Identification of Regions of Arrestin That Bind to Rhodopsin†

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ABSTRACT: Arrestin facilitates phototransduction inactivation through binding to photoactivated and phosphorylated rhodopsin (R*P). However, the specific portions of arrestin that bind to R*P are not known. In this study, two different approaches were used to determine the regions of arrestin that bind to rhodopsin: panning of phage-displayed arrestin fragments against R*P and cGMP phosphodiesterase (PDE) activity inhibition using synthetic arrestin peptides spanning the entire arrestin protein. Phage display indicated the predominant region of binding was contained within amino acids 90−140. A portion of this region (residues 95−140) expressed as a fusion protein with glutathione *S*-transferase is capable of binding to rhodopsin regardless of the activation or phosphorylation state of the receptor. Within this region, the synthetic peptide of residues 109−130 was shown to completely inhibit the binding of arrestin to rhodopsin with an IC_{50} of 1.1 mM. The relatively high IC_{50} of this competition suggests that this portion of the molecule may be only one of several regions of binding between arrestin and R*P. A survey of synthetic arrestin peptides in the PDE assay indicated that the two most effective inhibitors of PDE activity were peptides of residues 111−130 and 101−120. These results indicate that at least one of the principal regions of binding between arrestin and R*P is contained within the region of residues 109−130.

Rhodopsin is the G protein-coupled receptor that transduces light signals in photoreceptor cells. This receptor, coupled with the accompanying phototransduction cascade, is exquisitely sensitive, being capable of detecting single photons. The components involved in the activation and amplification cascade, which ultimately leads to a neural impulse, have been almost completely identified (see refs *1* and *2* for recent reviews).

Equally important to the efficient functioning of the transduction cascade is the inactivation process which allows the rapid recovery and repriming of the transduction components. For rhodopsin, the inactivation involves phosphorylation of the photoactivated rhodopsin on the C-terminus by rhodopsin kinase (*3*, *4*). This phosphorylation promotes the high-affinity binding of arrestin, thus blocking the binding and activation of the G protein (transducin), effectively completing the inactivation of rhodopsin (*5*, *6*). The binding of arrestin is very specific for rhodopsin that is both phosphorylated and photoactivated (*5*), with arrestin remaining bound to rhodopsin until the retinal chromophore is released (*7*).

In addition to rhodopsin, evidence is accumulating that many G protein-coupled receptors (GPCRs)[1] are desensitized using homologous kinase and arrestin proteins (*8*−*11*). Similarly, these activated GPCRs are phosphorylated by a G protein-coupled receptor kinase which promotes the binding of an arrestin-like protein. In these other receptor systems, arrestin-like proteins not only sterically block G protein binding but also can promote receptor sequestration through clathrin-mediated endocytosis (*9*, *10*, *12*−*14*).

The availability and abundance of rhodopsin and arrestin in retina have allowed these retinal proteins to become one of the principal model systems for understanding the regulation of other G protein-coupled receptor systems. Studies in which synthetic peptides matching the sequence of rhodopsin were used have determined that arrestin binds principally to the cytoplasmic loop between transmembrane segments V and VI of rhodopsin, and also to a lesser extent to the cytoplasmic loop between transmembrane segments I and II (*15*). Some specific mutations in loop I−II (N73A) and loop III−IV (P142A and M143A), however, cause a substantial decrease in the extent of arrestin binding (*16*), suggesting that these two cytoplasmic loops may be more important in arrestin binding than indicated by the peptide competition studies. Several research groups have independently established that a cluster of positively charged amino acids near the middle of the arrestin polypeptide are important for the ability of arrestin to discriminate phosphorylated and photoactivated rhodopsin from photoactivated rhodopsin

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[1] Abbreviations: DHPP, 4-(1′,1′-dimethyl-1′-hydroxypropyl)phenoxyacetyl-alanyl-aminomethyl resin; Fmoc, 9-fluorenylmethoxycarbonyl; GPCR, G protein-coupled receptor; GST, glutathione *S*-transferase; HOBt, 1-hydroxybenzotriazole; MCS, multiple cloning site; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDE, phosphodiesterase; R, rhodopsin; R*, photoactivated rhodopsin; RP, phosphorylated rhodopsin; R*P, phosphorylated and photoactivated rhodopsin; ROS, rod outer segments; TFA, trifluoroacetic acid.

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(*17*−*21*). Furthermore, it is clear that the carboxyl terminus of arrestin is also important in keeping arrestin in an inactive state, allowing arrestin to bind to photoactivated rhodopsin only when it has been exposed to the phosphorylated C terminus of rhodopsin (*22*−*25*). However, the portions of arrestin that promote the high-affinity binding (K_d = 20−50 nM; *26*, *27*) to rhodopsin have not been identified.

In this study, two different techniques were used to examine the sequence of arrestin that binds to R*P. First, random portions of arrestin were displayed on the surface of the filamentous M13 phage and allowed to bind to R*P to identify regions of arrestin that could bind to R*P. This technique has been used previously to identify binding domains in other proteins, such as between c-myb and its coactivator CBP (*28*), and to map monoclonal antibody binding epitopes (*29*). The second approach made use of a library of two sets of overlapping synthetic 20-residue peptides covering the entire sequence of arrestin. These peptides were used to compete with transducin binding to rhodopsin in a cGMP PDE assay. These two techniques both indicate that the region of residues 101−130 of arrestin contains a principal binding domain for rhodopsin.

EXPERIMENTAL PROCEDURES

Vector Construction. The pCANTAB 5E phagemid vector, which contains the cDNA encoding the protein III coat protein of the filamentous M13 phage, was obtained from Pharmacia linearized at the *Not*I and *Sfi*I sites (Recombinant Phage Antibody System Kit, Pharmacia). A multiple cloning site (MCS) was added to this vector in two stages. First, an *Sfi*I−*Xho*I−*Sac*I−*Not*I linker was prepared using two complementary synthetic oligonucleotide primers (5′-GAGGCCCAGCCGGCCTCGAGCTCACTCAGTCTCCAGCGGCCGCAC and 5′-GTGCGGCCGCTGGAGACTGAGTGAGCTCGAGGCCGGCTGGGCCTC). This linker was cloned into the commercial pCANTAB 5E. Subsequently, a blunt cloning site was desired; consequently, the modified phagemid was linearized at the *Sac*I site to allow the insertion of an *Eco*RV site. A palindromic *Sac*I−*Eco*RV−*Sac*I oligonucleotide primer was synthesized (5′-GCGAGCTCGATATCGAGCTCGC), self-annealed, digested with *Sac*I, and ligated into the modified phagemid. The resultant phagemid with an *Sfi*I−*Xho*I−*Sac*I−*Eco*RV−*Sac*I−*Not*I MCS (pCAN-ECO253) was used in all subsequent experiments.

