Insertional Mutagenesis and Immunochemical Analysis of Visual Arrestin Interaction with Rhodopsin*

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Visual arrestin inactivates the phototransduction cascade by specifically binding to light-activated phosphorylated rhodopsin. This study describes the combined use of insertional mutagenesis and immunological approaches to probe the structural determinants of arrestin function. Recombinant arrestins with insertions of a 10-amino acid c-Myc tag (EQKLISEEDL) were expressed in yeast and characterized. When the tag was placed on the C terminus after amino acid 399, between amino acids 99 and 100 or between residues 162 and 163, binding to rhodopsin was found to be very similar to that of wild-type arrestin. Two stable mutants with Myc insertions in the 68–78 loop were also generated. Binding to rhodopsin was markedly decreased for one (72myc73) and completely abolished for the other (77myc78). Limited proteolysis assays using trypsin in the absence or presence of heparin were performed on all mutants and confirmed their overall conformational integrity. Rhodopsin binding to either 162myc163 or 72myc73 arrestins in solution was completely inhibited in the presence of less than a 2-fold molar excess of anti-Myc antibody relative to arrestin. In contrast, the antibody did not block the interaction of the 399myc or 99myc100 arrestins with rhodopsin. These results indicate that an interactive surface for rhodopsin is located on or near the concave region of the N-domain of arrestin.

Although each amplification point of the signaling cascade has its own mechanism that allows the system to return to the original inactive state, a regulatory process at the receptor level is crucial to ensure that GPCR signaling is effectively quenched (3). Many GPCRs are inactivated through a two-step process beginning with phosphorylation by a member from a distinct class of serine-threonine kinases known as GPCR kinases (GRKs) that interact specifically with activated receptors (4). Phosphorylation is an important chemical modification that allows the activated receptors to be further recognized by proteins from the arrestin family (5). Arrestins stop the signal transduction quickly by directly preventing the G-protein access to the cytoplasmic loops of activated receptors (6). This leads to a diminished cellular responsiveness to a specific stimulus, a phenomenon known as homologous desensitization.

Phototransduction in retinal rod cells has been a useful model system for the study of arrestin-mediated desensitization of GPCRs (7, 8). Both visual arrestin and rhodopsin, the GPCR that initiates phototransduction, can be isolated in comparatively large quantities from bovine retinas. Crystal structures at 2.8 Å resolution are available for both proteins (9–11). Quenching of the phototransduction cascade starts when light-activated rhodopsin becomes phosphorylated on serine and threonine residues within its C terminus (12–14). Next, visual arrestin binds with high affinity and specificity to light-activated, phosphorylated rhodopsin by a mechanism that is incompletely understood (15, 16). Experimental evidence suggests that in addition to phosphate binding residues, arrestin contains elements that enable it to specifically recognize the loops of the photoactivated rhodopsin (17–19). It has been shown that arrestin is able to recognize the activated, phosphorylated rhodopsin or a C-terminal truncated form of the activated rhodopsin in the presence of a synthetic fully phosphorylated peptide corresponding to the last 19 amino acids of the C terminus of rhodopsin (20). Additionally, charge reversal or neutralization by mutagenesis of Arg-175 in arrestin produces mutants that are able to bind to light-activated rhodopsin in its unphosphorylated state, as does a splice variant of arrestin that is truncated at its C terminus (21–23). It is not known what molecular constraints prevent wild-type arrestin from binding directly to light-activated unphosphorylated rhodopsin.

