were in the range of 211–578 Ci/mmol. The specific binding of the different arrestins varied from a high of 114,000 dpm to a low of 2,800 dpm. In order to compensate for the different amounts and specific activities of the various arrestins, all data are expressed as bound to free ratios. Although the binding of each arrestin was found to be concentration dependent, the selectivity pattern (*i.e.* relative binding to the different functional forms of rhodopsin) was not concentration dependent over the range of concentrations used here. All experiments were run in triplicate with the mean ± S.E. shown.

phosphorylated forms of rhodopsin (*i.e.* binding to P-Rh* and Rh* are comparable). ARRΔ(2–16) discrimination between dark and light-activated forms of rhodopsin is also somewhat reduced suggesting that the NH₂ terminus may also provide one of the "activation recognition" sites. The removal of the COOH terminus from ARRΔ(2–16) appears to, if anything, enhance its binding selectivity (Table I). This enhanced selectivity appears to be due predominately to an increased binding of ARR(Δ(2–16)-365) to P-Rh* as compared of ARR Δ(2–16). It is interesting that ARR(Δ(2–16)-365) also binds with higher specificity to rhodopsin than does ARR(-6-365).

Truncated arrestins ranging from ARR(1–218) to ARR(1–351) including ARR(Δ(2–16)-191) demonstrate very low binding to all four functional forms of rhodopsin as well as to phosphorylated and non-phosphorylated opsins (Table I).

However, it is not clear if this low binding is even specific since these mutants also bind comparably to liposomes (data not shown). We believe that the mutants of this group are unable to assume and/or maintain the proper conformation necessary for specific binding. This may in part be due to decreased thermostability of these mutants since we have found that ARR(1–355) is much less stable than full-length arrestin (see below).

A fifth group of mutants includes ARR(1–191) to ARR(1–167). Both ARR(1–191) and ARR(-6–191) display specific binding with a selectivity most similar to that of ARR(1–362) (*i.e.* P-Rh* > P-Rh > Rh* > Rh). In contrast, ARR(1–185) demonstrates binding specificity solely for the phosphorylation state of the receptor (P-Rh* ~ P-Rh > Rh* ~ Rh). ARR(1–167) is the shortest species that still discriminates between phosphorylated and nonphosphorylated rhodopsin, although its selectivity is profoundly diminished in comparison with ARR(1–185). Unfortunately, we were unable to resolve ARR(1–165) and ARR(1–158) (produced using a cDNA partially digested with *Ksp*632I) well enough to excise the individual bands. However, when excised together these arrestins display a slight preference for phosphorylated rhodopsin, which most likely can be ascribed to ARR(1–165).

The final group of mutants range from ARR(1–158) to the shortest mutant studied ARR(1–69). While these mutants do not distinguish the phosphorylation state of rhodopsin they unexpectedly have a preference for binding to the non-activated forms of rhodopsin (Rh and P-Rh) (Table I, Fig. 3). This interesting feature suggests that this stretch of arrestin contains at least one site involved in discriminating the activation state of rhodopsin.

These data corroborate our previous localization of both the "phosphorylation recognition" and activation recognition sites within the NH$_2$-terminal 191 amino acids of arrestin (28). (Note that we use these terms in a strictly functional sense, *i.e.* the activation recognition site designates any number of structural elements within the arrestin molecule that enable it to discriminate between dark and light-activated rhodopsin, while the phosphorylation recognition site includes all portions of arrestin that take part in discrimination between phosphorylated and non-phosphorylated forms of rhodopsin. We do not necessarily imply that there is one particular structural domain fulfilling either of these functions.) In general, our data allow us to conclude that the major phosphorylation recognition site is localized between residues 165–185 of arrestin although the NH$_2$-terminal residues 2–16 might also play a supportive role in this function. The activation recognition domain includes the NH$_2$-terminal residues 2–16 and at least one site between residues 16 and 145. In addition, the ability of ARR(-6–191) to bind preferentially to light-activated rhodopsin while ARR(-6–145) and shorter species bind better to dark rhodopsin suggests the participation of yet another site localized between residues 145 and 191.

