Immunolocalization of X-arrestin in human cone photoreceptors

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Abstract X-arrestin is a recently identified retina-specific gene of unknown function. Affinity-purified anti-peptide antibody to human X-arrestin was prepared, and used in Western blot analysis of human retinal proteins and for immunohistochemistry on human retinal sections. By Western blot analysis, the antibody specifically bound to an ~47 kDa protein, and by indirect immunofluorescence specifically labeled cone photoreceptors with greatest intensity in their outer segments. In single and double label experiments, the localization of X-arrestin immunoreactivity was compared with immunolabeling patterns obtained with antibodies to red/green cone opsin, rhodopsin, and S-antigen. The results showed that X-arrestin is expressed in red-, green- and blue-sensitive cones in the human retina.

Key words: X-Arrestin; Arrestin; Anti-peptide antibody; Western blot analysis; Immunofluorescence; Human cone photoreceptor

1. Introduction

Isolation and characterization of retina-specific genes have contributed greatly towards understanding of retinal biology and function, and pathogenesis of inherited retinal degenerations. Well-known examples of such genes include rhodopsin and peripherin [1,2]. Recently, S-antigen or retinal arrestin, which plays a role in desensitization of photoactivated rhodopsin in phototransduction [3], joined the ranks of retinal genes that can cause retinopathy; a mutation in this gene was shown to be involved in Oguchi disease, a form of congenital stationary night blindness [4].

In a project to isolate human retina-specific genes by a subtractive cDNA cloning strategy, we isolated a cDNA encoding a new type of retinal arrestin [5]. The cDNA encoded a protein of 388 amino acid residues which showed a 49–58% homology to β-arrestin and S-antigen, respectively, indicating its distinctness from the other arrestins. The gene was expressed in photoreceptors, as assessed by in situ hybridization, and was named X-arrestin due to its X chromosome localization [5]. Another group subsequently isolated this gene, which they named C-arrestin, from human, rat and *Xenopus laevis* [6,7] and predicted it to be cone-specific in expression on the basis of in situ hybridization in rat retina [6].

Precise cellular localization of the protein encoded by a newly isolated gene is an important step towards the goal of discovering its function, and is especially instructive in a compartmentalized tissue such as retina, with its layered organization of highly differentiated classes of neurons. In this paper we report the preparation of an anti-peptide antibody directed against a unique sequence in X-arrestin, and its use in immunohistochemical studies on human retinal sections, along with antibodies to red/green cone opsin, rhodopsin and S-antigen to demonstrate the localization of X-arrestin in the three classes of human cone photoreceptors.

2. Materials and methods

2.1. Preparation of X-arrestin anti-peptide antibody

The human X-arrestin sequence was analyzed for a unique region showing high antigenicity by GenAlign and Plotstructure computer programs, respectively (GCG, Madison, WI). A 13 amino acid sequence including an arbitrarily added cysteine (QKAVEAEGDEGSC, residues 377–388) was chosen from the carboxy terminus, and the peptide was synthesized and used to prepare an anti-peptide antibody as described [8]. Briefly, 5 mg of peptide was coupled to keyhole limpet hemocyanin using *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS), purified through Sephadex G-25, mixed with Freund's complete adjuvant, and used for immunization of a New Zealand white rabbit. Every 2–3 weeks thereafter, the rabbit was immunized with a half dose of antigen in incomplete Freund's adjuvant, and serum was obtained. The antiserum, along with preimmune serum, was tested for antibody activity by Western blot analysis. All use of animals (rabbits and rats) was in accordance with the guidelines established in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The antiserum was purified by affinity chromatography as described [8]. Briefly, an affinity column was prepared by coupling bovine serum albumin to the synthetic peptide using MBS, purifying it through Sephadex G-25, then coupling it to CNBr-activated Sepahrose 4B (Pharmacia Biotech, Piscataway NJ). The antiserum was passed through the affinity column, and the bound antibody was eluted with glycine after washing. IgG concentration was estimated by spectrophotometry (OD_{280}).