The crystal structure of visual arrestin has revealed that the molecule is composed of two domains, the N-domain (primarily residues 8–180) and the C-domain (primarily residues 188–392) followed by a carboxyl-terminal region (residues 372–404) that is also called the “C-tail” (9, 10). The three-dimensional structure is lacking information about the location of the last 10 amino acids of the C-tail. Some features of the arrestin-rhodopsin interaction have been proposed based on studies.
The 162myc163 and 72myc73 constructs were created by synthesizing two overlapping primers for each mutant, both containing the Myc epitope. Two separate PCR reactions were performed for each construct using one of the overlapping primers and the corresponding wild-type 5'-or 3'-end primers to generate two halves of the molecule. These were used in a third PCR to anneal the two halves together. The 77myc78 arrestin was made by "megaprimer" PCR, which consists of two PCR steps (36). The first PCR was performed using an internal mutagenic primer containing the Myc epitope sequence and a wild-type 5'-end primer. The second PCR used the wild-type 3'-end primer and the purified product of the first PCR as the 5'-end primer. All recombinant DNA sequences were confirmed using Sequenase v2.0 (USB) by the dideoxy chain termination method (37). The DNA constructs were linearized by digestion with the enzyme, electroeluted into yeast, expressed, and purified as previously described (38).

**Binding Assays of Mutant Arrestins to Rhodopsin**—Reaction mixtures were prepared containing wild-type or mutant arrestins (2 μM) and an excess of nonphosphorylated or phosphorylated rhodopsin (6 μM) in ROS membranes in 10 mM Hepes and 100 mM NaCl, pH 7. Half of the samples were exposed to white light for 2 min at room temperature and then all of the samples were centrifuged in the dark (40,000 × g for 30 min at 4 °C). The pellets were solubilized in 1× SDS-PAGE sample buffer and analyzed on 12% SDS-polyacrylamide gels under reducing conditions according to the method of Laemmli (39). Proteins were visualized by staining with Coomasie Brilliant Blue R-250 and quantified by scanning densitometry.

**Competition Binding Experiments with the Anti-Myc Antibody 9E10**—Each Myc-tagged or wild-type arrestin (2 μM) was preincubated with increasing amounts of purified anti-Myc antibody (up to 3.3 μM) in 10 mM Hepes and 100 mM NaCl, pH 7. Half of the samples were exposed to white light for 2 min. This was followed by the addition of the anti-Myc antibody (4 μM) to all samples and incubation. After centrifugation the pellets were analyzed as previously described.

**Centrifugation Binding assays of 162myc163 and 99myc100 arrestins to unphosphorylated rhodopsin from ROS membranes** were also performed in the presence of a synthetic fully phosphorylated peptide (7PP) consisting of the last 19 amino acids of rhodopsin C terminus (40). Each reaction mixture contained 6.7 μM arrestin and 6.5 μM phosphorylated rhodopsin in disc membranes. Half of the samples also contained 300 μM of the synthetic peptide 7PP. The assays were performed as described above in the dark or under illumination in the absence or presence of 3.3 μM anti-Myc antibody, and the pellets were analyzed on 12% SDS-polyacrylamide gels.

**Limited Proteolysis Analysis of Wild-type and Arrestin Mutants**—Wild-type and arrestin mutants were incubated with trypsin (TPCK-treated) in 50 mM Tris buffer, pH 8, containing 100 mM NaCl in the absence or presence of 0.13% heparin (w/w). The enzyme/protein ratio was 1% (w/w). Aliquots were withdrawn at various times, mixed with Laemmli sample buffer, and analyzed by 12% SDS-PAGE.

**RESULTS**

Five arrestin mutants were generated by placing the c-Myc tag (EQKLISEEDL) into different loop regions of the molecule and on its C terminus (Fig. 1). The insertion sites were selected from site-directed mutagenesis, limited proteolysis, synthetic peptides, phage display, and chemical modification of arrestin (24–30). These studies suggest that prior to rhodopsin binding, arrestin is maintained in an inactive conformation by multiple intramolecular electrostatic interactions among highly conserved residues from both domains and the C-tail. The interaction of rhodopsin phosphates with specific lysines and arginines in arrestin is thought to disrupt these intramolecular forces and to cause a conformational change in the molecule that leads to the exposure of currently unknown high affinity binding elements on arrestin.