Analysis of Interactions Involved in Arrestin Binding to Rhodopsin—In order to shed light on the nature of the interactions involved in arrestin binding to distinct functional forms of rhodopsin, we next studied the effect of ionic strength on these interactions. The binding of full-length ARR(-6–404) to P-Rh* is not particularly sensitive to inhibition by high ionic strength (Fig. 4A). In fact, its binding is slightly enhanced at physiological salt concentrations and then inhibited at higher salt with an IC$_{50}$ of ~800 mM for potassium acetate. Since hydrophobic interactions are enhanced by increasing ionic strength while hydrophilic interactions are inhibited, these data suggest that both hydrophobic and hydrophilic (most likely ionic) interactions contribute to the
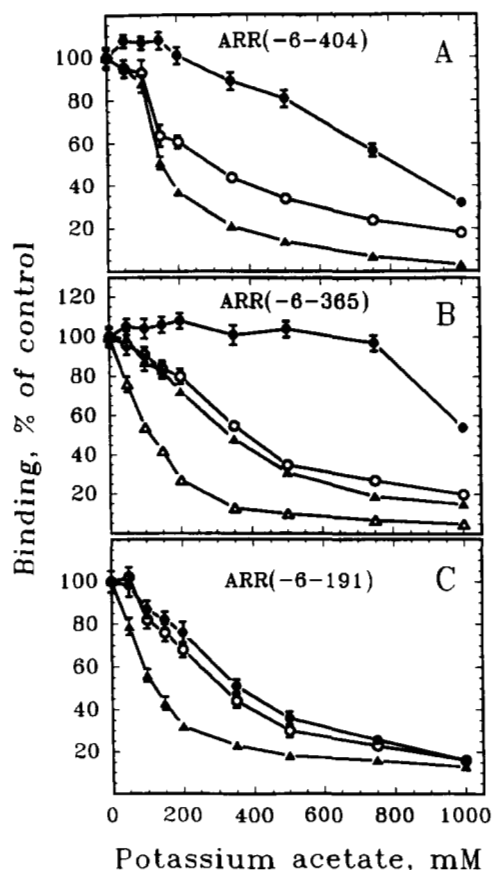

FIG. 4. **Inhibition of arrestin binding by ionic strength.** The effect of ionic strength on the binding of ARR(-6-404) (*A*), ARR(-6-365) (*B*), and ARR(-6-191) (*C*) to P-Rh (O), P-Rh* (●), Rh (△), and Rh* (▲) was measured. Samples containing 1.5 nM arrestin, 150 nM rhodopsin, and various concentrations of potassium acetate were incubated for 5 min at 37 °C. Arrestin binding was then determined by gel filtration on Sepharose 2B columns as described under "Experimental Procedures." The means ± S.D. of two independent experiments performed in duplicate are presented. The average control binding (no salt) in fmol to Rh*, P-Rh, and P-Rh*, respectively, for each arrestin was 10.2, 7.8, 83.6 for ARR(-6-404), 26.3, 21.3, and 33.5 for ARR(-6-365), and 13.5, 25.8, and 38.5 for ARR(-6-191). In addition, the control binding of ARR(-6-365) to Rh was 6.2 fmol.

binding of full-length arrestin to P-Rh*. We next probed whether this ionic interaction was involved in recognition of the phosphorylation state or activation state of rhodopsin. This was done by assessing the ability of salt to inhibit ARR(-6–404) binding to Rh* and dark P-Rh since in either case only one of these two interactions should be primarily involved. Unexpectedly, binding to both Rh* and P-Rh was strongly inhibited by salt, with IC$_{50}$ values (150 and 350 mM, respectively) indicative of the ionic nature of both interactions. These findings suggest the participation of an additional binding site, hydrophobic in nature, that is only mobilized during the interaction of arrestin with P-Rh*. When the same series of experiments was carried out using ARR(-6–365), similar results were obtained with the binding to P-Rh* being the least salt sensitive (Fig. 4B). The binding of ARR(-6–365) to Rh appeared to be the most sensitive with an IC$_{50}$ ~ 100 mM. Interestingly, the interaction of ARR(-6–191) with P-Rh* appeared to be a simple sum of two ionic interactions (with dark P-Rh and Rh*) with no hydrophobic interaction (Fig. 4C). These results suggest that the hydrophobic binding site is localized between residues 191 and 365.

Stability of Full-length and Truncated Arrestins—In arrestin/rhodopsin binding assays it is impossible to reach a true

