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UV-light-dependent binding of a visual arrestin 1 isoform to photoreceptor membranes in a neuropteran (*Ascalaphus*) compound eye[1]

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Abstract Arrestins are regulators of the active state of G-protein-coupled receptors. Towards elucidating the function of different arrestin subfamilies in sensory cells, we have isolated a novel arrestin 1, Am Arr1, from the UV photoreceptors of the neuropteran *Ascalaphus macaronius*. Am Arr1 forms a phylogenetic clade with antennal and visual Arr1 isoforms of invertebrates. Am Arr1 undergoes a light-dependent binding cycle to photoreceptor membranes, as reported earlier only for members of the arrestin 2 subfamily. This suggests a common control mechanism for the active state of invertebrate rhodopsins and G-protein-coupled receptors of antennal sensory cells. Furthermore, it implies that a strict correlation of distinct arrestin isoforms to distinct functions is not a general principle for invertebrate sensory cells.

Key words: Arrestin; UV-absorbing rhodopsin; Light-dependent arrestin binding; Vision; Sensory cell

1. Introduction

Arrestins constitute a family of regulatory proteins involved in the control of the active state of G-protein-coupled receptors (GPCRs). Arrestins may also act as clathrin adapters in the endocytosis of GPCRs, as has been shown for example for the β-adrenergic receptors (reviewed in [1,2]) and *Drosophila melanogaster* (Dm) rhodopsin (P) [3,4]. A new role for arrestin emerges from the finding that β-arrestin also functions as an adapter protein linking GPCR inactivation to the initiation of the MAP kinase signalling pathway [5,6]. In vertebrate photoreceptor cells arrestin binding to the light-activated state of P (metarhodopsin, M) is part of a sequence of regulatory reactions leading to the termination of visual responses. High-affinity binding of arrestin to bovine M is promoted by the P kinase-catalyzed phosphorylation of serine and threonine residues located near the C-terminus of P. The formation of a complex between arrestin and the activated P state represents the deactivation step which uncouples P from G-protein (transducin) activation (for summaries see [7,8]).

The necessity for an effective deactivation of the active P state is particularly evident in invertebrate photoreceptors in which light-absorption triggers the formation of a long-lived active M-state [9]. In dipteran flies (e.g. *Drosophila*, *Calliphora*) two genes encoding visual system-specific expressed arrestins, arrestin 1 (Arr1) and arrestin 2 (Arr2), have been isolated [10–13]. Analysis of *arr1*- and *arr2*-mutants in *Drosophila* showed that both arrestin isoforms contribute to the termination of the phototransduction cascade [14]. Arr2, the major arrestin form of the photoreceptor cell, binds light-dependently with high affinity to M [15–18]. Both arrestins are phosphorylated light-dependently by a calcium/calmodulin-dependent protein kinase [13,19,20]. In a distinct difference to the arrestin-mediated deactivation of vertebrate P, phosphorylation of M is not a prerequisite for binding of Arr2 [4,15,21]. It is rather so that binding of Arr2 is part of a mechanism that allows M to become phosphorylated by a P kinase [15] and that prevents P dephosphorylation by the receptor phosphatase RDGC [16,22,23]. As the interaction of M with Arr2 is the rate-limiting step in the over-all termination of the light response in *Drosophila* [14], it is of particular interest to know whether or not the formation of stable M–arrestin complexes is a general mechanism in the control of the active P state of invertebrate photoreceptors.

An insect P system with ideal properties for studying the light-dependent biochemical events in phototransduction is located in the UV-sensitive dorsal eye of the neuropteran *Ascalaphus macaronius* (Am). It has been shown that absorption of UV-light converts P (λ_{max} 345 nm) into a long-lived M (λ_{max} 480 nm). M can be completely photoreconverted into P, simply by irradiation with blue–green light [24,25]. In the present study we isolated the gene encoding an arrestin isoform from *Ascalaphus* with a high homology to *arr1* of *Drosophila* and to antennal arrestins. We show here for the first time that light-dependent-high-affinity binding of arrestin to M is not only a property of Arr2 isoforms but is also observed with the Arr1-related subtypes.

