Cloning and Functional Characterization of Salamander Rod and Cone Arrestins

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PURPOSE. To clone, localize, and determine functional binding characteristics of rod and cone arrestins from the retina of the tiger salamander (Ambystoma tigrinum).

METHODS. Two arrestins from salamander retina were cloned on the basis of their homology to known arrestins from other species. The expression pattern of these arrestins (SalArr1 and SalArr2) in the retina was determined by immunocytochemistry and in situ hybridization. SalArr1 and SalArr2 were expressed and functionally characterized.

RESULTS. Both immunocytochemistry and in situ hybridization show that SalArr1 and SalArr2 localized specifically to rod and cone photoreceptors, respectively. SalArr1 demonstrated a characteristic high selectivity for light-activated phosphorylated rhodopsin (P-Rh*) and significant species selectivity, binding preferentially to amphibian rhodopsin over bovine rhodopsin. Mutant constitutively active forms of SalArr1 demonstrated a 2- to 4-fold increase in P-Rh* binding (compared with wild-type protein) and an even more dramatic (up to 25-fold) increase in binding to unphosphorylated Rh* and dark P-Rh. Constitutively active SalArr1 mutants also showed a reduced specificity for amphibian rhodopsin. The ability of Escherichia coli-expressed SalArr1, SalArr2, and an SalArr1-3A (L369A,V370A,F371A) mutant to bind to frog Rh* and P-Rh* and to compete with tritiated SalArr1 for amphibian P-Rh* was compared. SalArr1 and its mutant form bound to amphibian P-Rh* with high affinity (Kᵢ = 179 and 74 nM, respectively), whereas the affinity of SalArr2 for P-Rh* was substantially lower (Kᵢ = 9.1 μM).

CONCLUSIONS. SalArr1 and SalArr2 are salamander rod and cone arrestins, respectively. Crucial regulatory elements in SalArr1 are conserved and play functional roles similar to those of their counterparts in bovine rod arrestin. Rod and cone arrestins are relatively specific for their respective receptors. (Invest Ophthalmol Vis Sci. 2000;41:2445–2455)

Many types of cells transduce a signal across the cell membrane to the cytoplasm where a response is mounted. Cells have devised a number of mechanisms to accomplish this process, many of which rely on specific receptor molecules that are matched to the particular signaling agent. G protein-coupled receptors (GPCRs) are a ubiquitous class of receptors responding to a wide variety of signals, including light, odorant molecules, neurotransmitters, and hormones. These receptors make seven passages through the cell membrane and couple to heterotrimeric guanine nucleotide-binding proteins (G proteins).

Rhodopsin is the prototypical GPCR, mediating the transduction of light into a neural signal in all known visual systems. All eukaryotic visual pigments are fundamentally the same, consisting of the apoprotein opsin coupled to a retinoid chromophore. The absorption of a photon by rhodopsin induces a series of conformational changes in the protein that culminate in rhodopsin assuming its active conformation (Rh*). Rh* then consecutively binds and activates dozens of molecules of visual G protein, transducin. Rh* also serves as a substrate for rhodopsin kinase. The incorporation of one or more phosphates into rhodopsin COOH terminus yields phosphorylated light-activated rhodopsin (P-Rh*), which is recognized by arrestin. Arrestin binds to this form of rhodopsin with marked selectivity. Tight binding of arrestin precludes further transducin interaction, apparently by steric exclusion, shutting down the signaling. Serious defects in the shut-off mechanism due to the absence of either rhodopsin kinase or arrestin itself in humans lead to Oguchi disease and autosomal recessive retinitis pigmentosa, as well as light-dependent retinal degeneration in transgenic mice, underscoring the importance of a timely shut-off for the function of photoreceptor cells.

Amphibian photoreceptors have served as a classic model for visual studies, in part because of the robustness of the eye tissue once the eye is opened. Salamander eyes have been very amenable to electrophysiological studies, because single large photoreceptors can be easily isolated. These cells provide stable recording for up to 48 hours, and the cells themselves can be maintained in culture for several weeks. The salamander retina contains at least two classes of rod photoreceptors, red and green. In addition, there are at least three classes of cone photoreceptors, red, blue, and ultraviolet.
Red rod opsin\textsuperscript{19} and red and blue cone opsins\textsuperscript{20} are the only molecules involved in phototransduction in the salamander to have been cloned to date. In a continuing effort to better understand the molecular basis of the visual process, we have cloned two arrestins from the salamander retina (SalArr1 and SalArr2) and characterized their cellular localization and binding properties. Please note that to avoid the already considerable confusion in the names of arrestin proteins, we have provisionally used the names SalArr1 and SalArr2 for rod and cone arrestins, respectively.