A highly cationic region beginning with Lys-163 located on the concave surface of the N-terminal domain of arrestin has been proposed to interact with rhodopsin phosphates by Palczewski et al. (31) who used the polyanion heparin to inhibit binding to the receptor. Also, polyclonal antibodies directed against peptide 170–182 were unable to bind arrestin complexed with light-activated, phosphorylated rhodopsin (32). Specific residues that may play a role in phosphate binding were identified by mutagenesis experiments. Among them, Arg-175 represents a key element (21). The interaction of rhodopsin phosphates with this residue is thought to be essential for inducing a high affinity binding conformation in arrestin (33, 34). However, this residue does not appear to be directly accessible in the inactive arrestin. It has been suggested that prior to contacting Arg-175 rhodopsin phosphates first interact with other specific positive charged residues from the concave surface of the N-domain (35).

The present study describes the combined use of insertional mutagenesis and immunoochemical approaches to further probe the structural determinants of arrestin function. Recombinant arrestins with insertions of the c-Myc epitope at various positions were generated based on the available crystal structures of arrestin, and each mutant was tested for its ability to specifically recognize light-activated phosphorylated rhodopsin. Competition experiments with the anti-Myc antibody were performed in solution to test which regions of arrestin, if blocked, would affect its function. The results show that this technique represents a useful approach for the study of rhodopsin binding to visual arrestin.

**EXPERIMENTAL PROCEDURES**

**Materials**—The hybridoma cell line expressing the anti-Myc antibody 1–9E10 was purchased from the American Type Culture Collection. The antibody was purified on a protein A-protein G column (Pierce). Restriction enzymes were from New England Biolabs and Promega. T4 DNA ligase was from Invitrogen. Cloned Pfu polymerase was from Stratagene. The TPCK-treated trypsin was from Sigma. Bovine arrestin and bovine rod cell outer segment (ROS) membranes were prepared as described previously (20).

**Creation and Expression of Recombinant Arrestins**—Bovine retinal arrestin was cloned into the EcoRI restriction site of the Pichia pastoris shuttle vector pPIC-ZA (Invitrogen). The synthetic oligonucleotides used for each construct are listed in Table I. The 99myc100 construct was prepared by cloning a double-stranded synthetic DNA encoding the Myc epitope sequence into the unique NarI site of the target DNA. The 399myc arrestin was made using PCR with a wild-type 5’-end primer and a mutagenic 3’-end primer containing the Myc epitope sequence.

**TABLE I**

| Construct | Mutagenic primer |
|-----------|------------------|
| 399myc    | 5'-CGAATTCTGACAGATCTTCCGAGATGAGCTTCTGCTCCTCCTGGTCTGTCTTCTCTTC-3' |
| 99myc100  | 5'-CGGGCAGAGATCTTCCGAGATGAGCTTCTGCTCCTCCTGGTCTGTCTTCTCTTC-3' |
| 162myc163 | 5'-GAGCCAGGATCTTCCGAGATGAGCTTCTGCTCCTCCTGGTCTGTCTTCTCTTC-3' |
| 77myc78   | 5'-GTCAGATTCCTCAGAGATGAGCTTCTGCTCCTCCTGGTCTGTCTTCTCTTC-3' |
| 72myc73   | 5'-GACGAAAAATTTATCTCCGAGGAAGATCTGGACGTGATGGGCCTCAGC-3' |
The c-Myc tag was chosen because it is recognized with high affinity. The c-Myc tag insertion site is located in another region before the C terminus, which can be traced up to amino acid Glu-399. Myc insertions at Asp-162/Lys-163 or Ile-72/Asp-73 resulted in constructs whose binding to light-activated phosphorylated rhodopsin was inhibited by the anti-Myc antibody. The ribbon structure is based on molecule A from (10).

The Myc-tagged arrestins were produced in yeast, and their binding to rhodopsin was compared with that of wild-type arrestin (Fig. 2). When the c-Myc tag was placed at the C-terminal end of arrestin following amino acid Glu-399 (399myc), replacing the last five amino acids, binding was found to be very similar to that of wild-type arrestin with respect to both the specificity for light-activated phosphorylated rhodopsin and the amount of binding (Fig. 2). When the anti-Myc antibody was used as a competitor for arrestin binding to light-activated phosphorylated rhodopsin, there was no competition, although there was clear binding of the antibody to the 399myc arrestin as evidenced by the specific presence of more antibody in the assay pellet for the light reaction (Figs. 3 and 4). This result indicates that the C terminus of arrestin is not a binding region for rhodopsin in agreement with previous data on truncated arrestins (22, 26). Insertion of the Myc tag between Leu-77/Ser-78 (72myc73) resulted in a construct with reduced binding to the light-activated phosphorylated form compared with the wild-type arrestin (22, 26). In the three-dimensional structure of arrestin the C terminus can only be traced as far as residue 393, and thus the precise position of the Myc tag in the 399myc construct is not known.