2.2. Western blot analysis

Retinas were obtained from human Eye Bank eyes, homogenized in single-detergent-lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% Triton X-100), and centrifuged to prepare retinal extract. Protein concentration was determined with Micro Protein Determination (Sigma, St. Louis, MO). 10 μg of retinal extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electroblotted onto an Immobilon membrane (Millipore, Bedford, MA). The membrane was blocked with non-fat dry milk, reacted with the whole antiserum or affinity-purified antibody (50–1000× dilution, 0.2–2.0 μg/ml, respectively), washed and reacted with biotinylated goat anti-rabbit IgG antibody. Bound antibody was visualized by reaction with streptavidin-alkaline phosphatase conjugate (Gibco BRL, Gaithersburg, MD), followed by washing and treatment with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt for color development.

2.3. Immunohistochemistry

Human Eye Bank eyes and pigmented (Long Evans) rat eyes were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and dissected portions of retina-lined eyecups were embedded in O.C.T. compound (Miles, Inc., Elkhart, IN). Cross-sections of retina (6 μm thickness) were prepared on a cryostat and used for immunostaining. Sections were first post-fixed in 4% paraformaldehyde, washed in phosphate-buffered saline (PBS) and blocked with 10% goat serum in PBS, then incubated for 1 h at room temperature with one of the following antibodies: (1) affinity-purified rabbit anti-human X-arrestin peptide antibody (5 μg/ml); (2) rabbit antibody generated

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against a 22-residue peptide in the N-terminal extension of human red and green cone opsins [9,10], 1:100 dilution; (3) MAbA9-C6 anti-bovine S-antigen monoclonal antibody [11], 1:100 dilution, or (4) G26c anti-bovine rhodopsin monoclonal antibody [12], 1:10 dilution. Following washing, sections were incubated with FITC-labeled goat anti-rabbit IgG, FITC-GARG (50× dilution; Organon Tecknika, Durham, NC) for the X-arrestin and red/green cone opsin antibodies, and FITC-labeled goat anti-mouse IgG, FITC-GAMG (50× dilution; BRL, Gaithersburg, MD) for the rhodopsin and S-antigen monoclonal antibodies. Slides were then washed and mounted in glycerol/PBS.

For double immunofluorescent histochemistry, human retinal sections were first processed for X-arrestin immunoreactivity as described above, except with the use of rhodamine labeled goat anti-rabbit IgG (Rh-GARG) (50× dilution; Boehringer, Indianapolis, IN) as the initial secondary antibody. Sections were then washed, post-fixed in cold methanol for 10 min (only for subsequent reaction with the red/green cone opsin antibody, as recommended by John C. Saari, Ph.D) and further reacted with the second specific antibody, i.e. either anti-human red/green cone opsin or anti-bovine S-antigen antibody. Following the wash step, sections were finally reacted with the appropriate FITC-tagged secondary antibody, i.e. FITC-GARG (for opsin) or FITC-GAMG (for S-antigen). Control sections for the double staining experiments were reacted with both secondary antibodies i.e. Rh-GARG and either FITC-GARG or FITC-GAMG. All slides were examined in a Zeiss Photomicroscope III by phase contrast and fluorescence microscopy using filter sets selective for rhodamine and fluorescein.

3. Results

In this study, a rabbit polyclonal antibody was prepared against a 13-amino acid peptide chosen from the carboxy-end of the human X-arrestin sequence. Sequence homology is extensive among all members of the arrestin family [5]; because the carboxy end was one of the few unique regions of X-arrestin, the peptide sequence was derived from this region. By Western blot analysis of human retinal homogenates, the whole rabbit antiserum showed activity predominantly against a protein with an apparent size of ~47 kDa (data not shown). The anti-peptide antibody was purified by affinity chromatography, and on Western blot analysis, the purified X-arrestin antibody recognized a single band of ~47 kDa in human retinal extracts. This band was not reactive with the preimmune serum or the flow-through fraction from the affinity purification (Fig. 1).