2. Materials and methods

2.1. Cloning and sequencing of arrestin cDNA

cDNA clones encoding arrestin were isolated from an oligo(dT)-primed cDNA library of *Ascalaphus* heads cloned in the vector Lambda ZAP II (Stratagene). Screening of the library was performed at low-stringency conditions [26] with *Calliphora vicina arr1* (*Cvarr1*)

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[1] The cDNA sequence of the *Ascalaphus* Am Arr1 gene has been submitted to the EMBL data library under the accession number AJ303080

Abbreviations: Am, *Ascalaphus macaronius*; Arr1, arrestin 1; Arr2, arrestin 2; Cv, *Calliphora vicina*; Dm, *Drosophila melanogaster*; GPCR, G-protein-coupled receptor; Hv, *Heliothis virescens*; M, metarhodopsin; P, rhodopsin

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cDNA as a probe. Sequencing of the coding and reverse strand was performed by the didesoxy-chain-termination method.

2.2. Northern blot analysis and in vitro translation of arrestin

Northern blot analysis was performed with digoxigenin-labelled cRNA as described previously [26]. ^{35}S-labelled arrestin was synthesized by in vitro translation from 2 μg DNA per 50 μl reaction volume using the TNT™ coupled reticulocyte lysate system (Promega). Non-incorporated label was removed by centrifugation on a microcon 10 microconcentrator (Amicon).

2.3. Preparation of visual-pigment extracts and spectrophotometry

Specimens of *Ascalaphus* (= *Libelluloides*) *macaronius* (Neuroptera) were collected in Slovenia. Compound eyes were cut from the head with a razor blade, and retinas were dissected from the eyes in red light. The dorsal UV-light-sensitive retina was separated from the lateral retina. The distal part of the retina, in which the P-containing rhabdomeres are located, was removed from the remaining tissue with a forceps and was kept on ice or frozen in liquid nitrogen and then stored at −80°C. Photoreceptor membranes were prepared for spectrophotometric measurements by homogenizing isolated dorsal retinas in PBS (0.2 M sodium phosphate buffer, 0.1 M NaCl, pH 6.3). Membranes were pelleted by centrifugation and washed three times with PBS before extraction of the visual pigment with 4% (w/v) digitonin in PBS, pH 6.3. Recording of difference spectra was performed at 4°C with a Kontron spectrophotometer. Conversion of P to M was carried out by irradiation of visual-pigment extracts with monochromatic UV-light (345 nm), conversion of M into UV-P was performed by irradiating extracts with monochromatic blue light (482 nm).

2.4. Arrestin binding

For arrestin-binding experiments, isolated dorsal retinas were irradiated with UV-light (345 nm) to photoconvert P into M (P→M). Washed photoreceptor membranes were subsequently prepared in the dark as described above. For releasing arrestin from membranes, M, produced in situ by conversion of P→M, was photoreconverted into P by irradiating the isolated photoreceptor membranes with blue light (M→P) (see also legend to Fig. 4). Membranes were then sedimented by centrifugation and the supernatant was analyzed for arrestin by SDS–PAGE and Western blotting.

2.5. SDS–PAGE and Western blot analysis

The protein pattern of photoreceptor membranes and the light-dependent binding of proteins were analyzed by SDS–PAGE on 12% gels and by immunoblotting. For immunoblots, proteins were electroblotted onto PVDF membranes in 50 mM Tris buffer pH 8, containing 20% methanol and 0.1% SDS. Immunolabelling was performed with polyclonal antibodies raised against recombinately expressed Arr1 and Arr2 of *Calliphora* followed by binding of ^{125}I-labelled secondary antibody. Autoradiography was performed with Kodak X-ray film (X-OMAT-AR).