When the Myc tag was inserted into two loops in the N-terminal domain, either between Asp-162/Lys-163 (162myc163) or Ala-99/Thr-100 (99myc100), the resulting constructs were fully functional (Fig. 2). In the 162myc163 construct, the insert site is located in a concave region formed by four β-strands and loop 68–78 from the N-domain. In the 99myc100 construct the Myc tag insertion site is located in another region before the α-helix formed by residues 102–109 (Fig. 1). The effect of the anti-Myc antibody on the interaction of these mutants with rhodopsin was tested by preincubating the same amount of arrestin with increasing concentrations of the antibody in solution (Fig. 4). The anti-Myc antibody had a strong inhibitory effect on the binding of 162myc163 to light-activated phosphorylated rhodopsin. Binding in this case was completely abolished in the presence of less than a 2-fold molar excess of antibody relative to mutant arrestin. In contrast the antibody did not affect the 99myc100-rhodopsin interaction (Figs. 3 and 4). More antibody was found specifically in the assay pellet for the light reaction compared with the dark one, a consequence of its specific binding to 99myc100. Binding assays were also conducted in which the antibody was added after the 162myc163 arrestin had been allowed to react with light-activated phosphorylated rhodopsin. The antibody was found predominantly in the supernatant and had only a slight inhibitory effect on the preformed 162myc163-rhodopsin complex (Fig. 4, asterisk). This suggests that the Myc tag in 162myc163 is not accessible to the antibody when arrestin is already bound to rhodopsin in solution.

Two arrestins with Myc insertions in the loop region 68–78 were also generated, and the effects on rhodopsin binding were tested. Insertion of the Myc tag between Leu-77/Ser-78 (77myc78) produced a completely inactive arrestin protein, whereas insertion of the same tag between Ile-72/Asp-73 (72myc73) resulted in a construct with reduced binding to the receptor (41% relative to wild-type), although the specificity for the light-activated phosphorylated form was retained (Fig. 2). Rhodopsin binding to 72myc73 was completely inhibited in the presence of less than a 2-fold molar excess of antibody relative to arrestin, similar to the case of the fully functional 162myc163 mutant.

The 162myc163 and 99myc100 arrestins were also tested for their ability to bind the light-activated unphosphorylated form of rhodopsin (R+) in the presence of the 19-amino-acid, fully phosphorylated peptide (7PP) corresponding to the C terminus of rhodopsin. It is thought that the 7PP interaction with arrestin promotes a conformational change in the molecule, which then allows the protein to recognize R* (38). Binding of 162myc163 or 99myc100 to R* was substantially enhanced in the presence of the 7PP (Fig. 5), an effect similar to that previously reported for wild-type arrestin (20). Binding of 162myc163 to R* was inhibited by the anti-Myc antibody, whereas that of 99myc100 was not affected. The antibody was present in the assay pellet for the light reaction with 99myc100 and R*, indicating that it was able to bind the arrestin-rhodopsin complex.

Experiments were also performed to test if the anti-Myc antibody affects the activation of arrestin as measured by the interaction between 162myc163 and the synthetic 7PP. The arrestin-7PP interaction can be detected by monitoring the reactivity of the three sulphydryl groups in arrestin. It has been shown that the rate of the reaction of the three sulphydryl groups in arrestin to 5,5′-dithiobis(2,2′-nitrobenzoic acid) (DTNB) reagent is very slow and that the presence of 7PP increases the rate of the reactivity of one sulphydryl group.