**METHODS**

**DNA Cloning**

Frozen retinas from larval tiger salamanders (*Ambystoma tigrinum*) in the aquatic phase were kindly provided by Sergei Nikonov and Edward N. Pugh, Jr, (University of Pennsylvania, Philadelphia). Poly(A)	extsuperscript{+} RNA was isolated from four retinas (MicroQuick Prep; Pharmacia, Piscataway, NJ) and converted to first-strand cDNA (First-Strand cDNA Synthesis kit; Pharmacia), priming reverse transcription with 25 picomoles oligo(dT) (\textcopyright 5-AACTGGAAATTCGCGGCCAGAAATTTTTTTTTTTTTTTTTTTTTTTTTTTTT). Arrestin cDNA was amplified from this first-strand cDNA in polymerase chain reaction (PCR), using degenerate oligonucleotide primers designed against conserved arrestin sequences (PH1127: 5\textsuperscript{\prime}-GCAATTTGCTTGCCTTCG; PH129: 5\textsuperscript{\prime}-GTGATYTCACGCGGGC; PH130: 5\textsuperscript{\prime}-GAYGGIGTIGYTTIGTIGAYCC). For initial PCR we used 2 U Pfu polymerase (Promega; Bio-Metra, Göttingen, Germany) in 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% Triton X-100, 1.5 mM MgCl\textsubscript{2}, 0.2 mM dNTPs, and 30 picomoles of degenerate oligonucleotide primer (94°C, 45 seconds; 45°C, 1 minute; 72°C, 3 minutes; 35 cycles). Amplified products were cloned into the pCR2.1 (Invitrogen, San Diego, CA) and sequenced.\textsuperscript{21} Exact sequence. SalArr1 sense (PH336: 5\textsuperscript{\prime}-GAAATTCATGAGCAC-GCGAATTCCTTAG-5\textsuperscript{\prime}; PH129: 5\textsuperscript{\prime}-GCGAATTCATGAGCA-GCGAATTCCTTAG-5\textsuperscript{\prime}) primers, and SalArr2 sense (PH337: 5\textsuperscript{\prime}-GCAATTTGCTTGCCTTCG; PH132: 5\textsuperscript{\prime}-GCAATTTGCTTGCCTTCG-5\textsuperscript{\prime}) primers were isolated and sequenced on both strands to confirm the sequence. After identifying the open reading frame (ORF) start and stop sequences by conceptual translation, primers containing restriction sites were designed to amplify the entire coding sequence. SalArr1 sense (PH336: 5\textsuperscript{\prime}-GCGAATTCATGAGCAG-CAAGATGGAAGGCCTG-5\textsuperscript{\prime}) and anti-sense (PH342: 5\textsuperscript{\prime}-GCAAGCTTCAAGCAAGGACGGATCAAAAGTATTAC-5\textsuperscript{\prime}) primers, and SalArr2 sense (PH337: 5\textsuperscript{\prime}-GCGAATTCATGAGCAG-CAAGATGGAAGGCCTG-5\textsuperscript{\prime}) and anti-sense (PH335: 5\textsuperscript{\prime}-GCGAATTCATGAGCAG-CAAGATGGAAGGCCTG-5\textsuperscript{\prime}) primers were used in PCR with Pfu polymerase (Stratagene, La Jolla, CA) to minimize DNA replication errors. These reactions were performed in 10 mM KCl, 10 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 20 mM Tris-HCl (pH 8.75), 2 mM MgSO\textsubscript{4}, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin (BSA), and 0.2 mM dNTPs with 2.5 U Pfu polymerase.

The nucleotide sequences have been submitted to the GenBank/EMBL Data Bank with accession numbers AF203327 (SalArr1) and AF203328 (SalArr2).