Insert Preparation and Library Construction. Random fragments of bovine arrestin cDNA were prepared for insertion as follows. The coding region for bovine arrestin cDNA was obtained by polymerase chain reaction (PCR) using primers that specifically annealed to the 5′- and 3′-ends of the coding region of arrestin cDNA (5′-GCGAATTCGCTAGCCATATGAAGGCCAATAAGCC and 5′-CTAAGCTTTCACTCATCCATAGCCGC, respectively). These PCR amplifications were performed using *Pfu* polymerase (Stratagene) to minimize the number of errors introduced during DNA replication in the PCR process. Bovine arrestin cDNA was gel purified (Qiaex II, Qiagen) and sonicated for 2 min with a microprobe sonicator (Microzoon ultrasonic cell disrupter with a 2 mm sonicator tip) at 45% maximum output to produce randomly sized fragments of arrestin cDNA. Agarose electrophoresis of an aliquot of the cDNA before and after sonication showed that of the original 2 μg sample, approximately 5% remained full-length and the majority of the sonicated fragments were in the 200−600 bp range. These sonicated fragments were then ligated into 1.3 μg of pCAN-ECO253 phagemid that had been linearized with *Eco*RV and dephosphorylated by treatment with calf intestinal alkaline phosphatase. The ligated constructs were used to transform competent *Escherichia coli* TG1 cells (13 mL) and plated onto 13 separate plates of SOB medium (*30*) containing 2% glucose and 100 μg/mL carbenicillin (Sigma). The suppressor strain TG1 of *E. coli* was selected since the pCANTAB 5E parent phagemid contains an amber stop codon downstream of the *Eco*RV insertion site, thus allowing for the complete formation of protein III. The resultant library contained 3.8×10^3 colony-forming units with a background of 5% of nonrecombinant colonies. Glycerol stocks were prepared from these plates by scraping the colonies from the plates in 25 mL of 2× yeast-tryptone medium (2×-YT; *30*), adding glycerol to a final concentration of 15%, and freezing aliquots (−80 °C).

To ensure that there was not an inherent bias in the library, the library was probed with defined fragments of arrestin cDNA to obtain an estimate of the representation of the different portions of arrestin in the library. Approximately 1500 colony-forming units were plated on each of nine separate 2×-YT plates (150 mm) containing 100 μg/mL carbenicillin and 2% glucose (w/v) and grown overnight. Colonies on each plate were transferred to nylon membrane (Magna Nylon, Micron Separations, Inc.), denatured by autoclaving for 3 min, and the DNA was linked to the membrane by exposure to ultraviolet light (120 mJ/cm^2, Stratalinker, Stratagene). Membranes were then individually probed with a specific portion of the arrestin cDNA prepared as follows. Portions of arrestin cDNA approximately 100−200 bp in size were amplified by PCR (1−150, 5′-GCGAATTCATGAAGGCCAATAAGCCCGC and 5′-CTCAGGATCCACCAGCACGA; 68−250, 5′-CCATCTACCTGGGGAAGAG and 5′-GAGGTCCCTGAGGAAGCTGA; 246−410, 5′-CCTCTACTTCTCCCAGGTCCAGGT and 5′-TGCGGAGCTGGCTGCAGCAT; 409−612, 5′-CAAGATGTGGGCAAGAGCTG′ and 5′-GCAAGCTTGCGCAGGGGCTTGTCCGA; 547−761, 5′-CGGGTACCGATATGGGTCCCCAGC and 5′-GCAAGCTTATAATCACTCGAGTAGAG; 653−839, 5′-AACCCATTCCTGTGACCGTG and 5′-GCAAGCTTCGTCAGCGTCTTGGT; 810−935, 5′-AAACAGCTCGCTGACCAAGA and 5′-ATGATGGTGCTGGAGGCCAG; 938−1107, 5′-AAGGAGGGCATCGACCGGACCGTC and 5′-GCAAGCTTTCAGGCACTTTCCTTGGCGGTATCTG; and 1092−1215, 5′-GATACCGCCAAGGAAAGTTT and 5′-GCGAATTCTCACTCATCCATAGCCGCCTC. Each PCR mixture contained 1 ng of bovine arrestin cDNA in a plasmid vector, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 15−35 pmol of each oligonucleotide primer, and 2 units of *Tbr* polymerase (PrimeZyme, Biometra), and the mixtures were amplified for 35 cycles as follows: 94 °C for 45 s, 45 °C for 45 s, and 72 °C for 45 s. Following amplification, the PCR products were gel purified (QIAEX II, Qiagen), and 50 ng of each reaction product was labeled with [α-^{32}P]dCTP (Oligolabelling Kit, Pharmacia). Probes were hybridized to the colony lifts in aqueous Denhardt's

solution as previously described (*31*), and the number of positive colonies was counted. The number of colonies for each probe was normalized by dividing the number of colonies by the probe length.

It should be noted that the fragmentation of the arrestin cDNA for insertion into the phagemid vector was originally performed with DNaseI in the presence of Mn^{2+} (*32*). However, the phage libraries produced from these fragments showed large biases toward some portions of the arrestin cDNA, possibly due to secondary structure in the cDNA.

Phage Production and Isolation. For recombinant phage production, an aliquot of the library was thawed and added to 10 mL of 2×-YT and the mixture incubated for 45 min at 37 °C while it was being shaken. The culture was then diluted to an OD_{600nm} of 0.3 in 2×-YT with 2% glucose and 100 μg/mL carbenicillin, and allowed to incubate an additional 60 min. Helper phage carrying kanamycin resistance (M13K07, Pharmacia) were added to the culture at an approximate multiplicity of infection of five helper phage per each bacterial cell. After the helper phage were allowed to infect the *E. coli* for 1 h, the culture medium was changed to 2×-YT with carbenicillin (100 μg/mL) and kanamycin (50 μg/mL), and the mixture was cultured overnight (37 °C). The use of the phagemid vector and helper phage generate phage that on average have zero to one copy of recombinant protein III among the five copies of protein III that are present on the mature phage particle.

The supernatant containing the secreted recombinant phage was collected by centrifugation, filtered (0.45 μm) to remove unsedimented bacteria, and phage precipitated by the addition of $^1/_5$ volume of 20% polyethylene glycol 8000 (w/v) with 2.5 M NaCl. These precipitated phage were pelleted (10000*g* for 20 min at 4 °C) and resuspended in panning buffer [equal parts of 2×-YT and 1% bovine serum albumin (w/v) in phosphate-buffered saline [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.4)], using $^1/_{10}$ of the volume of the overnight phage culture volume. The resuspended phage were again filtered (0.45 μm) to remove any aggregates of phage particles.

Preparation of Rod Outer Segment (ROS) Membranes and Red Blood Cell Membranes for Use in the Phage Display Assay. Bovine rod photoreceptor cell membranes containing phosphorylated rhodopsin were prepared as described, measuring rhodopsin concentration spectroscopically with a molar extinction coefficient of 40 600 M^{-1} s^{-1} at 498 nm (*22*). Membranes from lysed red blood cells (ghosts) were prepared according to the methods of Hanahan and Ekholm (*33*). To obtain an approximately equivalent amount of membranes, the protein content of the ghosts was measured (Micro BCA protein analysis kit, Pierce), and the same quantity of protein in the ghosts was used as rhodopsin in disk membranes.