**Fig. 1. Position of the Myc insertion sites in the three-dimensional structure of visual arrestin.** This ribbon diagram of visual arrestin shows the position of four Myc insertion sites and that of the C terminus, which can be traced up to amino acid Glu-399. Myc insertions at Asp-162/Lys-163 or Ile-72/Asp-73 resulted in constructs whose binding to light-activated phosphorylated rhodopsin was inhibited by the anti-Myc antibody. The ribbon structure is based on molecule A from (10).

**Fig. 2. Binding of wild-type and Myc-tagged arrestin mutants to rhodopsin.** Binding assays were performed as described under “Experimental Procedures” with the following functional forms of rhodopsin from ROS membranes: unactivated, unphosphorylated (R), light-activated, unphosphorylated (R*), unactivated, phosphorylated (RP) and light-activated, phosphorylated (R*P). The results were normalized to the level of wild-type arrestin binding to R*P. Data shown represent the mean of three independent experiments ± S.E.
while inhibiting the reactivity of another (38). The effect of the 7PP on the sulphydryl reactivity of 162myc163 was found to be similar to that of wild-type arrestin, both in the absence and presence of the anti-Myc antibody, indicating that binding of the anti-Myc antibody to 162myc163 does not prevent the 7PP from interacting with arrestin (data not shown).

In addition to their binding properties, all Myc-tagged arrestin mutants were characterized by limited trypsinolysis assays as a tool to examine their conformational integrity. Insertions that affect protein folding usually produce unstable mutants that are either highly susceptible to proteolysis or form insoluble aggregates. If wild-type arrestin is partially denatured by heating at 70 °C, the change in protein conformation is readily evident from the altered trypsinolysis pattern (Fig. 6, lanes 11 and 12). Previous studies have shown that heparin and other negatively charged ligands such as light-activated phosphorylated rhodopsin or the synthetic 7PP are able to bind arrestin and induce a conformational change in the molecule (20, 26, 31, 38). Although it has been determined that the conformational changes induced by these ligands may not be identical, an enhanced susceptibility to proteolysis of arrestin C terminus is a common effect (38). Similar to the case of wild-type arrestin (Fig. 6, lane 3), heparin enhanced the proteolysis of the C terminus in all mutants, producing a distinct pattern as shown for 162myc163 and 72myc73 arrestins (Fig. 6, lanes 6 and 9). The lysine residue in the inserted Myc tag renders the modified arrestin loops susceptible to trypsin proteolysis (Fig. 6, lanes 5 and 8). Cleavage within the tag was confirmed for all mutants by Western blot analysis of the digested samples with specific antibodies (data not shown). In the presence of heparin the trypsinolysis of the inserted Myc tag in 72myc73 was enhanced (Fig. 6, lane 9), whereas that of 162myc163 was inhibited (Fig. 6, lane 6). A cationic region beginning with residue Lys-163 in bovine visual arrestin has previously been hypothesized to represent a heparin recognition site based on its similarity to heparin binding motifs found in other proteins (31).

**Discussion**

Several lines of study have focused on the identification of rhodopsin-binding sites on the arrestin molecule in order to understand the mechanism of its specific recognition of the light-activated and phosphorylated form of the receptor. Phage display and peptide competition studies have implicated both the N- and C-terminal domains of arrestin in the interaction with the receptor: residues 11–30, 51–70, 111–130 from the N-domain of the structured similar β-arrestin member in the interaction with the corresponding m2 muscarinic cholinergic receptor (43). Many residues in the 163–176 region of visual arrestin have been indicated to participate in phosphate recognition (21). Previous experiments using differential chemical modification of visual arrestin have shown that rhodopsin protects some lysine residues in both domains of arrestin from acetylation (27). It remains unclear how all the proposed binding elements

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**Fig. 3.** Binding of wild-type and three fully functional Myc-tagged arrestins to phosphorylated rhodopsin in the absence and presence of the anti-Myc antibody. Samples containing wild-type or Myc-tagged arrestin mutants (2 μM) in the absence or presence of anti-Myc antibody (3.3 μM) were used in centrifugation binding assays with phosphorylated rhodopsin (6 μM) from ROS membranes as described under “Experimental Procedures.” The pellets containing rhodopsin and bound proteins were analyzed by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Lanes 2, 6, 10, and 14 represent the binding of wild-type and that of the fully functional Myc-tagged arrestins to light-activated phosphorylated rhodopsin (R*P) in the absence of the anti-Myc antibody. Lanes 4, 8, 12, and 16 represent binding of the above arrestins to R*P in the presence of the anti-Myc antibody.