**Sequence Comparison and Phylogenetic Analysis**

Nucleotide and polypeptide sequences were aligned by computer (PC-Gene; IntelliGenetics, Mountain View, CA). For the phylogenetic reconstruction, all vertebrate arrestins, with arrestin from the *Caenorhabditis elegans* as an outgroup, were aligned using Clustal W.\textsuperscript{22} This alignment was then imported into MEGA\textsuperscript{23} and the phylogenetic tree constructed using neighbor-joining with p-distance and treating gaps in a pairwise fashion.

**Nonradioactive In Situ Hybridization Histochemistry Followed by Indirect Immunofluorescence**

The probes for in situ hybridization were prepared as follows. A *BglII-HindIII* fragment (260 bp) of SalArr1 cDNA and a *Balt-HindIII* (273 bp) fragment of SalArr2 cDNA were subcloned into a vector (pBluescript II KS; Stratagene) digested with *BamHI-HindIII* and SmaI-HindIII (constructs pSV1 and pSO1, respectively). Antisense and sense (control) probes were generated using T3 and T7 RNA polymerase, respectively. Salamanders were killed by cervical transection, eyes removed, and eyecups immersed overnight in cold 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2). After fixation, eyecups were cryoprotected in 30% sucrose and frozen on dry ice. All procedures were performed using 20-μm-thick sections cut on a cryostat and mounted on coated (Vecabond; Vector, Burlingame, CA) slides. Slides-mounted, rather than free-floating, sections were used to preserve the morphology. Nonradioactive in situ hybridization histology (ISHH) with digoxigenin-labeled probes was performed essentially as described earlier.\textsuperscript{24} A mixture of nitroblue tetrazolium (0.34 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.17 mg/ml; NBT/BCIP) (Boehringer-Mannheim, Indianapolis, IN) was used as a substrate. When the color developed, the sections were washed in Tris-EDTA, blocked as described,\textsuperscript{24} and incubated with F4C1 monoclonal antibody\textsuperscript{25} (1:500) overnight at 4°C. This antibody readily recognizes SalArr2 and does not recognize SalArr1 on Western blot analysis (Fig. 3). The sections were subsequently incubated with biotinylated secondary anti-mouse antibody (1:200; Vector) for 1 hour at room temperature, followed by avidin D conjugated with fluorescein, (1:100; Vector). Sections were imaged on a confocal microscope (Olympus, Lake Success, NY) in dual-channel mode. One channel registered fluorescence at 488 nm to visualize fluorescein, and the second channel used transmitted light to visualize mRNA reaction product.

**Expression of Arrestins**

SalArr1 and SalArr2 cDNAs were excised from pCR2.1 with *BspHI-HindIII* and *NcoI-HindIII*, respectively, and subcloned into the *NcoI-HindIII* digested pG260\textsuperscript{26} for in vitro transcription and into pTrcHisB (Invitrogen) for expression in *Escherichia coli*. In vitro transcription was performed, as described.\textsuperscript{26} Translation using rabbit reticulocyte lysate (RLS) was performed as described\textsuperscript{26} in 70% RRL, 120 mM potassium acetate, 30 mM creatine phosphate, 160 μg/ml creatine kinase, 200 μl/ml RNAsin, 0.1 μg/ml pepstatin, 0.1 μg/ml leupeptin, 0.1 mg/ml soybean trypsin inhibitor, 5 mM cyclic adenosine monophosphate (cAMP), 50 μM of 19 unlabeled amino acids, 40 to 50 μM [\textsuperscript{14}C]leucine (14,000–35,000 disintegrations per minute [dpm]/μl), and 0.3 to 0.4 μg [\textsuperscript{3H}]leucine (800,000–1,000,000 dpm/μl; leucine-specific activity 10–25 Ci/mmol). Translation was performed using 150 μg/ml of mRNA with an idealized 5\textsuperscript{\prime}-untranslated region (UTR)\textsuperscript{26} at 22°C for 2 hours. Adenosine triphosphate (ATP) and guanosine triphosphate (GTP; 1 mM each) were added following by incubation for 7 minutes at 22°C (runoff). Samples were cooled on ice, and all aggregated proteins were pelleted by centrifugation (200,000g, 100,000g).
Rhodopsin Preparation

Urea-stripped bovine rod outer segment (ROS) membranes were prepared, phosphorylated with purified rhodopsin kinase, and regenerated with 11-cis-retinal, as described. To obtain ROS from frogs (Rana catesbeiana) were prepared as described to provide an amphibian rhodopsin for binding assays. Frog rhodopsin was phosphorylated using endogenous rhodopsin kinase or added purified bovine rhodopsin kinase to assays. Frog rhodopsin was phosphorylated using endogenous rhodopsin kinase or added purified bovine rhodopsin kinase to an average stoichiometry of 0.9 or 3.4 and 6.4 moles phosphate per mole rhodopsin, respectively, and then regenerated with 11-cis-retinal, as described.