Panning and Rescue. Selection of phage, also known as panning or biopanning (*34*), was performed using 1 mL aliquots of the resuspended phage solution in microcentrifuge tubes that had been coated with 1% BSA in PBS prior to use. For selection of phage against photoreceptor cell disk membranes, 100 μg of phosphorylated rhodopsin was added to the phage suspension in dim red light, with the rhodopsin subsequently photoactivated by the exposure to flood lights at 20 cm for 1 min on ice. Selection of phage against ghosts was obtained by adding red blood cell membranes containing 100 μg of protein to the phage suspension. These panning mixtures were allowed to incubate for 2 h while being gently rotated in the dark at 4 °C. Phage that had bound to the membranes were separated from the unbound phage by centrifugation (30000*g* for 10 min at 4 °C). The membrane pellet was washed twice with 1 mL of PBS and twice with 1 mL of PBS containing 0.1% Tween 20, pelleting the membranes between each wash as described above. The membrane pellets obtained from these two reactions (photoreceptor and ghosts) were approximately equal in size, indicating that protein content is an adequate first approximation for achieving equivalent membrane amounts from photoreceptor disks and red blood cell ghosts.

Following the final wash, the phage remaining associated with each membrane pellet were acid eluted by resuspending in 400 μL of 0.5 M NaCl in 140 mM sodium phosphate buffer (pH 4.0) (15 min, 4 °C). This particular pH was empirically determined to give the highest yield of phage without substantial damage to the phage particles due to acid hydrolysis. The supernatant was collected following centrifugation (30000*g* for 10 min) and neutralized with an equivalent volume of alkaline PBS (pH 11.0). These phage were then mixed with an equal volume of 2×-YT medium, and the entire panning process was repeated three times. After the final pan, including the PBS and PBS/Tween washes, the phage bound to the disk membranes were added to 10 mL of TG1 *E. coli* culture that were in the logarithmic growth phase, and allowed to infect the cells for 1 h at 37 °C. Following infection, cells were plated on SOB plates (*30*) containing carbenicillin (100 μg/mL) and 2% glucose (w/v).

DNA Sequencing. The portion of arrestin being displayed on individual phage particles was determined by sequencing through the cloning site in the gene III cDNA to determine the portion of arrestin cDNA that had been randomly cloned. Individual colonies were cultured, and plasmid cDNA was isolated (Wizard Prep, Promega). Sequencing was performed using the dideoxy chain termination method (*35*; Sequenase v2.0, Amersham), separating the products by electrophoresis with an 8% denaturing polyacrylamide gel (Sequagel-8, National Diagnostics). Primers used for sequencing in the forward and reverse directions were 5′-CCAAGCTTTG-GAGCCTTTTTTTTGGAG and 5′-CTTTCAACAGTCTAT-GCGGCACG, respectively.

Creation and Expression of the Arrestin 95−140/GST Fusion Protein. A portion of arrestin spanning amino acids 95−140 was expressed as a carboxyl-terminal fusion protein with glutathione *S*-transferase. The cDNA construct was created using oligonucleotide primers to add an *Eco*RI site immediately upstream of the cDNA encoding Gly-95 (5′-GCGAATTCGTGGGGGCCTCGGGCGCCACCACG) and a *Sal*I site immediately downstream of the cDNA encoding Gly-140 (5′-GCGTCGACTGCCCACATCTTGCGGAGCTG-GCTG). Arrestin cDNA was amplified by PCR with *Pfu* polymerase (Stratagene) using these two primers and cloned into the *Eco*RI−*Sal*I sites of pGEX-4T-1 (Pharmacia). Proper construction of the fusion cDNA was confirmed by dideoxy sequencing (*35*). The fusion plasmid was transformed into *E. coli* strain BL21 (Novagen), and expression was induced by the addition of IPTG to a final concentration of 0.1 mM. The fusion protein (95−140/GST) was purified using glutathione−agarose (Pharmacia), eluting with 10 mM reduced glutathione (Pharmacia).

Binding and Peptide Competition Assays. Binding of arrestin and the 95−140/GST fusion protein to rhodopsin was assayed in a modified centrifugation assay (*36*). Briefly, 1.4 μM arrestin (purified from bovine retinas; *22*) or 1 μM 95−140/GST was mixed with excess nonphosphorylated or phosphorylated rhodopsin (8 μM) in ROS membranes in PBS containing 1 μM glyceraldehyde-3-phosphate dehydrogenase (Sigma) as an internal standard. The mixture was exposed to white light for 2 min, and then centrifuged in the dark (40000*g* for 30 min). For binding experiments, the pellet was solubilized in Laemmli sample buffer (*37*) and analyzed on a 12% SDS−PAGE. The relative ratio of arrestin and rhodopsin had previously been determined to give complete binding of the available bovine arrestin to photoactivated phosphorylated rhodopsin. An aliquot of phosphorylated ROS membranes was also treated with heat (5 min, 100 °C) to provide a sample of the lipid membrane as a control for nonspecific binding.

For peptide competition studies, the assay was carried out as described above except that synthetic peptides were added prior to illumination of the reaction mixture. The peptide of residues 109−130 of arrestin was synthesized (see Peptide Synthesis) with a serine in place of Cys-128 to improve solubility and handling properties (KKLGANTYPFLLTF-PDYLPSSV). A randomly scrambled peptide of residues 109−130 was also synthesized (KDYLVPLSSNAPLYLGT-FPFTK). In these competition experiments, an aliquot of the reaction supernatant was analyzed by SDS−PAGE so the amount of arrestin competitively released into the supernatant could be measured. Arrestin was quantified by scanning densitometry, normalizing each sample to the internal glyceraldehyde-3-phosphate dehydrogenase band.

Preparation of ROS for the PDE Assay. ROS for the PDE assay were prepared by sucrose density differential centrifugation in ROS buffer that contained 100 mM KCl, 50 mM NaCl, 10 mM $MgAc_2$, and 20 mM MOPS (pH 7.5). Frozen retinas were thawed and then homogenized in ROS buffer supplemented with 2 mM DTT and 1 mM benzamidine. The pellet collected after centrifugation was submitted to the ROS preparation as described previously (*38*). The ROS were collected by centrifugation and then suspended in the buffer used in the phosphodiesterase activity assay [100 mM KCl, 4 mM $MgSO_4$, and 10 mM HEPES (pH 8.0)] to a concentration of about 3 mg of rhodopsin/mL. Milliliter aliquots were frozen in liquid N_2 and stored at −78 °C wrapped in foil in the dark.