The affinity purified human X-arrestin anti-peptide antibody was used for indirect immunofluorescent staining of rat and human retinal sections. No detectable immunoreaction was seen in sections of rat retina using this antibody (data not shown). In sections from human retina, prepared from the far periphery, positive immunoreactivity was observed in widely spaced cells with the appearance of cones (Fig. 2a). A faint immunofluorescence reaction was visible throughout the length of these cells, from the synaptic termini to the outer segments, but the signal was generally most intense over their short outer segments (arrows, Fig. 2d). When X-arrestin immunostaining was performed on human retinal sections prepared from the cone-rich perifoveal region, positively stained cone-shaped cells, with intensely reactive outer segments were more abundant (Fig. 2b,e). Control sections, reacted with X-arrestin preimmune serum revealed no specific staining of any retinal cell type (Fig. 2c).

For comparison with the staining pattern seen with the X-arrestin antibody, adjacent sections from the same human retina were reacted with an antibody specifically recognizing opsin in red-and green-sensitive cone photoreceptors [9,10].

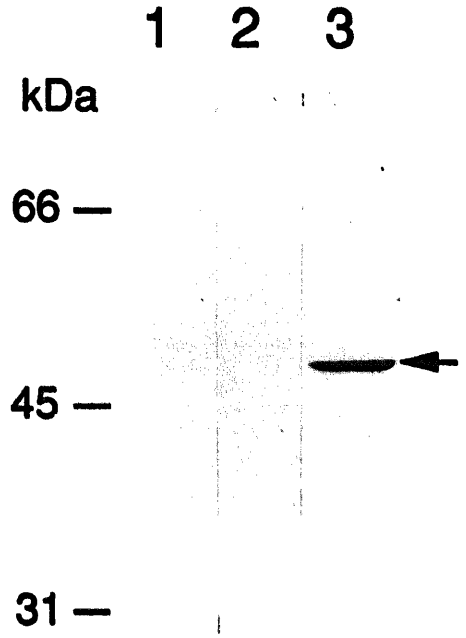

Fig. 1. Western blot analysis of human retinal homogenate proteins. Human retinal extracts were electrophoresed in 10% SDS-PAGE, transferred to a nylon filter and reacted with preimmune serum from the rabbit used for antibody production (lane 1), pass-through fraction from the affinity purification (lane 2), and affinity-purified, human X-arrestin anti-peptide antibody (lane 3). Bound antibody was visualized by the alkaline phosphatase color reaction. The X-arrestin anti-peptide antibody recognizes a protein in human retina of ~47 kDa (arrow). The size markers are bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa).

The results revealed a very similar pattern of immunostaining with either antibody (cf. Fig. 2e,f). Thus, the distribution of X-arrestin positive cells appeared to correspond closely to that of the red and green cones. By contrast, staining of adjacent sections with antibodies to rod-expressed proteins i.e. rhodopsin and S-antigen, revealed strikingly different labeling patterns. With the A9C6 monoclonal antibody against S-antigen, the entire rod photoreceptor layer was reactive, as previously described [11], and the positions of the nuclei and inner segments of the unstained cone cells were strikingly revealed as keyhole-shaped negatively stained areas between the strongly immunoreactive rod photoreceptors (arrows, Fig. 2g). As previously shown [12], the G26c anti-rhodopsin antibody specifically labeled just the outer segments of the rods, which appeared much longer and thinner than the outer segments of the cones (compare Fig. 2e,f with Fig. 2h,i).

In order to confirm the localization of X-arrestin in red and green cones, a double label procedure was performed using the antibodies against X-arrestin and red/green cone opsins on the same section (Fig. 3a,b). The result, visualized using filter sets selective for either rhodamine or fluorescein, revealed that each cone cell positive for the red/green cone opsin was also positive for X-arrestin, thus confirming expression of X-arrestin in red-and green-sensitive cone photoreceptors (Fig. 3a,b). Very minimal background staining was seen in control sections doubly reacted with only the two fluorescent secondary antibodies (Fig. 3c,d).