3. Results and discussion

3.1. Isolation of a gene encoding an Arr1-related protein

Arr1 isoforms have not yet been investigated with respect to the proposed interactions with a long-lived, active P state. Therefore, we focussed our study on the identification of a putative Arr1 homolog in UV photoreceptors of the neuropteran owlfly. Screening of a head-cDNA library of *Ascalaphus* at reduced stringency with a *Cvarr1* cDNA resulted in the isolation of a number of cDNA clones with inserts up to 1.5 kb. Sequencing revealed that the largest cDNA (1.5 kb) contained 105 bp of 5′-untranslated region with an in frame stop codon 10 nucleotides before the first ATG start codon, an open reading frame encoding an arrestin consisting of 381 amino acids (calculated MW = 42 780) and a 218-bp 3′-untranslated region. When aligned with known arrestins, the deduced amino acid sequence showed the highest amino acid identity (70.6%) to Hv Arr, an antennal arrestin from the moth *Heliothis virescens* (Hv) [27] (Fig. 1). The most

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  Am Arr1     (1) MVANFKVFKKSSPNGKITIYLGKRDFVDHVSGVEPVDGVLLLDDDYINQK
   Hv Arr     (1) MVYNFKVFKKCAPNGKITLYMAKRDFVDHISTVEPIDGVVLLDEEYVR--
  Dm Arr1     (1) MVVNFKVFKKCSPNNMITLYMNRRDFVDSVTQVEPIDGIIVLDDEYVRQ-
  Dm Arr2     (1) MVVSVKVFKKATPNGKVTFYLGRRDFIDHIDYCDPVDGVIVVEPDYLK--
Consensus     (1) ** ..***** **...* *   ***.*.   ..* **.  ..  *

  Am Arr1    (51) ERKVFGQIVCSFRYGREEDEVMGLNFRKDLTLVSEQIYPSNEK--VETTK
   Hv Arr    (49) GRKVFGQMVCTFRYGREEDEVMGLNFYKELFLASEQIYPPPEKRNYELSR
  Dm Arr1    (50) NRKIFVQLVCNFRYGREDDEMIGLRFQKELTLVSQQVCPPQKQ-DIQLTK
  Dm Arr2    (49) NRKVFGQLATTYRYGREEDEVMGVKFSKELILCREQIVPMTNP-NMEMTP
Consensus    (51)  **.*.* .  .*****.**..*. * *.* * ..*. *          .

  Am Arr1    (99) LQDRLIKKLGPNAIPFKFVLPPNAPATITLQPGAEDQGEPCGVQYFLKAF
   Hv Arr    (99) TQERLIKKLGDGAIPFRLTVPPGAPGSVILQPGLEDDGEPCGVQYYVKIF
  Dm Arr1    (99) MQERLLKKLGSNAYPFVMQMPPSSPASVVLQQKASDESQPCGVQYFVKIF
  Dm Arr2    (98) MQEKLVRKLGSSAHPFTFHFPPNSPSSVTLQQEGDDNGKPLGVEYTIRAF
Consensus   (101)  *..*. .*** * **    **  * .. **    * . *.**.*  . *

  Am Arr1   (149) VGESDTERPHARSSVSMQIRKIQYAPSKQG-RQPCTVVRKDFMLSPGDLE
   Hv Arr   (149) VGDSEIDRSHRRSTVALGIRKVQYAPAKPG-PQPCTVVRKDFVLSPGQLE
  Dm Arr1   (149) TGDSDCDRSHRRSTINLGIRKVQYAPTKQG-IQPCTVVRKDFLLSPGELE
  Dm Arr2   (148) VGDSEDDRQHKRSMVSLVIKKLQYAPLNRGQRLPSSLVSKGFTFSNGKIS
Consensus   (151) .*.*  .* * ** . . *.* **** . *  .*...*.*.* .*.* ..
                                                          .