equilibrium because of the transient nature of metarhodopsin II (12). However, previous studies have demonstrated that arrestin binding to rhodopsin stabilizes the metarhodopsin II state (12). Due to the relatively low levels of arrestins produced by *in vitro* translation, we were not able to measure the ability of the mutant arrestins to stabilize metarhodopsin II. However, we were able to study the stability of the complexes formed between several of the arrestins and either P-Rh* or Rh*. This was done by measuring the dissociation of the various arrestins from rhodopsin at low temperature (0 °C). We found that complexes of the full-length ARR(-6-404) and ARR(1-404) with P-Rh* (dissociation $t_{1/2}$ = 160 ± 12 and 123 ± 7 min, respectively) are substantially more stable than with Rh* ($t_{1/2}$ = 112 ± 9 and 68 ± 5 min, respectively). In both cases ARR(-6-404) appears to form a more stable complex than does ARR(1-404). Interestingly, a complex of ARR(-6-365) with P-Rh* has an ~13-fold longer half-life ($t_{1/2}$ = 2122 ± 114 min) than that of full-length arrestin, while the complex with Rh* has only an ~2-fold longer half-life ($t_{1/2}$ = 232 ± 11 min). ARR(-6-191) also forms a very stable complex with P-Rh*, with a half-life (2074 ± 236 min) comparable to that of ARR(-6-365). The substantially slower dissociation of the truncated arrestins from P-Rh* suggests that their affinity for rhodopsin is higher than that of full-length arrestin. However, our direct binding studies demonstrate that the truncated arrestins bind less well to P-Rh* when compared to full-length arrestin (as assessed by bound/free ratios). Several differences in the properties of these arrestins may account for this apparent contradiction. First, truncated arrestins exhibit much less discrimination between P-Rh* and phosphoopsin as compared to full-length arrestin (Fig. 3). Thus, the decay of metarhodopsin II would accelerate the dissociation of ARR(-6-404) to a substantially higher degree than that of ARR(-6-365) and ARR(-6-191). A second possible explanation is that the mutant arrestins are less stable at the conditions we use for our binding experiments (37 °C). Indeed, under incubation conditions (15 min at 37 °C) where the full-length arrestins are not inactivated (compared to samples kept on ice), ARR(-6-365) is inactivated 17 ± 4% while ARR(-6-355) and ARR(1-355) are inactivated 31 ± 6%. In contrast, ARR(1-191) and ARR(-6-191) display the same high stability as full-length arrestin. This suggests that the slow dissociation of both ARR(-6-365) and ARR(-6-191) from P-Rh* is predominately accounted for by their poor ability to discriminate between P-Rh* and phosphoopsin.

The decreased stability upon removal of 39 or 49 COOH-terminal residues of arrestin as compared to the high stability of ARR(-6-191) is also paralleled by the yields of the respective proteins in the *in vitro* translation system. Thus, ARR(-6-365), ARR(-6-355), and ARR(-6-191) are translated with ~2-, ~3-, and ~1.3-fold lower yields, respectively, as compared to ARR(-6-404). In addition, the percentage of these proteins remaining in the supernatant after high speed centrifugation also correlates with both their yields and thermostability (data not shown). RRL is known to contain a wide variety of heat shock proteins, including heat shock proteases, that are able to recognize and destroy denatured or incorrectly folded proteins. High speed centrifugation removes aggregated proteins that in most cases result from improper folding or denaturation. Therefore, the close correlation of stability, yield, and aggregation strongly suggests that while both full-length arrestin and ARR(-6-191) fold and maintain their conformations easily, ARR(-6-365) and ARR(-6-355) do not. This might suggest that the NH_2-terminal half of arrestin is not only a functional domain but a distinct structural domain as well.

Arrestin Binding to Nonphosphorylated Rhodopsin—Since the COOH terminus of rhodopsin serves as the major phosphorylation domain for rhodopsin kinase and βARK, this region likely serves as the phosphorylation recognition site for arrestin binding (3, 4, 33). To study the relative importance of this COOH-terminal phosphorylation domain, we assessed the ability of the various arrestins to bind to [329]G-rhodopsin. This truncated form of rhodopsin lacks its COOH-terminal 19 amino acids and no longer serves as a substrate for rhodopsin kinase (34) or βARK (35). Upon light activation, [329]G-Rh* binds ARR(-6-404), ARR(-6-365), ARR(-6-191), and ARR(-6-145) as well as light-activated full-length rhodopsin (Fig. 5). In contrast, when dark Rh and [329]G-Rh were tested, the binding of ARR(-6-404), ARR(-6-365), and ARR(-6-191) was significantly diminished by the absence of the rhodopsin COOH terminus while ARR(-6-145) was unaffected (Fig. 5). These results demonstrate that the nonphosphorylated rhodopsin COOH terminus plays a major role in arrestin binding to dark rhodopsin, most likely interacting with the arrestin phosphorylation recognition site (residues 165-191). This interaction is likely ionic since the rhodopsin COOH terminus has a net charge of -3 while arrestin residues 165-191 have a net charge of $+8$. In fact, ARR(-6-365) binding to Rh is extremely sensitive to salt inhibition (IC_{50} = 100 mM) (Fig. 4). In contrast, the interaction of arrestin with light-activated rhodopsin does not appear to involve the COOH terminus of rhodopsin. These results suggest that the ability of arrestin to weakly interact with dark nonphosphorylated Rh is not an artifact of mutagenesis, but a property of arrestin that may enable it to "probe" whether rhodopsin is activated and/or phosphorylated.