To compare the distribution of the two classes of retinal arrestins, double labeling was performed using the X-arrestin

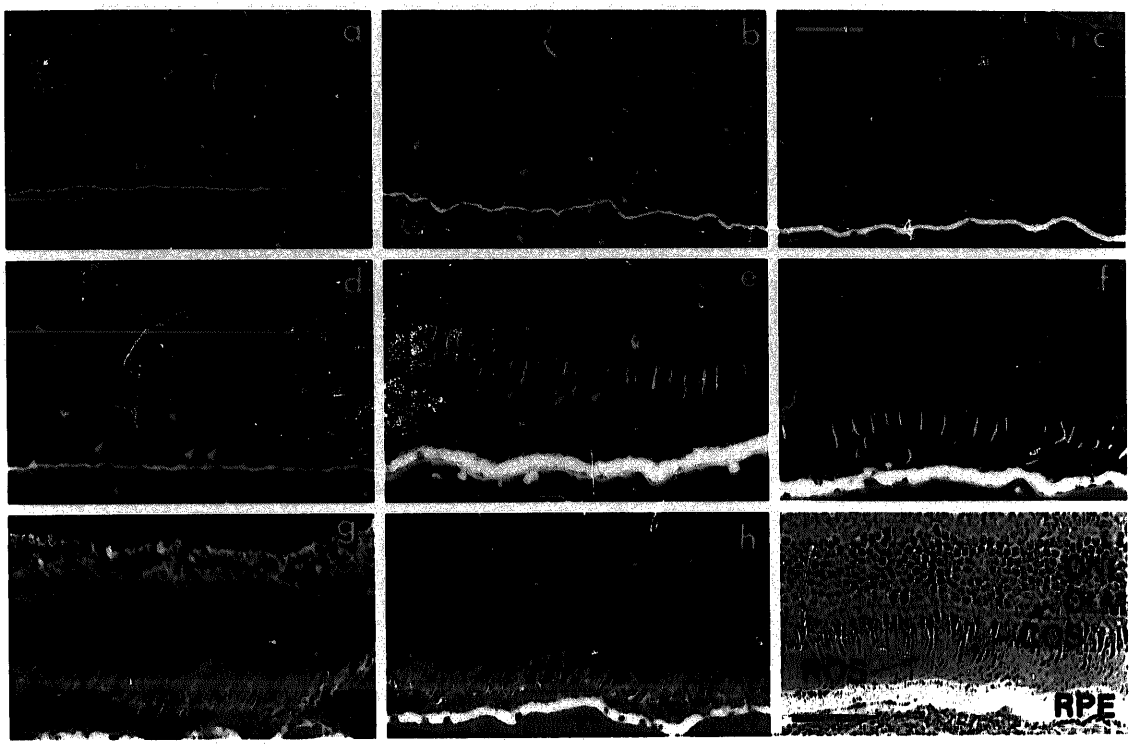

Fig. 2. Localization of X-arrestin in human retina by indirect immunofluorescence. Frozen sections of peripheral (a) and perifoveal (b) retina from two human donor eyes reacted with affinity-purified human X-arrestin anti-peptide antibody (a,b) or rabbit preimmune serum (c). The wavy horizontal line seen at low power in (a–c) is due to autofluorescence of lipofuschin in the aged retinal pigment epithelium. Images in (d,e) are higher magnifications of sections shown in (a,b), respectively. Arrows in (d,e) point to cone outer segments. There is a slight detachment of the retina in most areas of the perifoveal sections. Adjacent perifoveal sections (f–h) show immunostaining patterns with anti-human red/green cone opsin antibody (f), anti-bovine S-antigen antibody (g), and anti-bovine rhodopsin antibody (h). The phase contrast image of the field in (h) is shown in (i). ONL, outer nuclear layer; OLM, outer limiting membrane; COS, cone outer segment; ROS, rod outer segment; RPE, retinal pigment epithelium. Magnification (a–c) ×60; (d–i) ×150. Bar = 165 μm in (a–c) and 65 μm in (d–i).