  Am Arr1   (198) LEVTLDKQLYYHGEKIAVNICIRNNSNKVVKKIKAMIQQGVDVVLFQNGQ
   Hv Arr   (198) LELTLDKQLYIHGETVAVNMCVRNHSNKVVKKIKACIQQGVDVVLFQNGQ
  Dm Arr1   (198) LEVTLDKQLYHHGEKISVNICVRNNSNKVVKKIKAMVQQGVDVVLFQNGQ
  Dm Arr2   (198) LEVTLDREIYYHGEKTAATVQVSNNSKKSVKSIKCFIVQHTEITMV-NAQ
Consensus   (201) **.***...* ***. ... ...*.*.*.**.**. ..*........*.*

  Am Arr1   (248) YRTTIAHLETQEGCPIQPGSALQKVMYLLPSLQSNKDRRGIALDGQIKRQ
   Hv Arr   (248) YRNIVASIETQDGCPLQPGSSLQKVLHLTPTLAHNRDKRGIALDGQLKRS
  Dm Arr1   (248) FRNTIAFMETSEGCPLNPGSSLQKVMYLVPTLVANCDRAGIAVEGDIKRK
  Dm Arr2   (247) FSKHVAQLETKEGCPITPGANLTKTFYLIPLAANNKDRHGIALDGHLKDE
Consensus   (251)  .   *  ** .***  **. *.*. .* * .  * *. ***..*  *.

  Am Arr1   (298) DTNLASTTLLAN-PEQRDAFGIIVSYAVKVKLYLGALGGELAAELPFILM
   Hv Arr   (298) DTTLASTTLLLD-PDQRDAFGIVVSYSAKVKLYLGAISGELVAELPFILM
  Dm Arr1   (298) DTALASTTLIAS-QDARDAFGIIVSYAVKVKLFLGALGGELCAELPFILM
  Dm Arr2   (297) DVNLASSTMVQEGKSTGDACGIVISYSVRIKLNCGTLGGEMQTDVPFKLL
Consensus   (301) *. ***.*.       .**.** .** ...** .*...**. ...**.*.

  Am Arr1   (347) HPRPE-----R----NKFIHADSQADVEMFRQDTVDPSVDDPNY------
   Hv Arr   (347) HPKEG-----R----VKMIHADSQADVEMFRQDTVHHQESVEVY------
  Dm Arr1   (347) HPKPS-----R----KAQLEAEGSIEA----------------------
  Dm Arr2   (347) QPAPGTIEKKRSNAMKKMKSIEQHRNVKGYYQDDDDNIVFEDFAKMRMNN
Consensus   (351) .* .      *     .   .     .    ..

  Am Arr1   (382) -----
   Hv Arr   (382) -----
  Dm Arr1   (365) -----
  Dm Arr2   (397) VNMAD
Consensus   (401)
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Fig. 1. Amino acid sequence comparison of selected invertebrate arrestin isoforms. Amino acids are designated by single-letter code. *Ascalaphus* arrestin (Am Arr1) deduced from the cDNA sequence is compared to antennal arrestin of Hv (Hv Arr; [27]) and to visual arrestins from Dm (Dm Arr1, Dm Arr2 [10–13]). Asterisks and dots indicate amino acids conserved in all or in three sequences, respectively. The alignment was performed with the program 'Vector NTI' (Informax Inc.). For accession numbers see legend to Fig. 2.

closely related visual arrestin is the Arr1 isoform expressed in the photoreceptors of *Drosophila* (63% identity). The regions of high homology do not include the C-terminus, a domain known to show only weak homology amongst all known arrestins. Interestingly however, the C-tails of Am Arr1 and Hv Arr1 are rather highly homologous (67%) (Fig. 1).

Presently the number of arrestin polypeptides in databases comprises only about 10 entries for identified arrestin genes of invertebrates. The tree shown in Fig. 2 reveals that Arr1-related isoforms, including Am Arr1, constitute a phylogenetic clade which is distinct from other arrestin subfamilies, notably from that of the Arr2 isoforms (Fig. 5). As noted earlier [27], both invertebrate arrestin subgroups harbor an antennal arrestin. This suggests that not only photoreceptors but also antennal sensory cells have evolved mechanisms for GPCR inactivation which require the interaction of GPCRs with two arrestin isoforms.