**Fig. 4.** Competitive inhibition of binding between Myc-tagged arrestins and light-activated phosphorylated rhodopsin using the anti-Myc antibody. The same amount of each Myc-tagged arrestin (2 μM) was preincubated with increasing amounts of anti-Myc antibody (up to 3.3 μM) in solution. Binding assays to R*P (6 μM) in ROS membranes were subsequently performed (see “Experimental Procedures”). The monoclonal anti-Myc antibody inhibited the 162myc163 arrestin binding to R*P (solid triangles) in a concentration-dependent manner but did not block the binding of 99myc100 or 399myc arrestins to R*P (open circles and open triangles, respectively). Only a slight inhibitory effect was seen on the preformed 162myc163-rhodopsin complex (asterisk) in the presence of the antibody (4 μM). The results are expressed as the percentage of arrestin that bound to R*P in the absence of antibody. Each point represents the mean of three independent experiments ± S.E.

**Fig. 5.** Binding of wild-type and Myc-tagged arrestins to unphosphorylated rhodopsin from ROS in the presence of the synthetic 7PP. Centrifugation binding assays with unphosphorylated rhodopsin from ROS membranes were performed as described under “Experimental Procedures.” Samples containing wild-type or arrestin mutants 162myc163 and 99myc100 (6.7 μM) were mixed with unactivated or light-activated unphosphorylated rhodopsin in disc membranes (6.5 μM) in the absence or presence of 300 μM 7PP. The pellets containing rhodopsin and bound proteins were analyzed by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Lanes 4, 10, and 16 show the specific binding of 162myc163, 99myc100, and wild-type arrestin to light-activated rhodopsin (R*) in the presence of the synthetic 7PP. Lanes 6, 12, and 18 represent the binding of the above arrestins to R* in the presence of 7PP and the anti-Myc antibody (3.3 μM). The arrows indicate the specific presence of the anti-Myc antibody in the assay pellet for the light reaction of 99myc100 and R* (lane 12).
located on different regions of the arrestin molecule are approached by the light-activated phosphorylated rhodopsin. A large conformational change has been suggested to occur when rhodopsin binds to visual arrestin, which could assemble many recognition elements to create a continuous receptor-binding surface (44). However, the nature and the extent of this ligand-induced conformational change are not known.

The results presented in this report have been obtained by using an insertional mutagenesis and antibody approach to analyze the arrestin-rhodopsin interaction. The advantage of the insertional relative to the site-directed mutagenesis technique is the ability to scan a large molecular surface by positioning an epitope tag between amino acids situated on different regions of the protein of interest. This technique has been used in the past to analyze protein-protein interactions. For example the technique was used in a study on rhodopsin topography and transducin-binding sites (45). As mentioned before, a 10-amino-acid insertion such as the c-Myc tag is informative in both cases in which it either alters the binding interaction between proteins or in which it has no effect. If the insertion of the c-Myc tag in arrestin is placed within a region important for rhodopsin binding, a marked effect on the interactivity of the Myc tag in this loop in the presence of heparin becomes more exposed upon heparin binding. Interestingly, the cleavage of the Myc tag in 162myc163 by trypsin is apparently protected by the addition of heparin (see Fig. 6, lane 6). One possible interpretation of this result is that this positioning of the Myc tag could be at or near a heparin-binding site.