Peptide Synthesis. Peptide fragments of bovine arrestin were synthesized using a RAMPS Multiple Peptide Synthesis System (DuPont). "Wang" carboxylate resin cartridges with 0.1 mmol of first Fmoc amino acids on *p*-alkoxy benzyl alcohol resin (DuPont) were used for the synthesis of most peptides. For synthesis of peptides with a C-terminal Fmoc-Pro, DHPP resin (Bachem Bioscience, Inc.) was used. Sequential coupling of amino acids was carried out according to the RAMPS protocol using HOBt esters prepared in situ from Fmoc amino acids. Cleavage and deprotection from resin were carried out by incubating the resin for 3 h at room temperature in 90:5:5 (v/v) TFA/H_2O/ethanedithiol. Peptides were precipitated from solution using cold diethyl ether and lyophilized. Peptides were further purified by size fractionation using Biogel P-2 in 5% acetic acid. Most yielded correct amino acid analyses and with greater than 80% purity as assessed with an analytical HPLC profile. Using this protocol, two sets of arrestin peptides were synthesized. The first set contained peptides with bovine arrestin residues 1−20, 21−40, 41−60, 61−80, 81−100, 101−120, 121−140, 141−160, 161−180, 181−200, 201−220, 221−240, 241−260, 261−280, 281−300, 301−320, 321−341, 342−362, 363−383, and 384−404. The second set was comprised of overlapping peptides containing arrestin residues 11−30, 31−50, 51−70, 71−90, 91−110, 111−130, 131−150, 151−170, 171−190, 191−210, 211−230, 231−250, 251−270, 271−290, 291−310, 311−330, 331−351, 352−372, and 373−393. Some of the peptides were produced with either low yields or insufficient purity as judged by amino acid analysis. These peptides, as well as others used in this work, were synthesized by solid phase techniques using Fmoc derivatives of amino acids with an automatic synthesizer (model 431A from Applied Biosystems). The peptides were then purified by reverse phase HPLC.

Phosphodiesterase Assay. ROS (1 mL) were thawed; 50 μL of 1.9 mM GTP was added, and the mixture was incubated at 25 °C for 30 min. For each determination, 80 μL of ROS, 20 μL of 10 mM ATP (or buffer), 40 μL of 1.9 mM GTP and 1.1 mM GDP, and 360 μL of buffer (or 160 μL of buffer and 200 μL of ca. 0.8 mM peptide in buffer) were combined and incubated for 1 min. Data collection was started at time 0; cGMP (100 μL of a 27.2 mM solution) was added at either 0 or 60 s, and a light flash lasting $^1/_4$ s was applied 60−120 s after the addition of cGMP. The pH was measured using a Beckman F40 pH meter equipped with a Corning flat surface electrode. The data were collected and stored at 1 s intervals using a ComputerBoards CIO-DAS08-PGH analog input card and Control-CB data acquisition program on a Gateway 486-33 computer.

RESULTS

Phage Library Characteristics. A phage library using the M13 filamentous phage was constructed which displayed random portions of bovine retinal arrestin. In brief, the arrestin cDNA was sonicated to create random fragments, which were subsequently ligated into the gene III cDNA in the phagemid vector pCAN/ECO253 (a modified pCANTAB 5E vector, Pharmacia). Arrestin fragments displayed in this vector are fused with protein III in the N1 portion of protein III near the amino terminus. The primary library generated from 2 μg of arrestin cDNA contained 3.8×10^3 colony-forming units (cfu). Sixteen colonies were randomly selected, and the insert size was determined by PCR, using primers that flanked the insertion site. This analysis indicated that the average insert size was 200 bp, ranging from 15 bp to full-length cDNA.

Although the library generated in this manner should be random, it is conceivable that secondary structure in the arrestin cDNA or other factors might introduce a bias in the library. To assess this potential bias, defined portions of the arrestin cDNA, 100−200 bp in size, were used to probe colony lifts of the plated library and the relative frequency of each portion was then counted (Table 1). On average, each cDNA fragment hybridized to 137 colonies, which when corrected for the varying length of the cDNA probes gives 0.83 colony/bp length of cDNA probe [±0.18 (SD)]. With this value as an estimator, all portions of the arrestin cDNA

Table 1: Measurement of the Random Nature of the Arrestin Phagemid Library[a]

portion of the arrestin coding region (nucleotide)	fragment length (bp)	no. of colonies	no. of colonies/bp
1−150	150	132	0.88
68−249	182	120	0.66
246−410	165	131	0.79
409−612	204	181	0.89
547−762	216	156	0.72
653−837	185	147	0.79
810−935	126	108	0.86
938−1107	170	106	0.62
1090−1212	123	153	1.24
			0.83 ± 0.18 ($x \pm$ SD)

[a] Colony lifts were probed with nine portions of the arrestin cDNA, spanning the entire length of the cDNA, and the number of colonies to which each probe hybridized was counted. The final column indicates the number of colonies averaged by the length of each probe.

are represented within one standard deviation from the mean, with the exception of the last 120 bp of the cDNA which is over-represented, and the region spanning nucleotides 938−1107 which is slightly under-represented in the library. This analysis indicates that the primary library contains a reasonably random distribution of arrestin cDNA fragments. Since antibodies are not available for many of the different portions of arrestin, we are unable to address whether there is an *expression* bias in the library.

Panning against Disks and Ghosts. Portions of arrestin that bound to R*P were selected by using disk membranes prepared from bovine ROS. These disk membranes contained rhodopsin that had been previously phosphorylated, subsequently regenerated with 11-*cis*-retinal, and then photoactivated. As a control for phage that might bind nonspecifically to the membranes, phage were also panned against cell membranes prepared from lysed red blood cells (i.e., ghosts). Ideally, the best control for nonspecific binding would be disk membranes without rhodopsin. However, these membranes are very difficult to obtain in quantities suitable for these experiments. Nonetheless, even though the ghosts are not a perfect control for nonspecific binding of the recombinant phage, they at least provide a first approximation for identifying phage that might bind to the lipid membranes as opposed to the R*P contained in the disk membranes.

After the phage were allowed to bind to the different membranes, unbound phage were removed by extensive washing with PBS and PBS with detergent. The bound phage were then acid eluted from the membranes, and used in two additional rounds of panning to highly enrich for the phage that were specifically bound to each substrate. This method of acid elution (as opposed to phage rescue and regrowth of phage particles) was selected to minimize any potential bias introduced by differential phage infectivity and growth rates. Following the third panning cycle, phage remaining bound to the membranes were rescued by the addition of *E. coli* cells, allowing the phage particles to infect the bacteria, and then plating the bacterial solution. The gene III−arrestin fusions in isolated colonies were then sequenced to identify the portion of the arrestin being displayed.