and S-antigen antibodies. As predicted, the two immunofluorescent staining patterns obtained with this combination of antibodies were very different, with the X-arrestin antibody labeling the cones, and the S-Ag antibody labeling the rods in the same section (Fig. 3e–g). This experiment also documented that there was no overlap of the fluorescent signal emitted by the rhodamine-labeled cones when viewed through the fluorescein filter set, and similarly that the fluorescein-labeled rods could not be visualized with the rhodamine filter set (Fig. 3e,f). This control verified that the precise co-localization of X-arrestin and red/green cone opsin in same cone cells in the above double label experiment (Fig. 3a,b) was due to signal uniquely emitted by one or the other fluorescently tagged secondary antibody, with no overlap of emission spectra visible with filter sets used.

The experiments described above clearly indicated expression of X-arrestin in red-and green-sensitive cones and not rods. The human retina also contains blue-sensitive cones, which are relatively scarce compared with the red/green cones [13,14]. The A9C6 S-antigen anti-peptide antibody used in this study has been conclusively shown to recognize not only the rods, but also the blue cones [15,16]. We took advantage of this characteristic of the antibody to investigate whether X-arrestin is expressed in blue cones. To do so, sections double immunolabeled with the X-arrestin and the A9C6 antibodies were carefully examined at high power to look for evidence of cones that were double labeled with X-arrestin and A9C6. Indeed, while the great majority of the cones was not labeled by A9C6, a few double labeled cones could be found, three examples of which are shown in Fig. 4. These cells were therefore blue cones that were expressing X-arrestin. Conversely, since the anti-peptide antibody recognizing a sequence in red and green cone opsin specifically labels red and green cones [9,10], it would be expected from the double label experiment with the X-arrestin and red/green cone opsin antibodies that a small percentage of the cones, representing the blue cones, would be positive for X-arrestin but negative for red/green cone opsin. This was also found to be the case, as shown in Fig. 5, which is from an adjacent section of the same retina shown in Fig. 4. This data thus provided further indirect evidence for the expression of X-arrestin in blue cones.

4. Discussion

Our initial attempt to localize X-arrestin within the retina by in situ hybridization gave equivocal results [5]. Despite repeated attempts using a carefully chosen ^{35}S-labeled riboprobe specific for X-arrestin, silver grains were heavily distributed over inner segments of both rods and cones in sections of human retina following liquid emulsion autoradiography. Using this technique and this particular probe, we could only conclude that X-arrestin is expressed predominantly in photoreceptors in the human retina. Craft et al. [6] also used in situ hybridization to localize the expression of X-arrestin (named C-arrestin by them), and although the precise sequence of their riboprobe was not described, they reported