DISCUSSION

We have previously utilized *in vitro* translation to express visual arrestin and characterize its binding to rhodopsin (28). In the present work we have produced and characterized 33 arrestin mutations using the *in vitro* translation system. The binding characteristics of the various arrestin mutants as well as the sensitivity of several of the mutants to ionic strength enables us to further localize the functional domains on
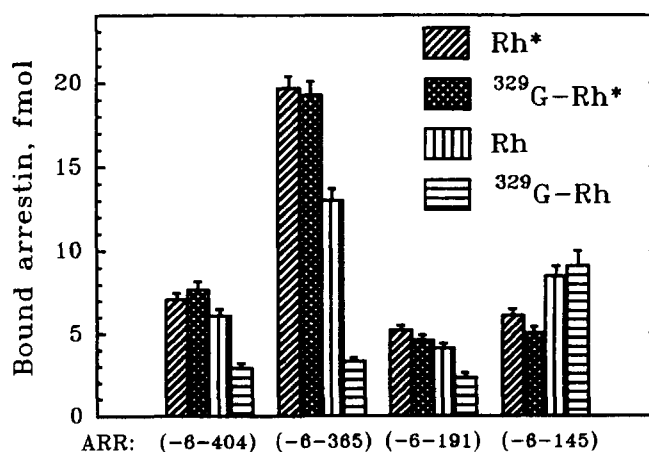

FIG. 5. **Full-length and truncated arrestin binding to [329]G-rhodopsin.** Samples containing 2 nM of the indicated arrestins and 150 nM of the different rhodopsins were incubated at 37 °C for 5 min. Arrestin binding was determined by gel filtration on Sepharose 2B columns as described under "Experimental Procedures." The specific activities of the different arrestins were 1221 dpm/fmol for ARR(-6-404), 1098 dpm/fmol for ARR(-6-365), 510 dpm/fmol for ARR(-6-191), and 384 dpm/fmol for ARR(-6-145). All experiments were performed two times in triplicate with the mean ± S.E. shown.

arrestin which contribute to the exquisite specificity of arrestin/rhodopsin interaction.

Phosphorylation Recognition Site—Our data enable us to localize a domain on arrestin that interacts with the phosphorylated COOH terminus of rhodopsin between residues 158 and 185. This is based on the finding that ARR(1–185) binds to P-Rh and P-Rh* ~6-fold better than to Rh and Rh* while ARR(1–165) and ARR(1–158) cannot discriminate between phosphorylated and non-phosphorylated rhodopsin. These findings support the previous hypothesis that this stretch of basic residues in arrestin (amino acids 163–182) is involved in binding to phosphorylated rhodopsin (19). However, our data also suggest that the NH2-terminal domain of arrestin (residues 2–16) might also be involved in phosphorylation recognition. This again is based on the finding that ARRΔ(2–16) does not discriminate between phosphorylated and non-phosphorylated rhodopsin. However, when the binding of ARR(Δ(2–16)-365) was tested it was found to discriminate very well between Rh* and P-Rh*. Thus, the COOH-terminal domain of arrestin appears to somehow inhibit the ability of ARRΔ(2–16) to recognize the phosphorylation state of rhodopsin. The primary structure (basic residues) of regions 2–16 and 158–185 of arrestin as well as the acidic nature of the phosphorylated COOH terminus of rhodopsin suggests the involvement of ionic interactions. Indeed, the binding of both arrestin ARR(-6–404) and ARR(-6–191) to dark P-Rh, which presumably is mediated predominantly by the phosphorylated COOH terminus of rhodopsin, is inhibited by high salt with IC_{50} values of ~350 mM (Fig. 4).

Activation Recognition Site—Unlike the phosphorylation recognition domain that is localized in one or possibly two discrete regions on arrestin, the activation recognition domain remains poorly defined. However, several lines of evidence suggest that the ability of arrestin to discriminate the activation state of rhodopsin is largely localized within the first 191 residues of arrestin. The amino terminus of arrestin appears to be one of the domains involved in activation recognition. Several of the arrestins containing 6 additional residues at the amino terminus, followed by the native bovine arrestin sequence, demonstrate better discrimination between light-activated and dark rhodopsin than their counterparts that have an alanine residue inserted between the first and second residues of native arrestin (*e.g.* ARR(-6–404) and ARR(-6–191)). In addition, deletion of the NH2 terminus of arrestin (residues 2–16) also reduces its ability to discriminate activation state. However, since ARRΔ(2–16) and ARR(Δ(2–16)-365) are still able to discriminate between light-activated and dark rhodopsin this suggests that this region may play more of a structural role in activation recognition.