Since Am Arr1 shows the highest degree of homology to an antennal arrestin and its molecular mass deviates from that of visual arrestins, we investigated whether *Amarr1* is expressed in the *Ascalaphus* eye at all. Northern blot analysis showed that an arrestin transcript of about 1.4 kb, a size correspond-

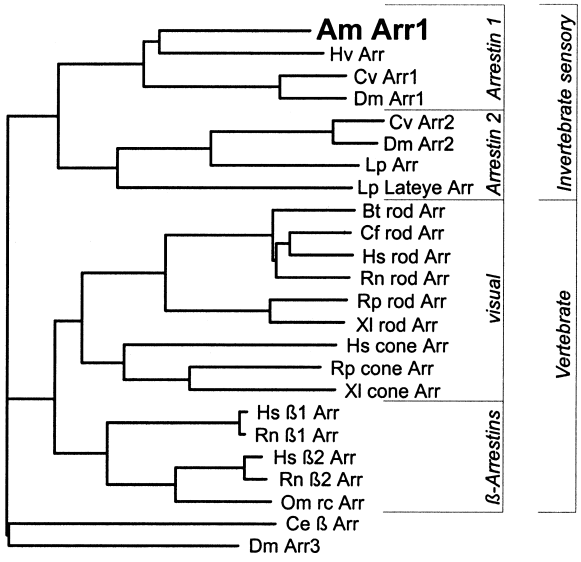

Fig. 2. Evolutionary relationship of representative arrestins. The phylogenetic tree of selected arrestin isoforms was calculated by pairwise alignment of amino acid sequences of Am Arr1, antennal arrestin from *H. virescens* (Hv Arr) [27] and arrestins from the following species: *Homo sapiens* (Hs), *Canis familiaris* (Cf), *Rattus norvegicus* (Rn), *Bos taurus* (Bt), *Rana pipiens* (Rp), *Xenopus laevis* (Xl), *Oncorhynchus mykiss* (Om), *Limulus polyphemus* (Lp), *C. vicina* (Cv) and *D. melanogaster* (Dm). The SwissProt or EMBL accession numbers for the respective arrestins are: Hv Arr, P55274; Cv Arr1, X79072, Dm Arr1, P15372; Cv Arr2, X79073; Dm Arr2, A34856; Lp Arr, U08883; Lp lateye Arr, P51484; Bt rod Arr, A28404; Cf rod Arr, Q28281; Hs rod Arr, P10532; Rn rod Arr, NP 037155; Rp rod Arr, S68173; Xl rod Arr, P51477; Hs cone Arr, P36575; Rp cone Ar, S68172; Xl cone Arr, L40463; Hs β1 Arr, NP004032; Rn β1 Arr, NP037042; Hs β2 Arr, NP004304; Rn β2 Arr, NP037043; Om rc Arr, U48410; Ce β Arr, AAA82342; Dm Arr3, AAF32365. *Ascalaphus* arrestin forms a clade with antennal arrestin from *Heliothis* and Arr1 isoforms expressed in fly photoreceptors.

ing to the size of the sequenced cDNA, is expressed in the dorsal, UV-light-sensitive retina (Fig. 3). This transcript is not present in RNA prepared from thorax tissue. RNA from antennae could not be obtained in amounts sufficient to test for *Amarr1* expression.

3.2. Light-dependent-high-affinity binding of arrestin to photoreceptor membranes

In order to obtain direct evidence for the presence of an arrestin in the UV photoreceptors of *Ascalaphus*, we performed binding assays as previously employed to demonstrate the light-reversible-high-affinity binding of Arr2 to *Calliphora* photoreceptor membranes (Cv Arr2) [15,16]. Fig. 4 shows the changes in absorbance induced by irradiation of a visual-pigment extract from dark-adapted *Ascalaphus* photoreceptor membranes with UV-light which converts UV-P (λ_{max} 345 nm) into M (λ_{max} 480 nm) (P→M; spectrum 1). Conversion of M to P is achieved by irradiation with blue–green light (M→P; Fig. 4, spectrum 2). As has been reported previously [24,25], long-lived M can be fully photoreconverted into P. The same irradiation program (P→M) was applied to induce protein binding to membranes in the isolated retina and, after isolation of membranes and removal of soluble proteins, to release bound proteins from the membrane by photoconversion of M→P. This photoconversion cycle leads, as clearly revealed by SDS–PAGE (Fig. 5a, lane D), to the release of a