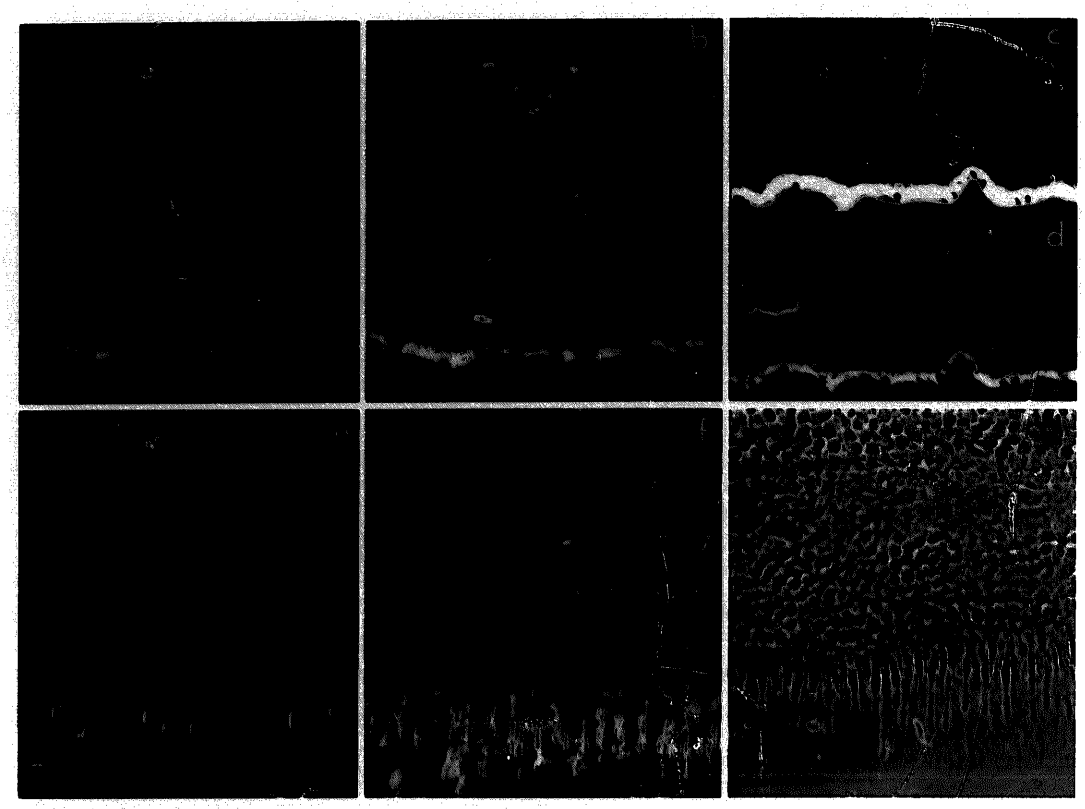

Fig. 3. Double immunofluorescent localization of X-arrestin in human retina. (a,b) Double immunolabel with X-arrestin and red-green cone opsin antibodies. A section of perifoveal human retina was sequentially reacted with rabbit anti-X-arrestin antibody, Rh-GARG, rabbit anti-red/green cone opsin antibody and FITC-GARG. Note the precise correspondence of positively stained cones, doubly labeled with the X-arrestin antibody (viewed with the Rh filter set in a) and with the red/green cone opsin antibody (viewed through the FITC filter set in b). (c,d) Adjacent control section from retina shown in (a,b) doubly reacted with Rh-GARG (c) and FITC-GARG (d) alone, viewed with corresponding filter sets. (e,f) Double immunolabel with X-arrestin and S-antigen antibodies. Specific localization of X-arrestin to Rh-tagged cones is seen in (e), whereas S-Ag distribution is confined to the FITC-tagged rods in the section, shown in (f). (g) Phase contrast image of the section shown in (e,f). Magnification (a,b,e–g) ×230; (c,d) ×150. Bar = 165 μm in (c,d) and 43 μm in (a,b,e–g).