Direct Binding Assay

This assay was performed essentially as described. In vitro translated arrestins (100 femtomoles, specific activity 100–140 Ci/mmol) were incubated in 50 µl of 50 mM HEPES-KOH (pH 7.5), 50 mM potassium acetate, 0.5 mM MgCl2, 1.5 mM dithiothreitol (DTT) with 0.6 µg of various functional forms of frog or bovine rhodopsin for 5 minutes at 22°C. The samples were then cooled on ice and loaded onto 2-ml Sepharose 2B columns equilibrated with 10 mM Tris-HCl (pH 7.4) and 100 mM NaCl buffer. Bound arrestin elutes with the receptor-containing membranes in the void volume (between 0.5 and 1.1 ml). Nonspecific binding (in the absence of rhodopsin) was subtracted.

RESULTS

Cloning and cDNA Analysis

Degenerate oligodeoxynucleotide primers were designed against sequences that are well conserved among all vertebrate visual and nonvisual arrestins. Primer pairs PH130/PH127 and PH129/PH1127 were used to amplify cDNA from reverse-transcribed salamander poly(A)+ RNA, resulting in the expected 700- and 500-bp products, respectively. The sequences of these fragments showed the greatest similarity to members of the arrestin superfamily in the GenBank database. The two sequences (designated SalArr1 and SalArr2) share only 64% identity in the overlapping region. These two clones were used to design exact oligonucleotide primers to amplify the remaining 5′ and 3′ portions of the cDNAs.

SalArr1 cDNA (Fig. 1A) is 1897 nucleotides in length, containing a single long ORF, where there are two potential initiating ATG codons. Because both ATG codons have Kozak’s consensus sequence, we used the first ATG as the initiating codon in the conceptual translation. The presence of an in-frame stop codon upstream of the putative initiation codon indicates that we obtained the 5′ end of the ORF for this cDNA. The absence of a consensus polyadenylation signal and the absence of a strong polyadenylated region suggest that the 3′-UTR is incomplete.

SalArr2 cDNA (Fig. 1B) is 1641 nucleotides in length, containing a single long ORF. Translation initiation for the conceptual polypeptide is assumed to begin with the first ATG in the ORF. The nucleotides just upstream of this initiating codon (AATC) agree well with Kozak’s consensus. There is an in-frame stop codon 18 nucleotides upstream from the putative initiating ATG and a consensus polyadenylation signal 20 nucleotides upstream of the poly(A)+ tail. These landmarks suggest that we cloned the full-length cDNA for SalArr2. SalArr1 and SalArr2 ORFs show 63.9% identity.

Polypeptide Comparison

SalArr1 encodes a 398-amino acid protein with a predicted mass of 45,056 Da. SalArr2 codes for a 392-amino acid protein with a predicted molecular mass of 43,895 Da. SalArr1 and 2 polypeptides are only 57.1% identical with each other. Both proteins, however, show conservation of amino acids that are recognized as hallmarks of the arrestin superfamily, particularly Arg-174 (SalArr1) and Arg-166 (SalArr2) which are homologous to Arg-175 of bovine rod arrestin. This arginine has been demonstrated to be a key element of arrestin’s phosphate sensor. Phyletic comparisons indicate that SalArr1 clusters with the amphibian rod arrestins and SalArr2 clusters with the amphibian cone arrestins (Fig. 2).