While ARR(-6–191) and ARR(1–191) still bind preferentially to the activated state of rhodopsin, ARR(-6–145), ARR(1–145), and shorter arrestins preferentially bind to the dark state of rhodopsin. This preference for activation state is also seen with truncated [329]G-rhodopsin where ARR(-6–404), ARR(-6–365), and ARR(-6–191) prefer light-activated rhodopsin, while ARR(-6–145) prefers dark rhodopsin (Fig. 5). This demonstrates that residues 145–191 of arrestin also participate in activation recognition and contain a site or sites necessary for normal arrestin selectivity. This brings the minimal number of sites involved in activation recognition to three: one at the NH2 terminus (residues 2–16), one between residues 17 and 145, and yet another between residues 145 and 191. Moreover, the finding that ARR(-6–404) and ARR(-6–191) binding to Rh* are equally inhibited by ionic strength (IC_{50} ~ 150 mM) suggests not only that hydrophilic interactions of moderate strength are involved in activation recognition but also that additional activation recognition sites COOH-terminal to residue 191 are unlikely.

Hydrophobic Interaction Site—Since the ability of arrestin to individually recognize the activation state or phosphorylation state of rhodopsin is inhibited by high salt, the binding of arrestin to P-Rh* would be expected to be somewhat less sensitive to high salt inhibition due to the cooperative nature of the two-site interaction. While this is exactly what is observed for ARR(-6–191) binding, this is not seen with ARR(-6–365) and ARR(-6–404) (Fig. 4). In fact, the binding of ARR(-6–404) to P-Rh* is stimulated at physiological ionic strength (~150 mM) and then declines at substantially higher concentrations ($IC_{50} > 800$ mM). The binding of ARR(-6–365) is even less sensitive to inhibition by salt, being stimulated at up to 500 mM before slowly declining with an $IC_{50} > 1$ M. These results suggest that the binding of ARR(-6–404) and ARR(-6–365) to P-Rh* involves both hydrophobic and ionic interactions. This hydrophobic interaction is not observed for ARR(-6–191) binding to P-Rh* nor for any arrestin binding to either dark P-Rh or Rh* (Fig. 4).

Based on these results we hypothesize that there is a hydrophobic binding site on arrestin, localized between residues 191 and 365, that becomes accessible to rhodopsin only upon simultaneous binding of the activation and phosphorylation recognition sites on arrestin. This rearrangement of the arrestin molecule, that exposes the hydrophobic or "booster" site, is most likely the major conformational change involved in arrestin binding that was predicted by Schleicher and co-workers (12) on the basis of very high Arrhenius activatiᴏn energies for binding. This substantial conformational change, triggered by the simultaneous binding of the activation and phosphorylation recognition sites which mobilizes the booster site, appears to be the mechanism that ensures the strict selectivity of full-length arrestin binding to P-Rh* (see below). While the contribution of the booster site to the stability of the arrestin-rhodopsin complex at high salt concentrations is clearly suggested by our data, the direct contribution at physiological salt concentrations is unknown. In addition, the same conformational change that mobilizes the booster site may also lead to an increased affinity of the activation and/ or phosphorylation recognition sites for rhodopsin. It is also worth noting that although arrestin interaction with Rh* was previously demonstrated, this interaction did not induce extra metarhodopsin II formation (39, 40). Thus, it seems likely that arrestin must undergo a conformational rearrangement, which occurs only upon interaction with P-Rh*, to stabilize the metarhodopsin II state.