The insertional mutagenesis technique eliminates the need to generate high affinity antibodies of different specificities against arrestin. Most of the currently available anti-arrestin antibodies are able to function well in Western blotting or enzyme-linked immunosorbent assays when arrestin is bound to a solid phase. However, these antibodies show either poor reactivity or fail to bind to arrestin in solution; consequently, they cannot be used as tools for inhibitory studies with rhodopsin. An antibody aimed at interfering with rhodopsin binding should have a high affinity for its epitope comparable with that of the arrestin-rhodopsin interaction for which the dissociation constant \( K_D \) is on the order of 50 nM (44). Previous studies have determined that the anti-Myc antibody has a high affinity for the c-Myc peptide tag \( K_D = 80 \text{nM} \). Consequently it can be used efficiently in competition experiments (42).

The anti-Myc antibody was able to block the binding interaction between 162myc163 and light-activated phosphorylated rhodopsin in a concentration-dependent manner, a behavior consistent with a competitive inhibition pattern (46). Further, the anti-Myc antibody was unable to disrupt the preformed 162myc163-R*P complex indicating that binding of light-activated, phosphorylated rhodopsin and that of the anti-Myc antibody to 162myc163 are mutually exclusive. The anti-Myc antibody also had an inhibitory effect on the 72myc73-rhodopsin interaction. These results suggest that the antibody binding to 162myc163 and 72myc73 occurs close to a region important for receptor recognition. Based on the crystal structure of arrestin, a possible common region blocked by the anti-Myc antibody in both constructs lies on or near the concave surface of the N-terminal domain of the molecule (see Fig. 1). In contrast, when the Myc tag was placed on a different region of the N-terminal domain of arrestin between amino acids 99 and 100 the antibody did not interfere with the arrestin-rhodopsin interaction.

Previous studies have indicated that arrestin contains elements for recognition of the photoactivated rhodopsin that are distinct from the phosphate-binding elements (17, 22, 47). As mentioned before, the concave surface of the N-domain contains a cationic region that has been implicated in the recognition of the phosphorylated state of rhodopsin (21, 35). Thus, in the case of the 162myc163-R*P interaction, the anti-Myc antibody may exert its inhibitory effect by shielding this cationic region. However, the anti-Myc antibody does not interfere with the interaction between the 162myc163 and the 7PP as indicated by the sulphydryl reactivity experiments. Consequently, in the case of the 162myc163-R* interaction in the presence of 7PP, the antibody must exert its inhibitory effect by directly blocking access to those arrestin elements responsible for recognition of the photoactivated but not phosphorylated rhodopsin.

Because all vertebrate arrestins share a high degree of sequence similarity, the study of visual arrestin-rhodopsin interaction is also relevant for understanding the mechanism of

![Fig. 6. The limited proteolysis pattern of wild-type and mutant arrestins. Digestion by trypsin was performed on wild-type and Myc-tagged arrestins in 50 mM Tris buffer, pH 8, containing 100 mM NaCl. The enzyme/arrestin ratio was 1% (w/w). Samples are shown untreated, treated with trypsin, and treated with trypsin in the presence of heparin (0.13% w/v). After 30 min the fragments were analyzed by 12% SDS-PAGE and visualized with Coomassie Brilliant Blue. In addition, the trypsinolysis pattern of wild-type arrestin preheated at 70 °C for 10 min is shown (lanes 11 and 12). The molecular mass markers (MW) are shown on the left. In the absence of heparin, trypsin cuts at the lysine of the Myc tag in both 162myc163 and 72myc73 mutants as indicated by arrows (lanes 5 and 8). In the presence of heparin, cleavage at the C terminus occurs in wild-type (lane 3) and in the Myc-tagged arrestins (lanes 6 and 9).](image-url)
desensitization in other GPCRs. Indeed, the recently published crystal structure of β-arrestin at 1.9 Å resolution has revealed that its three-dimensional structure is very similar to that of visual arrestin (43). The research presented in this report shows for the first time that an antibody against a stable fully functional arrestin mutant can efficiently inhibit the visual arrestin-rhodopsin interaction in solution. The antibody competition results combined with the insertional mutagenesis data represent direct evidence for the location of a rhodopsin-binding region on or near the concave surface of the N-terminal domain of visual arrestin. This technique can also be used to probe other potential receptor interactive regions in visual and nonvisual arrestins.

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