Tissue Distribution

Based on the polypeptide comparison and phyletic analysis, it appears that SalArr1 and SalArr2 are probably rod and cone arrestins, respectively. To verify that we cloned visual arrestins and to conclusively identify the cell type with which each arrestin is associated, we performed in situ localization, using probes that were specific to the 3′-UTR of each cDNA. Both probes labeled the photoreceptor layer (Figs. 3A, 3B). The SalArr1 probe (pSV1) labeled a virtually contiguous layer at the junction of inner and outer segments of photoreceptors, whereas the SalArr2 probe (pSO1) labeled individual widely separated cells at the same level. The F4C1 antibody, which recognized SalArr2 and not SalArr1 (Fig. 4), labeled separated cells with typical cone morphology (Fig. 3C through 3F). These same cells were clearly also labeled with pSO1 (Figs. 3D, 3F), but not with the SalArr1 probe (Fig. 3C).

Cell-Free Expression and Binding Characteristics

To test the binding characteristics of salamander arrestins, we subcloned both ORFs under control of the SP6 promoter into pG2S6I vector. This pGEM2-based vector adds an idealized 5′-UTR to ORF that enhances translation efficiency. Both constructs were linearized with HincIII (downstream from the stop codon) and transcribed using SP6 RNA polymerase. The translation of SalArr1 mRNA in rabbit reticulocyte lysate in the presence of radiolabeled leucine yielded a single protein band with expected electrophoretic mobility. To our surprise, the translation of SalArr2 mRNA using the same standard method repeatedly failed to yield any product. To exclude any errors in the ORF, we excised SalArr2 ORF from an expression pTrc vector (see description later) and subcloned it into pG2S6I. Transcription and translation of this construct still failed to yield any product, suggesting that SalArr2 does not fold properly in lysate (as a rule, misfolded and denatured proteins in reticulocyte lysate are rapidly degraded by abundant heat-shock proteases).

Using in vitro translated proteins we compared bovine visual arrestin and SalArr1 in the direct binding assay (Fig. 5A).
Bovine arrestin demonstrated characteristic highly selective binding to P-Rh*, whereas SalArr1 did not bind appreciably to any functional form of bovine rhodopsin. To test whether this apparent inactivity can be explained by species specificity, we performed similar experiments using frog rhodopsin with various stoichiometries of phosphorylation (Figs. 5B, 5C), because we were unable to obtain sufficient quantities of salamander rhodopsin. Arrestins from both species bind to frog rhodopsin, demonstrating the expected preference for P-Rh*. Both bovine and salamander arrestin bind well to rhodopsin, which has just one phosphate on average per rhodopsin molecule. Additional phosphorylation (up to 6.4 moles phosphate per mole rhodopsin) does not substantially change the binding.

Several elements in bovine arrestin have been found to be responsible for its strict selectivity toward P-Rh*. To test whether the mechanism ensuring the selectivity for P-Rh* is conserved in SalArr1 we introduced several mutations (homologous mutations in bovine arrestin shown in parentheses):...
R174E (R175E) and E373Ter (E379Ter) and 3A mutations L369A, V370A, and F371A (F375A, V376A, and F377A). The mutants were then tested in the direct binding assay with bovine and frog rhodopsin (Figs. 6A, 6B). All these mutations substantially increase SalArr1 binding to P-Rh* and to a proportionally greater extent to dark P-Rh and unphosphorylated Rh* (Fig. 6B).

Expression of Salamander Arrestins in E. coli

Many functional assays require substantial quantities of purified arrestin proteins that cannot be produced in cell-free translation 27,28. To establish a preparative expression system for both arrestins and to characterize SalArr2 that failed to express in the in vitro translation, we subcloned the ORFs of both salamander arrestins and that of SalArr1(3A) mutant into the E. coli expression vector pTrcHisB. SalArr1, SalArr2, and SalArr1(3A) expressed reasonably well in BL21 cells, yielding after our standard purification procedure 28 34, 5, and 2 mg of pure arrestin, respectively. To test the ability of purified proteins to bind to frog rhodopsin, we used a centrifugation assay 6 with subsequent quantitative Western blot analysis 27,28 (Fig. 7). We found that 80% of SalArr1, 82% of SalArr1(3A), and 44% of SalArr2 bound to 1.5 mg of frog P-Rh*. Only SalArr1(3A) demonstrated substantial binding to Rh* (Fig. 7), in agreement with our direct binding data (Fig. 6). To estimate the percentage of functionally active arrestins in our preparations, we performed similar experiments with 4.5 mg P-Rh* per assay, and found that 100%, 98%, and 85% of SalArr1, SalArr1(3A), and SalArr2, respectively, bound under these conditions. It is worth noting