Regulatory Region—Truncation of the acidic COOH-terminal region of arrestin (residues 355–404) dramatically reduces the selectivity of arrestin binding, suggesting that the COOH terminus plays an important regulatory role. Our studies suggest that this region is involved in maintaining the overall tertiary structure of arrestin, specifically the conformation of the COOH-terminal half of the molecule and its orientation to the NH2-terminal half. This is suggested by the relative instability of ARR(-6–365) and ARR(-6–355) compared with the high stability of both full-length arrestin and ARR(-6–191). The central portion of this region (residues 365–391) may be involved in maintaining a rigid conformation which contributes to both the stability and the exquisite selectivity of arrestin. This selectivity is imparted by the inability of full-length arrestin to bind appreciably to either Rh* or P-Rh and thus to induce conformational changes and mobilize the booster site. In contrast, the binding of ARR(-6–365) to either Rh* or P-Rh appears to partially mobilize the booster site. This is suggested by the ~2-fold
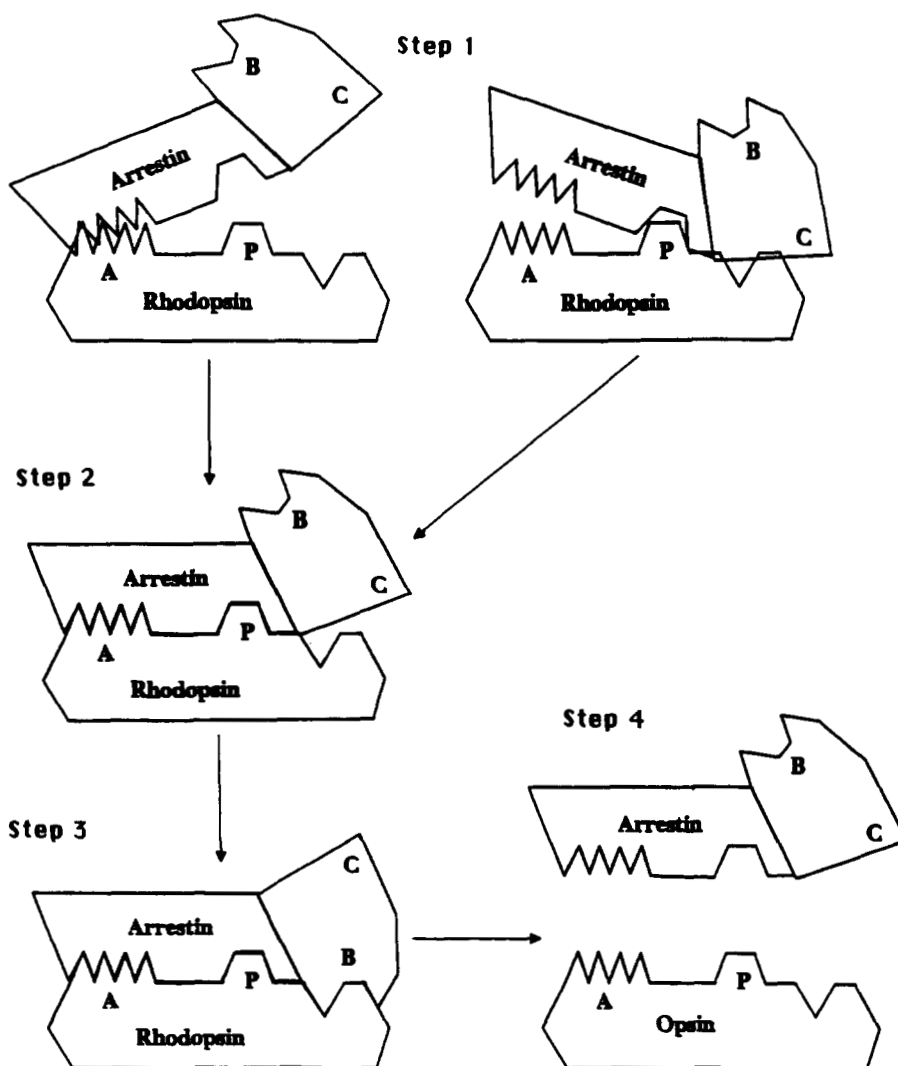
FIG. 6. **Model of arrestin-rhodopsin interactions.** The portions of the proteins involved are marked as follows. *A*, domain(s) on rhodopsin that changes conformation upon light-activation; *P*, phosphorylated rhodopsin COOH terminus; *B*, arrestin booster site; *C*, arrestin carboxyl terminus. *Step 1*, arrestin binds via its activation or phosphorylation recognition site to the respective portion of rhodopsin. *Step 2*, if rhodopsin is both activated and phosphorylated, the second of these two arrestin sites binds to rhodopsin. *Step 3*, the binding of arrestin to both sites on rhodopsin promotes a conformational rearrangement of arrestin that makes the booster site available. *Step 4*, the decay of metarhodopsin II to opsin accelerates arrestin dissociation. See text for details.

lower salt sensitivity of ARR(-6–365) binding to Rh* ($IC_{50} \sim$ 350 mM) as compared to ARR(-6–404), that cannot mobilize the booster site, or ARR(-6–191), that lacks the booster site, binding to Rh*. ARR(-6–365) binding to P-Rh* is also much less sensitive to salt inhibition than is full-length arrestin. Conceivably this is due to the increased flexibility of ARR(-6–365) enabling it to better accommodate binding to all forms of rhodopsin. The reduced selectivity of ARR(-6–365) is also demonstrated by its ability to bind to dark Rh. Thus, the COOH-terminal region of arrestin helps to maintain the overall arrestin conformation and control functionally relevant conformational changes. Given the importance of this region it is also a likely target for putative regulators of arrestin function such as phosphorylation. Finally, in order for this acidic regulatory region to react to the occupancy status of the activation and phosphorylation recognition sites, it may well interact with other domains on arrestin involved in binding selectivity (*e.g.* the basic NH2-terminal region).