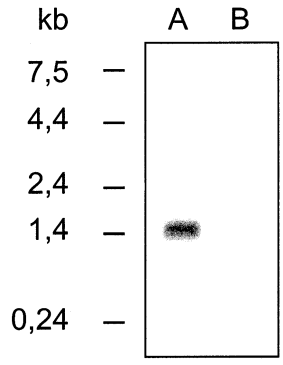

Fig. 3. Northern blot analysis of *Amarr1* gene expression. Total RNA from (A) dorsal eye and (B) thorax tissue (2 μg/lane) of *Ascalaphus* was probed with *Amarr1* antisense cRNA. An 1.5-kb transcript is detected in RNA prepared from the dorsal eye, but not in thorax tissue.

protein of about 45 kDa. Consequently, the *Ascalaphus* dorsal, UV-sensitive retina contains a 45-kDa protein which resembles known visual arrestins in as it binds to the photoreceptor membrane upon conversion of UV-P into M. The 45-kDa protein is released again from the membrane after photoregeneration of M to UV-P.

3.3. Western blot analysis of arrestin

Further information on the putative arrestin nature of the 45-kDa protein was obtained by immunoblotting. On Western blots of extracts containing the 45-kDa protein, antibodies directed against Arr1 and Arr2 of *Calliphora* photoreceptors cross-react with a protein (Fig. 5b, lanes C and D) co-migrating closely with Cv Arr2 (lane B). A protein corresponding in its mass to Cv Arr1 (lane A) was not detected. Since the data obtained for the primary structure of *Ascalaphus* arrestin suggested that Am Arr1 should be smaller by about 2.2 kDa than Cv Arr2 we translated the *Amarr1* cDNA in vitro. The electrophoretic mobility of the ^{35}S-labelled Am Arr1 protein corresponds to that of the protein cross-reacting with anti-Cv Arr1 and anti-Cv Arr2 (compare Fig. 5b lane E with lanes B and C). As the Western blots alone did not allow us to unequivocally decide whether or not the protein released by M→P conversion represented the arrestin isoform sequenced in the present study we excised the band from the gel and

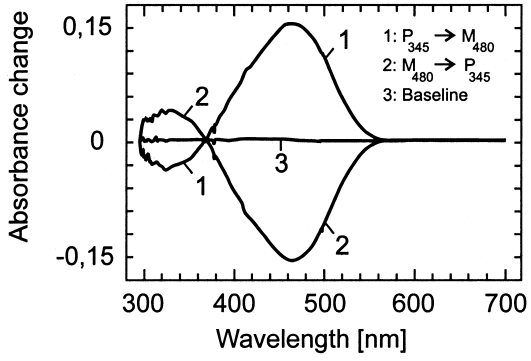

Fig. 4. Spectral properties of *Ascalaphus* UV-P. Difference spectra were recorded from digitonin extracts prepared from four dorsal retinas. Spectrum 1 was recorded after converting P→M by irradiation with UV-light (350 nm), spectrum 2 was recorded after M→P conversion by blue light (482 nm). Curve 3, baseline.