FIGURE 1. (Continued)
FIGURE 2. Multiple alignment of vertebrate rod and cone arrestins. *Identical amino acids. Amino acid positions for the bovine arrestin polypeptide are indicated above the sequences. All sequences used in the alignment were obtained from GenBank: human rod, X12453; mouse rod, M24086; rat rod, X51781; bovine rod, J02955; dog rod, X98460; pig rod, S82664; SalArr1, AF203327; Xenopus rod, P51477; R. pipiens rod, U30269; R. catesbeiana rod, U30267; killifish rod, AB002554; SalArr2, AF203328; R. pipiens cone, U30270; R. catesbeiana cone, U30268; Xenopus cone, L40463; human cone, S66793; and killifish cone, AB002555.
Figure 2. (Continued)
that comparable amounts of SalArr1 and SalArr1(3A) mutant bound to 1.5 and 4.5 μg of P-Rh*, whereas the percentage of bound SalArr2 increased dramatically with the threefold increase in P-Rh* concentration. These data suggest that the affinity of SalArr1 for P-Rh* is substantially higher than that of SalArr2. Because 85% to 100% of E. coli–expressed arrestins were found to be functionally active, we next attempted to obtain a more quantitative estimate of their relative affinities for P-Rh* in a direct binding assay. In this assay [3H]SalArr1 served as radioligand, and purified E. coli–expressed arrestins served as competing ligands (Figs. 8A, 8B). We also performed a similar series of experiments using [3H]bovine visual arrestin (not shown). Under the conditions used, SalArr1 and SalArr1(3A) inhibited [3H]SalArr1 binding with Kᵢ of 179 ± 27 and 74 ± 12 nM, respectively (corresponding Kᵢ for the inhibition of [3H]bovine arrestin binding were 540 ± 120 and 63 ± 9 nM, respectively). SalArr2 exhibited a dramatically lower affinity for frog P-Rh*, with Kᵢ for the inhibition of [3H]SalArr1 and [3H]bovine arrestin binding being 9.1 ± 3.3 and 25.7 ± 2.6 μM, respectively. Thus, regardless of the radiolabeled arrestin used, the affinity of SalArr2 for frog P-Rh* was approximately 50 times lower than that of SalArr1, in agreement with results in centrifugation experiments.

**DISCUSSION**

Several lines of evidence indicate that SalArr1 and SalArr2 are rod and cone arrestins, respectively. Sequence alignment

![Figure 3](Image 64x370 to 302x726)

**Figure 3.** Localization of SalArr1 (pSV1) and SalArr2 (pSO1) mRNA in the salamander retina. (A, B) Bright-field microphotographs of the salamander retina stained for pSV1 (A) or pSO1 (B) mRNA. The sections were not counterstained. mRNA is seen as dark blue-purple product of alkaline phosphatase reaction. Both mRNA species were localized to the photoreceptor layer. pSV1 reaction product was deposited in a layer parallel to the retinal surface that runs almost uninterrupted between the outer and inner segment layers (arrow in A). pSO1 reaction product were represented by discrete small columns of color product perpendicular to the retinal surface (arrows in B point to examples). (C, D) Digital overlays of low power confocal images collected in the dual-channel mode. One channel was set to register fluorescein fluorescence (green) and the second to register transmitted light (gray). In these photographs mRNA appears black. Nonradioactive ISHH for (C) SalArr1 mRNA (bright field) and for (D) SalArr2 mRNA (bright field) overlaid with SalArr2-specific immunohistochemistry (green). SalArr1 mRNA and F4C1 immunoreactivity did not overlap but instead localized to separate populations of cells. In contrast, SalArr2 mRNA and F4C1 immunoreactivity were localized to the same cells. (E) Cells stained with F4C1 antibody displayed the morphology of cones. Free-floating 40-μm-thick sections were used in this experiment to demonstrate the morphology of immunoreactive cells. The highest concentration of F4C1 immunoreactivity was found in the proximal portion of the cone outer segment. (F) High-power confocal image displaying an overlap of SalArr2 mRNA and F4C1 immunoreactivity. SalArr2 mRNA was localized to the proximal portion of the cone outer segments that also had the highest concentration of SalArr2 protein. In this case immunoreactivity is somewhat inhibited compared with cells displayed in (E), because mRNA product interferes with the immunoreaction. However, colocalization of the two stains is evident. RPE, retinal pigment epithelium; OS, outer segments; IS, inner segments; OPL, outer plexiform layer (denoted with asterisks); INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglionic cell layer.