NH2-terminal Region—The deletion of residues 2–16 in arrestin leads to both a significant decrease in affinity for rhodopsin as well as impaired selectivity. The behavior of ARRΔ(2–16) most closely resembles that of COOH-terminal truncated arrestins such as ARR(1–369). This is manifested as both a reduced binding to P-Rh* and an increased binding to Rh*. However, unlike the COOH-terminal-truncated arrestins, ARRΔ(2–16) maintains substantial specificity for P-Rh*

in relation to P-Rh. When the COOH terminus is truncated from ARRΔ(2–16), producing the mutant ARR(Δ(2–16)-365), its selectivity is, if anything, enhanced. Since removal of the COOH-terminal region of ARRΔ(2–16) does not dramatically alter its binding selectivity this suggest that residues 2–16 may be involved in interaction with the regulatory COOH-terminal region. In addition, the extremely low affinity and selectivity of arrestin (Δ(2–16)-191) suggests that residues 2–16 in the NH2-terminal domain may also play a role in maintaining the functional conformation of this domain.

Our results suggest that residues 2–16 of arrestin play both a structural as well as a functional role relaying information about the occupancy status of the primary binding sites on arrestin to the COOH-terminal domain. It is conceivable therefore that the Δ(2–16) deletion impairs both activation and phosphorylation recognition without directly participating in either one of these functions, although at present we cannot rule out a direct role for residues 2–16 in arrestin/rhodopsin binding. A series of more subtle mutations in this region will be necessary to address this issue.

Rhodopsin Phosphorylation Level Required for Arrestin Binding—In an effort to assess the effect of phosphorylation level on arrestin binding we utilized two types of phosphorhodopsin in our experiments. One preparation contained a medium level of phosphorylation ($\sim$2.1–2.2 phosphates/rhodopsin) while the other contained a low level of phosphoryl-

ation (~0.6 phosphates/rhodopsin). Due to the apparent stochastic nature of rhodopsin phosphorylation, rhodopsin containing ~2.1 mol/mol is likely a mixture of mono-, di-, and triphosphorhodopsins, with small amounts of highly phosphorylated (>3 mol/mol) and non-phosphorylated rhodopsin. The lower phosphorylation level preparation should contain predominately rhodopsin and monophosphorhodopsin in an ~1:1 ratio, with small amounts of di- and possibly triphosphorhodopsin (*cf.* in 33). The binding of full-length arrestin to the low stoichiometry P-Rh* is much lower than expected if a single phosphate is sufficient for the change of arrestin affinity from that characteristic for Rh* to that for P-Rh*. In fact, the difference in full-length arrestin binding between the low stoichiometry phosphorhodopsin and rhodopsin appears only upon activation of the latter and most, if not all, of this difference could be accounted for by a small (5–10%) fraction of polyphosphorhodopsin (Fig. 3). Conceivably, when the regulatory COOH-terminal domain controls binding specificity, the interaction of the phosphorylation recognition site with monophosphorhodopsin is too weak to promote significant binding to dark P-Rh. Thus, the weak binding would be insufficient to mobilize the booster site and bring about the conformational change necessary to overcome the constraint from the regulatory region. Both ARR(-6–191) and ARR(1–191) also clearly discriminate between phosphorhodopsin and rhodopsin. Again, the binding of truncated arrestin to P-Rh* containing low phosphate levels is significantly less than expected if one phosphate is sufficient to promote complete interaction.

Arrestin Interaction with Opsin—In 1987 it was predicted that arrestin was involved in the regulation of rhodopsin dephosphorylation (41). Indeed, arrestin was found to be the most slowly released protein among all that bind to rod outer segment membranes in a light-dependent manner (42). It has been shown that arrestin specifically inhibits the ability of protein phosphatase 2A to dephosphorylate freshly bleached phosphorhodopsin while it has no effect the dephosphorylation of dark phosphorhodopsin (43). Recently, it was reported that arrestin binding to opsin requires the presence of all-*trans*-retinal, forming a product that is spectrally indistinguishable from metarhodopsin II. This product does not activate transducin, although it still binds arrestin and serves as a substrate for rhodopsin kinase (44). The authors hypothesize that arrestin dissociation (and subsequent rhodopsin dephosphorylation) requires prior dissociation of all-*trans*-retinal and its reduction to retinol, which does not interact with opsin.