From the original 2.2×10^{13} cfu, 2.4×10^{3} cfu were obtained from the phage that were panned against ROS disk membranes, and 300 cfu were obtained from the phage that

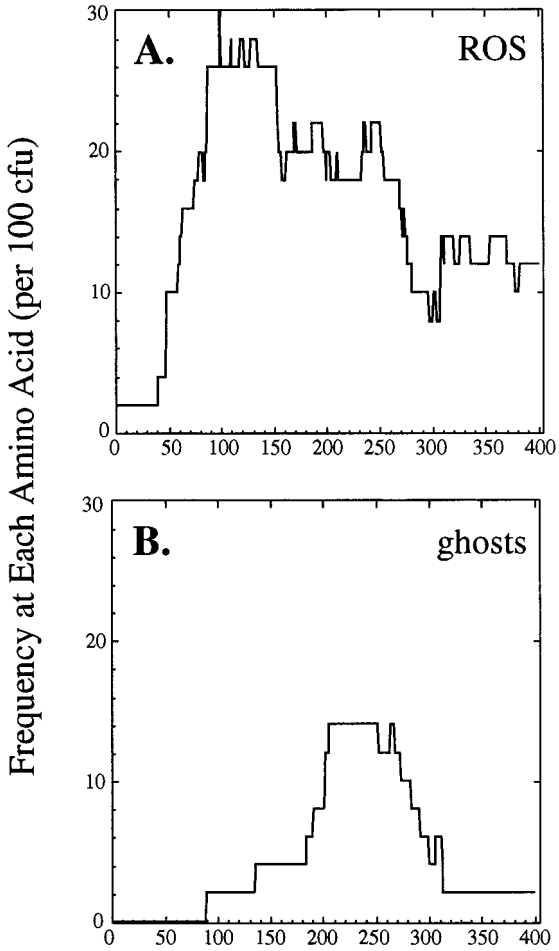

FIGURE 1: Binding of phage displaying portions of arrestin to rhodopsin. The frequency histogram indicates the number of times [per 100 colony-forming units (cfu)] any given amino acid in the sequence of retinal arrestin was represented in a peptide displayed on a phage that bound to photoreceptor disk membranes containing photoactivated and phosphorylated rhodopsin (A) or to ghost membranes prepared from red blood cells (B).

were panned against ghosts. A control tube containing no cell membranes yielded no colonies, indicating that the phage were not binding nonspecifically to the tubes, or aggregating to the extent that they would be pelleted at the centrifugation speeds used in this study. The phagemids were sequenced from 100 colonies from ROS disk panning and 50 colonies from ghost membrane panning. Any clones that were out of frame with the protein III reading frame were discarded from the data set, even though frame shifting is well-documented in M13 phage particularly when a stop codon is encountered (*39*, *40*). This exclusion was deemed the most parsimonious treatment of the data since it is impossible to be certain where the frame shift occurs, and thus, the actual translated product is unpredictable. Using this criterion, 11% of the clones were discarded from the data set.

The remaining clones of arrestin fragments that were displayed which showed binding to R*P in ROS disk membranes are shown in Figure 1A as a frequency histogram for the number of bound clones displaying any given amino acid. This plot shows the highest frequency for phage that displayed amino acids 90−140. For phage that bound to red blood cell membranes (Figure 1B), the highest frequency was obtained for the region of residues 200−280. Note that the peak of phage binding to ROS disk membranes (between

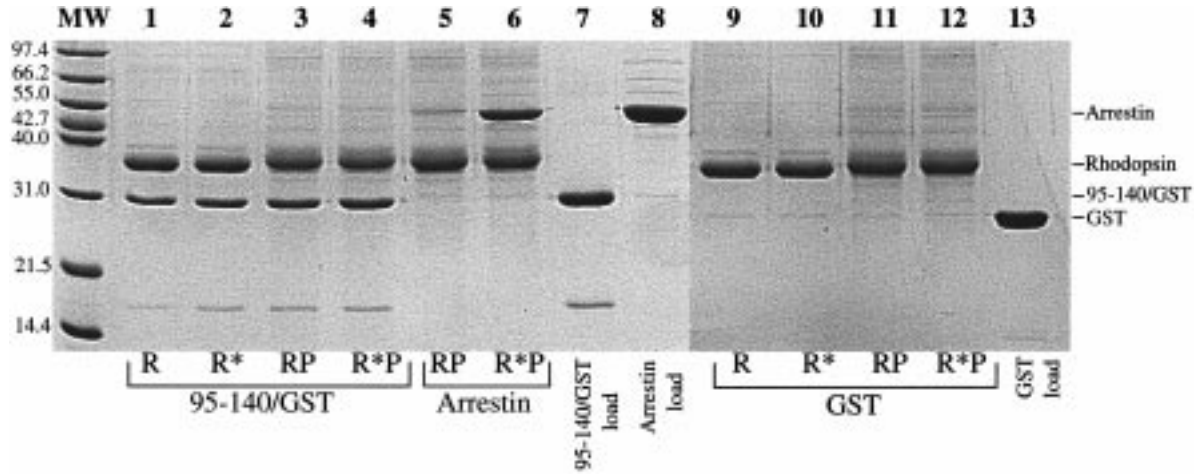

FIGURE 2: Binding of 95−140/GST, arrestin, and GST to various states of rhodopsin. Disk membranes containing rhodopsin were incubated in the presence of the 95−140/GST fusion protein (lanes 1−4), arrestin (lanes 5 and 6), or GST (lanes 9−12). The reaction mixture was centrifuged and the pellet containing rhodopsin and bound proteins submitted to 12% SDS−PAGE and stained with Coomassie blue. Membranes were prepared with rhodopsin (R) or phosphorylated rhodopsin (RP) kept in the dark or illuminated with white light for 2 min to provide activated rhodopsin (R*) and activated phosphorylated rhodopsin (R*P). Lanes 7, 8, and 13 show the amount of purified 95−140/GST, arrestin, and GST, respectively, added in each of the binding reactions. Molecular mass standards (MW) are indicated in kilodaltons.

residues 90 and 140) is almost absent in the phage binding to ghosts. In addition, the relatively few colonies obtained from the phage panned against ghosts indicates that nonspecific binding probably does not contribute significantly to the binding against ROS disk membranes.

The profiles shown in Figure 1 are qualitatively similar to the profiles generated in two similar panning experiments (data not shown), indicating the reproducibility of this technique. The previous two pannings were performed using phage rescue between each of the three rounds of panning as opposed to acid elution at high salt concentrations. The latter set of data is presented since it removes any potential bias that could be introduced by the recombinant phage having differential infectivity and growth rates, a problem previously documented (*41*, *42*).

Binding of the 95−140/GST Fusion Protein. To further investigate the potential contribution of the region of residues 90−140 of arrestin to the binding to R*P, a large portion of this region (95−140) was expressed as an aqueous-soluble fusion protein with glutathione *S*-transferase (95−140/GST; see Experimental Procedures). Binding assays using 95−140/GST and disk membranes containing rhodopsin revealed that this fusion protein bound to rhodopsin regardless of its photoactivation or phosphorylation state (Figure 2, lanes 1−4), albeit with a slightly enhanced level of binding to R*P (lane 4) compared to R (lane 1). Glutathione *S*-transferase without the arrestin peptide does not bind to any form of rhodopsin in disk membranes to a measurable extent (Figure 2, lanes 9−12).