![Figure 4](Image 323x193 to 559x392)

**Figure 4.** Specificity of F4C1 antibody for cone arrestin SalArr2. Western blot of bovine visual arrestin (lanes 1 and 4), SalArr2 (lanes 2 and 5), and SalArr1 (lanes 3 and 6) probed with F4C1 (lanes 1 through 3) and H11A2 (lanes 4 through 6) monoclonal antibodies, which in bovine arrestin recognize epitopes (corresponding amino acid numbers indicated in parenthesis) DGVVLVD (42-48) and NLASSTIIKE and DGVVLVD and NLASSTIIKE (305-314), respectively. In SalArr1 and SalArr2 the sequences homologous to F4C1 epitope are DGVVLVD and DGVVLVD, respectively (mismatches are italic), whereas the sequences homologous to H11A2 epitope are NLASSTIIKE and NLASSTIIKE. Equivalent amounts of lysate of overexpressing E. coli cells (1–4 μg protein/lane), each containing 10 ng of corresponding arrestin, were loaded. Arrows: positions of markers. Note that F4C1 recognized bovine arrestin and SalArr2 and did not recognize SalArr1, whereas H11A2 recognized all three arrestins.
shows that SalArr1 and SalArr2 clustered with the amphibian rod and cone arrestins, respectively (Fig. 2). Both in situ hybridization and immunocytochemistry demonstrated that SalArr2 was expressed in photoreceptor cells with cone morphology, whereas SalArr1 was expressed in rods (Fig. 3). Each type of cells appeared to express only one of these two arrestins. Of note, within rod cells SalArr1 mRNA was concentrated in the distal part of the inner segment, whereas in cones, SalArr2 message appeared to be localized in or near the proximal part of the outer segment. In other words, in both types of photoreceptors, arrestin message was localized right next to the compartment where arrestin functions.

**Binding Characteristics of SalArr1 and Key Structural Elements in Its Molecule**

SalArr1 had a clear preference for frog over bovine rhodopsin, whereas bovine arrestin did not demonstrate significant species specificity (Fig. 5). In bovine arrestin, mutations of the phosphorylation-sensitive trigger Arg175, deletion of the arrestin COOH terminus, as well as elimination of a cluster of bulky hydrophobic residues FVF (375–377) all yielded arrestin proteins with relatively high binding to unphosphorylated Rh* and dark P-Rh. Homologous mutations reduced the selectivity of SalArr1 in the same fashion, suggesting that the mechanisms responsible for arrestin's preferential binding to P-Rh* are conserved, at least among vertebrates. In sharp contrast to wild-type SalArr1, the mutant forms of this protein also demonstrated dramatically reduced species specificity, binding surprisingly well to bovine rhodopsin, especially P-Rh* (Fig. 6A). It is worth noting that mutation-induced increase of arrestin binding to frog P-Rh* paralleled the increase of its binding to frog Rh* and dark P-Rh, as well as to bovine P-Rh* (the order of
context of a sequential multisite binding model. The SalArr1(1-373) was relatively selective for P-Rh*. 

bovine Rh*, so that in the case of bovine rhodopsin, even the binding to frog Rh* to a substantially greater degree than to formation by several constraining intramolecular interactions are simultaneously disrupted only when arrestin that change conformation on light activation. All constraining interactions are necessary to elucidate whether both types of salamander rods express SalArr1 and whether all types of cones express SalArr2.

**Acknowledgments**

The authors thank Jeffrey L. Benovic for purified rhodopsin kinase, Larry A. Donoso for monoclonal antibodies F4C1 and H11A2, Toshimichi Shinohara for bovine visual arrestin cDNA, and Rosalie K. Crouch for 11-<i>cis</i>-retinal.

**References**


7. Krupnick JG, Gurevich VV, Benovic JL. Mechanism of quenching of phototransduction: binding competition between arrestin and