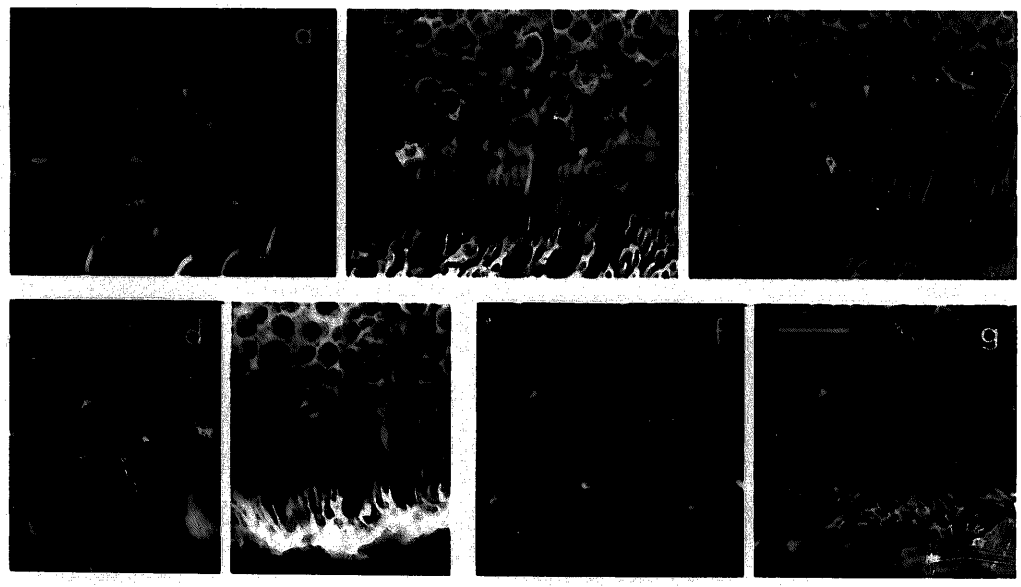

Fig. 4. Localization of X-arrestin in blue cones. High magnification micrographs of the section shown in Fig. 3e–g, double stained with X-arrestin (a,d,f) and A9C6 S-Ag (b,e,g) antibodies. The panels show three examples of X-arrestin positive cones (arrows) showing immunoreactivity with A9C6, thereby identifying them as blue cones. Adjacent X-arrestin positive cone cells, negative for A9C6 (asterisks) are red/green cones. Magnification, all panels ×375. Bar = 27 μm.

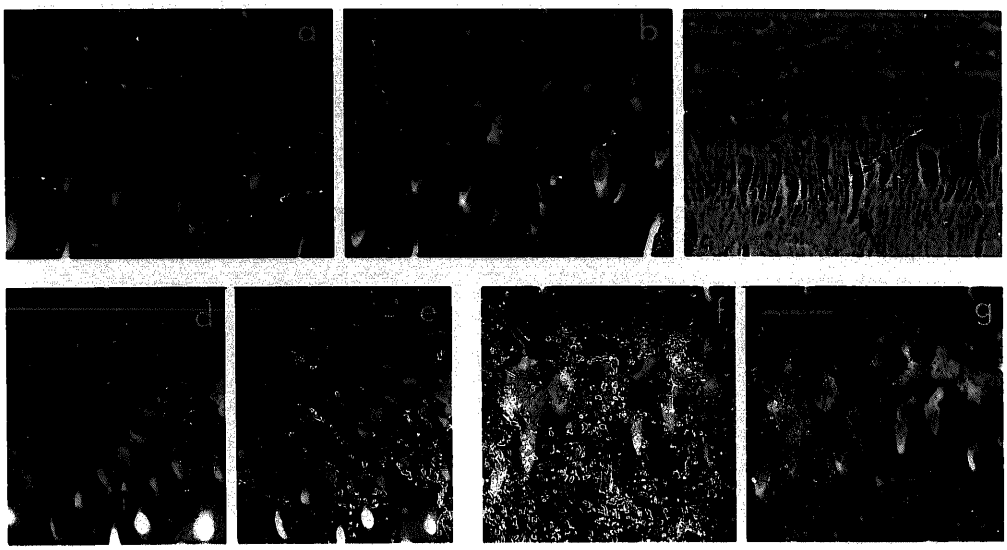

Fig. 5. Same section shown in Fig. 3a,b, showing double immunostaining of human retina with X-arrestin (a,d,f) and red/green cone opsin (b,e,g) antipeptide antibodies. Image in (c) is the phase contrast image of the section shown in (a,b). Arrows indicate three examples of X-arrestin positive cones that are negative for red/green cone opsin, and are therefore assumed to be blue cones. Magnification, all figures ×375. Bar = 27 μm.