To control for the possibility that the 95−140/GST fusion protein is binding nonspecifically to the lipid portion of the disk membranes, the binding experiment was repeated using disk membranes that had been heated to denature the rhodopsin. Neither arrestin, 95−140/GST, nor GST bound to these denatured membranes (data not shown). Binding is also abolished if 95−140/GST is boiled prior to adding the fusion protein to the disk membranes.

Peptide Competition. To better resolve the binding domain in the region of residues 95−140, overlapping synthetic peptides 20 amino acids in length corresponding to the sequence of arrestin spanning the region of residues 91−150 were used in the centrifugation assay to compete for the binding of arrestin to R*P. Each of these peptides was used at a concentration of 700 μM to compete for the binding of arrestin to R*P (Table 2). From these results, it appears that the most effective competitor peptide in this region is the peptide of residues 111−130 (38% inhibition of binding), with less competition by flanking peptide of residues 101−120 (21% inhibition of binding) or 121−140 (11% inhibition of binding). Additionally, peptides of residues 161−180 and 171−190 were tested for competitive effects because a previous study (*18*) had shown that the peptide corresponding to residues 170−182 could compete for the binding of arrestin to R*P. No effect on binding was seen with either of these two peptides.

Table 2: Inhibition of Arrestin Binding to R*P Using Synthetic Arrestin Peptides[a]

peptide (amino acids)	bound arrestin (% of control ± SD)
91−110	98.3 ± 3.8
101−120	79.4 ± 4.2
111−130	62.2 ± 6.2
121−140	89.1 ± 2.1
131−150	99.6 ± 4.4
161−180	100.3 ± 1.7
171−190	99.8 ± 2.3

[a] Photoactivated ROS membranes containing 8 μM R*P were incubated with 1.4 μM arrestin in the presence of 700 μM synthetic peptides. Following centrifugation, the extent of inhibition of arrestin binding was measured by analyzing the amount of arrestin remaining in the supernatant using SDS−PAGE. Arrestin was quantified by scanning densitometry, normalizing arrestin to an internal standard (glyceraldehyde-3-phosphate dehydrogenase). The means (±SD) from three experiments are shown.

Because peptide residues 111−130 showed limited solubility in PBS, the peptide was resynthesized to include two of arrestin's lysine residues (109 and 110) at the amino terminus (i.e., residues 109−130). In addition, the single cysteine residue in this peptide was also replaced by a serine to avoid any potential for covalent interaction and to further improve solubility. A dilution series of this peptide was used to compete for the binding of arrestin to R*P (Figure 3).

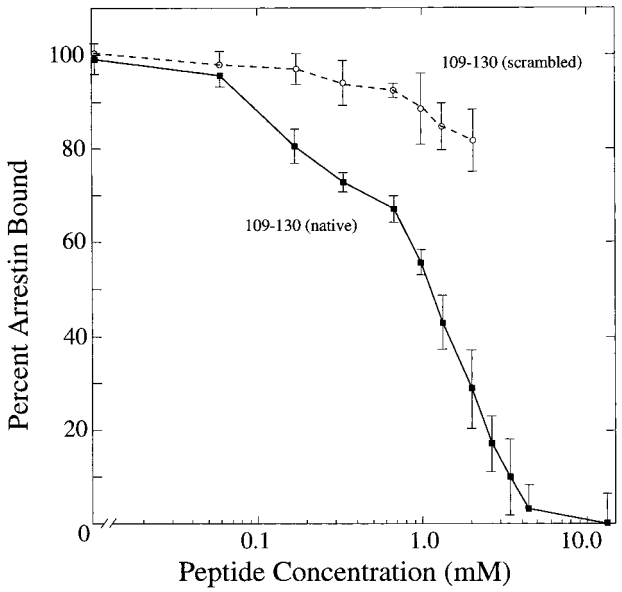

FIGURE 3: Inhibition of arrestin binding to R*P with the peptide of amino acids 109−130. Incubations containing 1.4 μM arrestin, 8 μM R*P in ROS membranes, and the indicated amount of the synthetic peptide of amino acids 109−130 (■) or the scrambled peptide of amino acids 109−130 (○) in the presence of 1 μM glyceraldehyde-3-phosphate dehydrogenase were prepared as described. The amount of arrestin competitively released into the supernatant was measured by SDS−PAGE, stained with Coomassie blue, and quantified by scanning densitometry. The curves represent the means (±SEM) from six experiments.

This peptide can completely inhibit the binding of arrestin to R*P (IC_{50} = 1.1 mM). A scrambled peptide with the same amino acid composition is not an effective competitor at these concentrations; at the IC_{50} for the peptide of residues 109−130, the scrambled peptide gives only a 13% reduction in the level of binding of arrestin to R*P (Figure 3).

PDE Assay. Photoactivated rhodopsin binds to and activates the G protein transducin which in turn stimulates cGMP phosphodiesterase (PDE). Upon hydrolysis of cGMP, H^+ is released which is measured as the light-induced PDE activity. In the presence of ATP and arrestin, the light-induced PDE activity is reduced because the activated rhodopsin is phosphorylated and binds arrestin which prevents further activation of transducin. In vivo, arrestin binds only after the activated rhodopsin is phosphorylated. Experimental evidence has shown, however, that the phosphorylated C terminus of activated rhodopsin serves to "activate" the arrestin so that it binds to other regions of rhodopsin (*22*). Furthermore, the arrestin mutant R175E binds to and quenches photoactivated rhodopsin that is not phosphorylated (*20*, *21*). These observations suggest that peptides containing regions of arrestin that bind to rhodopsin may not require that rhodopsin be phosphorylated to bind to rhodopsin. Because of this binding property, arrestin peptides could modulate the rhodopsin−transducin interaction in two distinct ways. First, the peptide could bind to photoactivated rhodopsin and block transducin binding (i.e., act like arrestin). In this situation, the effect of the peptide would be seen as a decrease in the PDE activity when assayed in the absence of ATP where the endogenous arrestin present in the ROS would have no effect on PDE activity. The peptides comprising residues 91−110, 101−120, and especially 111−130 (black bar) all inhibit the PDE activity in the absence of ATP (Figure 4A). Using the