In our studies, hydroxylamine treatment of opsin (to eliminate bound all-*trans*-retinal (44)) reduced arrestin binding to opsin to negligible levels (data not shown). In contrast, arrestin binding to phosphoopsin (the physiologically relevant species) was reduced only 2.5–3-fold by hydroxylamine treatment and was still higher than the binding to dark P-Rh. The binding of ARR(-6–365) to opsin and phosphoopsin was found to be even less sensitive to retinal dissociation. While the kinetics of retinal dissociation and reduction, arrestin dissociation, and phosphoopsin dephosphorylation remain to be determined, it is likely that arrestin is an important regulator of phosphoopsin dephosphorylation, acting even after all events relevant to the activation cascade and quenching have ended.

Current Model of Arrestin-Rhodopsin Interaction—Based on all of the available data, we propose the following multistep model of arrestin interaction with rhodopsin (Fig. 6). Arrestin is able to weakly bind to any given functional form of rhodopsin, enabling it to probe the functional state of the latter.

Thus, arrestin can bind to either to the phosphorylated COOH terminus of rhodopsin via phosphorylation recognition sites or to regions of rhodopsin that change conformation upon activation via activation recognition sites (*step 1*). If rhodopsin is either light-activated or phosphorylated, this ionic binding of moderate strength enables arrestin to rapidly dissociate. However, when rhodopsin is both phosphorylated and light-activated, arrestin binds to both sites on rhodopsin (*step 2*). This simultaneous binding of the phosphorylation and activation recognition sites of arrestin to their respective counterparts on rhodopsin triggers a substantial conformational change in arrestin (12). This conformational change appears to be controlled by the COOH-terminal region of arrestin possibly via its interaction with the NH$_2$-terminal domain. This promotes the binding of a third domain on arrestin, termed the booster site, that is hydrophobic in nature. This conformational change also appears to increase the affinities of both primary binding sites to their respective counterparts on rhodopsin (*step 3*). Following this conformational rearrangement, the binding of arrestin to rhodopsin becomes stronger and is not readily reversible. Arrestin remains bound at least until metarhodopsin II decays and, most likely, even longer. Since arrestin binding to phosphoopsin is of lower affinity (Fig. 3), the decay of metarhodopsin II accelerates arrestin dissociation (*step 4*). Our data suggest that the affinity of arrestin for phosphoopsin increases with increased phosphorylation level. Thus, arrestin is more readily released from opsin with lower levels of phosphorylation.

Future Prospects—We have identified multiple functional domains on arrestin that are involved in its specific binding to phosphorylated light-activated rhodopsin. The phosphorylation recognition domain on arrestin is localized predominately between residues 158–185, the most basic amino acid stretch in the arrestin molecule. It remains to be determined which of the nine positive charges in this region of arrestin are involved in binding to P-Rh*. Our data do not allow us to determine whether the positively charged amino-terminal domain of arrestin (residues 2–16) is also directly involved in phosphorylation recognition. In addition, it is presently unclear how many phosphates are required for high affinity arrestin binding to P-Rh* although our data suggest that it is likely more than one. It will be interesting to determine whether the affinity of arrestin for rhodopsin increases gradually with increased phosphate level or whether the binding is triggered at some set minimum number of phosphates.

There appear to be multiple domains on arrestin that are involved in activation recognition. These include at least three regions within the NH$_2$-terminal 191 residues of arrestin. The high conservation of residues 100–120 among all of the arrestins (17, 45–50) suggests that this may serve as a structural basis for the demonstrated arrestin interchangeability (51). In contrast, the relative variability of the NH$_2$-terminal sequences of the arrestins (17, 45–50) might play a role in receptor specificity (45, 52). The presence of both activation and phosphorylation recognition sites on the NH$_2$-terminal half of arrestin as well as its high thermostability suggests that this region of arrestin may form a separate structural as well as functional domain. In fact, a similar conclusion was previously made on the basis of the accessibility of different regions of arrestin to limited proteolysis (19).

We also identified a third domain of interaction between arrestin and rhodopsin that is localized between residues 191 and 355 on arrestin. This region was termed the booster site since it is mobilized as the result of the simultaneous binding of the two primary sites (activation and phosphorylation recognition) to their counterparts on rhodopsin. More accu-

rate localization of the booster site remains to be determined. However, several relatively hydrophobic stretches (amino acids 193–197, 218–227, 238–255, 267–286, 304–311, and 319–339) are possible candidates. It also seems plausible that this "double-trigger" mechanism of binding that ensures the strict selectivity of arrestin for P-Rh* (*i.e.* the binding of a protein to "indicator" sites on another protein triggers conformational changes that promote additional binding), might serve as a general mechanism for controlling protein/protein interactions.

It remains to be determined whether β-arrestin (45), as well as other arrestins (46–50), utilize a mechanism similar to visual arrestin to ensure receptor binding selectivity. We believe that the cell-free expression system and direct binding studies of the radiolabeled arrestins serve as a powerful tool for future characterization of the various natural, mutant, and chimeric arrestins.

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