that the probe was sufficiently specific to demonstrate cone-specific expression in rat retina. The rat is a challenging experimental model for study of cones, however, because cones are very scarce in the rat retina and closely resemble the rods in shape. By light microscopy of 1 μm thick plastic sections of mouse and rat retina fixed for electron microscopy with glutaraldehyde and paraformaldehyde, cones can be distinquished from rods primarily by their nuclear morphology, and location of the nuclei predominantly in the outer one third to one half of the outer nuclear layer (ONL), often close to the outer limiting membrane [17,18]. However, in situ hybridization is typically carried out using 6–8 μm frozen or paraffin sections of retina immersion fixed only with paraformaldehyde; under these conditions morphological preservation is quite poor and subtleties of nuclear morphology are lost (e.g. see Fig. 5a of our previous study [5] and Fig. 5 of [6]). Additionally, the resolution of this technique is limited by scatter of the signal, inherent in use of ^{35}S-labeled riboprobes and liquid emulsion autoradiography. Previous evidence in support of C-arrestin expression in cones [6] has been limited to demonstration in rat retina of very sparse label associated with a small percentage of the nuclei in the ONL, mainly located near the outer limiting membrane. Surprisingly, no silver grains were seen over the inner segments, the site of translation. The localization of C-arrestin was postulated to be in cones, based on the number and position of the labeled nuclei seen by in situ hybridization [6], but as discussed above, without definitive identification of the cone nuclei, it is not clear how it could be concluded that the signal was originating from cones.

In marked contrast to the technique of in situ hybridization, which at best enables the localization of expression of an mRNA to a particular cell type in the retina, immunohistochemistry allows for precise subcellular localization of the protein at the light microscopic level, as shown in this study. Anti-peptide antibodies, specifically designed to recognize unique antigenic determinants within proteins, are especially powerful tools that can enable even closely related proteins to be distinguished and localized by immunohistochemisty. The affinity purified anti-peptide antibody prepared in this study against a unique 13 amino acid sequence in the carboxy terminus of human X-arrestin showed specific activity against a single band of ~47 kDa on Western blots of human retinal homogenate. When used for immunolocalization in human retina along with antibodies recognizing other cone-and rod-specific proteins, this antibody conclusively demonstrated X-arrestin to be expressed in cone photoreceptors. The pattern of immunolabeling of cones in sections doubly reacted with the X-arrestin and red/green cone opsin antibodies clearly indicated that X-arrestin is expressed in the red-and green-sensitive cones. The data from the double label experiments with the two combinations of antibodies, i.e. X-arrestin + A9C6 and X-arrestin + red/green cone opsin confirmed the localization of X-arrestin in blue-sensitive cones. Thus it can be concluded that X-arrestin is expressed in all three classes of cones in the human retina. The predominant localization to the outer segments of these cells is consistent with a putative role for X-arrestin in cone phototransduction. The fainter reaction of the X-arrestin antibody in other parts of the cone photoreceptors, e.g. the synaptic termini, could be an artifact resulting from postmortem diffusion of the molecules. Alternatively, this result may reflect bona fide presence of X-arrestin or a similar molecule in other compartments of the cell, as suggested for S-antigen in rod photoreceptors [19]. Further studies using experimental animals with retinas amenable to study of cones will be needed to pursue this question.

Arrestins are thought to work by competing with the relevant G-protein for interaction with the receptor in a signal transduction cascade [3]. The carboxy terminal region has been shown to be unique and functionally important for each type of arrestin [20]. The carboxy-terminal region of X-arrestin is quite different from that of the other retinal arrestin, i.e., S-antigen, and in fact, is slightly more similar to β-arrestin [5]. Thus, it is interesting that the present data suggests on the basis of predominant localization in the cone outer segments that X-arrestin might interact with cone opsins

to effect their desensitization. In that case, X-arrestin might be expected to share more homology with S-antigen, which interacts with the rod visual pigment, rhodopsin. On the other hand, the dissimilarity may indicate that cone phototransduction involves a pathway different from that of rods. As demonstrated here for X-arrestin, use of an antibody prepared against the α subunit of cone transducin has provided evidence for expression of the same transducin subunit in all three types of human cones [9]. Expression of recombinant X-arrestin for use in functional studies may clarify the specific pathway in which this new arrestin participates.

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