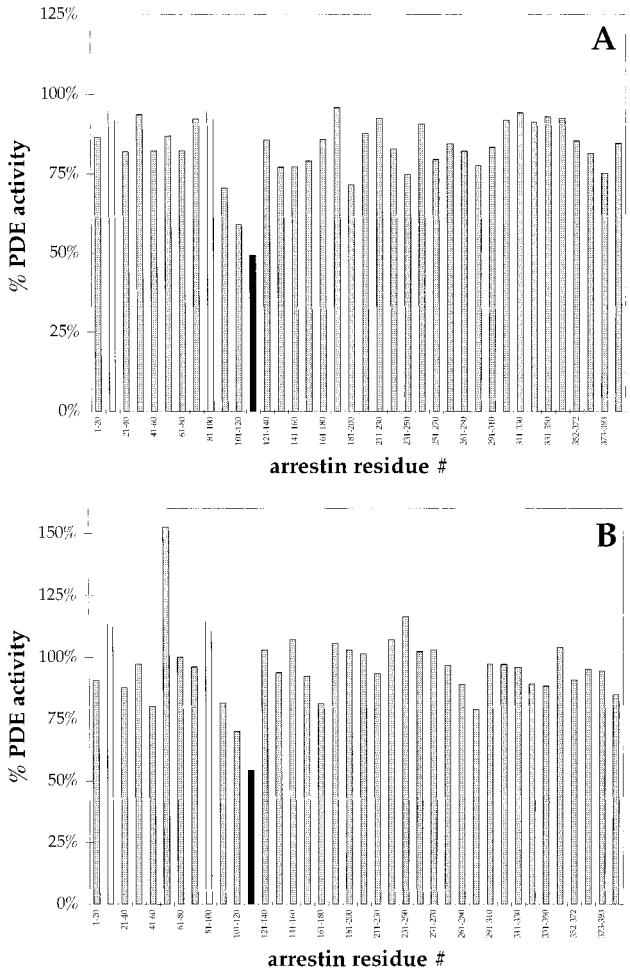

FIGURE 4: Phosphodiesterase activity of rod outer segments in the presence of arrestin peptides without ATP (A) and with ATP (B). The percent measured is the cGMP hydrolyzed at the end of the 4 min experiment in the presence of the peptide compared to its absence with the curves normalized to 0 at the light flash.

synthetic peptide of residues 109−130, with serine substituted for cysteine at position 128, resulted in as effective inhibition of the PDE activity as occurred in the presence of ATP (Figure 5). The scrambled peptide of residues 109−130 had little effect. These data suggest that this region (residues 101−130) binds to photoactivated rhodopsin and successfully competes with transducin binding, inhibiting transducin activation. In these experiments, since there is no ATP present arrestin has little effect on the PDE activity.

The alternative effect of the arrestin peptides is if the peptides could bind to R*P in such a manner that transducin binding is not inhibited, but that blocks the ability of arrestin to bind to R*P (i.e., an arrestin competitor). In this case, the effect of the peptide would be seen as an increase in PDE activity in the presence of ATP since the peptide would be blocking the binding of endogenous arrestin to R*P. This effect is observed for peptides of residues 51−70 and, to a lesser extent, the peptide of residues 231−250 (Figure 4B).

In addition to the effect seen with these peptides, two peptides (residues 11−30 and 81−100, white bars) were found to stimulate PDE activity both in the absence and in the presence of ATP (Figure 4A,B). Tests with isolated PDE showed that this result was a direct effect on the PDE (data not shown), though the reason for this has not been determined.

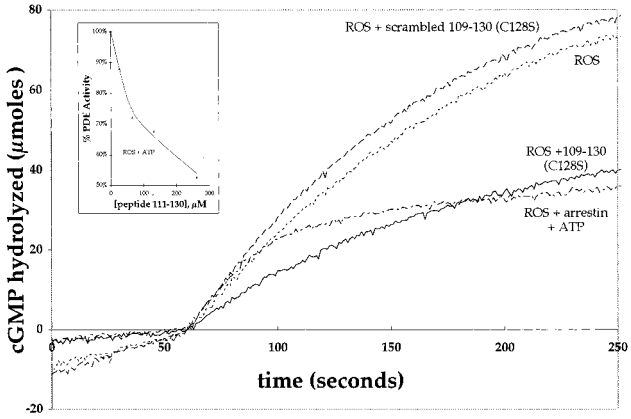

FIGURE 5: Phosphodiesterase activity in the presence of the arrestin peptide of amino acids 109−130(C128S) (solid line) and the scrambled peptide of amino acids 109−130 (long-dash line). Also shown for reference is the PDE activity in the presence (long-dash short-dash line) and absence (short-dash line) of ATP which demonstrate the effect of endogenous arrestin. The inset shows the effect of increasing amounts of the peptide of amino acids 111−130 on the PDE activity in the absence of ATP. The horizontal dashed line indicates the decrease in PDE activity obtained using endogenous arrestin in the presence of ATP.

DISCUSSION

The data obtained from these studies indicate that the principal region of arrestin that binds to R*P is contained in amino acids 109−130. The supporting evidence is fourfold. First, recombinant phage particles displaying portions of arrestin containing amino acids 90−140 are enriched for binding to ROS membranes containing R*P (see Figure 1). These same phage particles do not show a significant level of binding to cell membranes prepared from lysed red blood cells, indicating that the binding of the phage-displayed arrestin is likely specific to the rhodopsin in the membranes and not to the lipid bilayer.

The second line of evidence that supports our conclusion is that a fusion protein containing only arrestin amino acids 95−140 fused with glutathione *S*-transferase binds to rhodopsin (see Figure 2). This ability to bind rhodopsin, albeit without specificity for activation or phosphorylation state, indicates that a domain of arrestin that binds to rhodopsin is contained within amino acids 95−140. The absence of specificity for the different forms of rhodopsin is not surprising since recent studies indicate that other portions of the arrestin protein contain important regulatory elements. For example, Arg-175 has been demonstrated to be a key amino acid involved in the recognition of the phosphorylation state of the photoactivated rhodopsin (*43*). Replacement of Arg-175 with a neutral or negatively charged amino acid results in an arrestin that will bind to photoactivated rhodopsin regardless of its phosphorylation state. Removing amino acids 2−16 has a similar effect on the specificity of arrestin, creating an arrestin that binds equally well to both phosphorylated and nonphosphorylated photoactivated rhodopsin (*19*). In addition, the carboxy terminus also contains important regulatory residues in amino acids 361−404 (*44*). All of these regulatory elements are missing from the 95−140/GST fusion protein, so perhaps it is not surprising that this fusion protein shows binding to several forms of rhodopsin. Similarly, in a study of various arrestin polypeptide truncations (*19*), arrestin that was truncated at amino acid 145 (i.e., the polypeptide consisted of only amino acids 1−145) showed essentially no discrimination between the activation and phosphorylation states of rhodopsin. It is important to note that although the rhodopsin was in membranes, the binding of the 95−140/GST fusion was likely specific for rhodopsin because the fusion protein was unable to bind to membranes that had been boiled to denature the rhodopsin. Additionally, this fusion protein was also unable to bind to lipid membranes prepared from lysed red blood cells.

For the third line of evidence, using the synthetic peptide of amino acids 109−130 of arrestin, we were able to completely block the binding of arrestin to R*P in an in vitro binding assay (see Figure 3). This ability to specifically inhibit binding with a synthetic peptide is strong evidence that the region of amino acids 109−130 contains the principal binding region for R*P. Although the IC_{50} is relatively high, the competition is clearly specific since a scrambled peptide with the same amino acid composition was not an effective competitor. A likely interpretation for the high IC_{50} of this competition is that arrestin may have multiple points of contact with R*P. This interpretation would be consistent with observations from other laboratories.

The final line of evidence that indicates that the peptide of amino acids 109−130 contains a binding site for R*P is that the synthetic peptide of amino acids 109−130 is at least as active as endogenous arrestin and ATP in preventing activation of the cGMP phosphodiesterase (see Figure 5). Endogenous arrestin quenches the PDE activity by about 40%. This same level of quench is obtained using the peptide of amino acids 111−130 at a concentration of 200 μM (see Figure 5). The scrambled peptide of amino acids 109−130 was not effective at inhibiting the PDE activity. The PDE assay in the presence of arrestin peptide also gives some indication of two other regions of arrestin that may be involved in binding, namely, amino acids 51−70 and 231−250.

Recently, a three-dimensional structure of arrestin was presented at 3.3 Å resolution (*45*). According to this model, the region of amino acids 109−130 comprises the end of the only α-helical coil in arrestin (109 and 110), a short random coil (111−114), a β-strand (115−120), and another length of random coil (121−129). This region is located adjacent to the β-strand that contains Arg-175, an amino acid that has been clearly implicated in arrestin's recognition of the phosphorylation state of activated rhodopsin. In their paper, Granzin et al. (*45*) suggest a likely docking location for R*P onto arrestin. This location appears to be conjectural since no data or rationale is given as to why R*P would bind to the site chosen except that it is close to Arg-175. Our experimental data suggest that the binding pocket for R*P is in the N-terminal half of the arrestin molecule, as suggested by Granzin et al., but on the side of the molecule opposite of that indicated in their model. This location would still maintain the binding pocket in close proximity to Arg-175. Interestingly, the region of amino acids 109−130 overlaps one of the four regions that Craft and Whitmore (*46*) showed had the highest degree of conservation across members of the arrestin superfamily, suggesting that the results obtained here for arrestin may apply to the other members of the superfamily as well.

The identification of amino acids 109−130 as the principal binding region of arrestin to R*P is consistent with some

earlier observations made by other laboratories. This part of arrestin is contained within a region that had been identified in earlier studies (*19*, *25*) as the "activation-recognition" region, a portion of the molecule spanning amino acids 29−163 that is involved in the recognition of the photoactivated state of rhodopsin. In these studies, serial truncations of the arrestin polypeptide showed that removal of the region of amino acids 29−163 produced a protein that was greatly diminished in its ability to distinguish the activation state of rhodopsin. In addition, there was also a significant decrease in the total level of binding of these truncated arrestins. The data presented here do not contradict these studies, but rather indicate that this portion of arrestin may be much more important for binding to R*P than previously recognized, and more narrowly define the critical amino acids within this region. Interestingly, studies of the accessibility of the lysine residues in arrestin for acetylation of the amine group showed no difference for the exchange rate for Lys-109 and Lys-110 in the presence or absence or R*P (*47*). Since there is no change in acetylation, perhaps the two lysines are inaccessible in the inactive conformation of arrestin, and then are also blocked by R*P when arrestin is bound. Alternatively, the binding pocket may simply not involve these two flanking residues.

Other Regions of Arrestin. In addition to the primary binding region identified in the phage display and PDE assay, several other regions of arrestin were identified that appear to have a modest contribution to the binding to R*P. In the phage display study, phage displaying residues 160−195 were found to be represented in the phage binding to R*P at approximately 77% of the frequency of those displaying residues 90−140. This region has been previously demonstrated by others to be important for the recognition of the phosphorylation state of the photoactivated rhodopsin (*17*−*19*, *25*). Kieselbach et al. (*18*) have reported that a synthetic peptide spanning amino acids 170−182 can compete for the binding of arrestin to R*P. In contrast, we were unable to find any competitive effects using a synthetic peptide of amino acids 161−180 or 171−190 in binding assays even at concentrations of 1 mM. PDE assays showed no effects with amino acids 171−190, though a small inhibition was observed with amino acids 161−180 in the presence of ATP. Consequently, the contribution of this region to the binding of arrestin to R*P remains somewhat ambiguous, although the phage display study would suggest that in addition to its regulatory role, this region may also make some contribution to the binding to R*P.

In close proximity to this region, amino acids 225−270 also appeared to contribute to phage binding to ROS membranes at approximately 73% of the frequency of those phage displaying amino acids 90−140. This portion of arrestin is contained in the broad region of residues 195−360 that others have suggested contributes to the binding via a secondary binding site (*19*, *25*). Within this region, we observed a modest effect on PDE activity using the peptide of amino acids 231−250. These combined results suggest that this region may indeed contribute to the binding to R*P, although the interaction is weaker than that found for amino acids 109−130.

A final region identified in the phage display study spans amino acids 305−404, but is much reduced in frequency when compared to the binding of phage displaying amino acids 90−140 (49% of average frequency). The 40 C-terminal amino acids of arrestin contain a significant number of negatively charged amino acids, giving this region of the protein a net charge of −8. This C terminus has been implicated in previous studies to be important in the regulation of the binding specificity of arrestin for phosphorylated rhodopsin (*22*−*25*, *44*). The current understanding of this regulation is that the C terminus in some manner is responsible for blocking access of activated, unphosphorylated rhodopsin to the high-affinity binding site. Upon binding to phosphorylated rhodopsin, arrestin undergoes a molecular rearrangement, as evidenced by a high Arrhenius activation energy (*26*), which likely involves the mobilization of the C terminus. Artificial deletion of this region, either by proteolysis (*23*) or by in vitro translation of truncated cDNAs (*25*), or a naturally occurring splice variant of arrestin that lacks this C-terminal region (*24*), produces arrestin that binds to photoactivated rhodopsin regardless of its phosphorylation state.

In addition to these regions where we observed binding of the phage or effects of the synthetic peptides on PDE activity, others have also identified the amino terminus of the molecule as being important in the regulation of arrestin−rhodopsin binding. The first 30 amino acids in the amino-terminal region are rich in positively charged residues (+7 net charge), which apparently are important in regulating the specificity of arrestin for the phosphorylated form of photoactive rhodopsin, possibly through interaction with the negatively charged C terminus (*19*, *23*, *25*, *44*, *48*). Our phage display method and survey of peptides in the PDE assay do not identify any significant binding or effects of the N-terminal or C-terminal peptides, in agreement with these regions being primarily involved in the regulation of binding specificity and not directly in binding to R*P.

In summary, phage display of random arrestin polypeptides shows that the primary region of interaction between arrestin and R*P is contained within amino acids 90−140. Within this region, peptide competition in binding studies and in the cGMP PDE assay shows that amino acids 109−130 encompass the principal binding region for R*P. The relatively high IC_{50} of the binding competition suggests that this portion of the molecule may be only one of several binding regions between arrestin and rhodopsin.

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