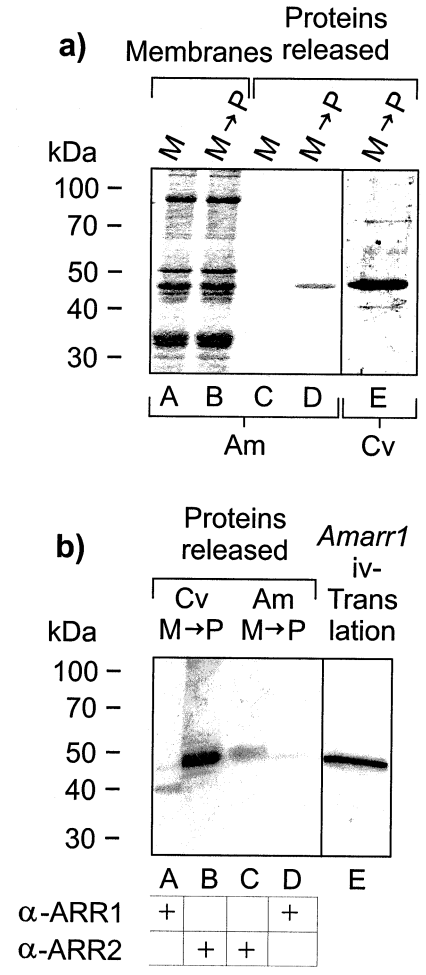

Fig. 5. (a) Light-induced-reversible-high-affinity binding of *Ascalaphus* arrestin to photoreceptor membranes. Visual pigment of Am photoreceptor membranes (lanes A–D) and Cv rhabdomeric membranes (lane E) was photoconverted into M-state as described under Section 2. SDS extracts of washed membranes (lanes A, B) or PBS-supernatants which contained released proteins (lanes C, D) were analyzed by SDS–PAGE. (A) *Ascalaphus* M-state membranes, (B) *Ascalaphus* membranes after conversion of M→P. (C) The absence of proteins in the PBS-extract from M-state membranes (lane A) indicates, that no protein is extracted from membranes remaining in the M-state. (D) PBS-extract from membranes after conversion of M→P (lane B), demonstrating the release of a 45-kDa protein. (E) PBS-extract from *Calliphora* rhabdomeric membranes obtained after M→P conversion, demonstrating the release of Arr1 (41 kDa) and Arr2 (45 kDa). (b) Antibody cross-reactivity of *Ascalaphus* arrestin. Western blot analysis of arrestin-containing samples obtained as in a, lanes D and E. Bound antibodies were visualized using ^{125}I-labelled secondary antibodies and subsequent autoradiography. (A, B) Controls showing cross-reactivity of anti-Cv Arr1 (A) and of anti-Cv Arr2 (B) with arrestins released from *Calliphora* membranes after M→P conversion. (C, D) Cross-reactivity of proteins released from *Ascalaphus* dorsal eye membranes after M→P conversion with anti-Cv Arr2 (C) and anti-Cv Arr1 (D). (E) in-vitro-translated *Ascalaphus* arrestin demonstrating co-migration of Am Arr1 with arrestin released by M→P conversion (lanes C, D). in-vitro-translated Am Arr1 was labelled with ^{35}S-methionine and visualized by autoradiography.

subjected it to Edman degradation. Gas chromatography indicated a N-terminal sequence of V-A-N-F-K-V-F-K-K-S-S-P-N-G-K for the major protein present in the excised band. This amino acid sequence corresponds to positions 2–16 of the N-terminus of Am Arr1 (see Fig. 1). Microsequencing furthermore revealed that the N-terminal methionine is not present in the mature arrestin of *Ascalaphus*, as has been shown previously to be the case for Cv Arr2 [16]. Thus, the 45-kDa protein isolated from the UV-sensitive *Ascalaphus* dorsal eye is the product of the *Amarr1* gene.

Am Arr1 shares the property of high-affinity binding to the receptor, which was previously reported only for Arr2 isoforms. In *Drosophila*, the release of Arr2 from the photoreceptor membrane occurs only if arrestin is phosphorylated by a calcium/calmodulin-dependent protein kinase [18]. A phosphorylation site at Serine-360 close to the major phosphorylation sites identified in Dm Arr2 (ser366) by site-directed mutagenesis is conserved in Arr1 of *Ascalaphus* and *Heliothis*. The arrestin isoform isolated in the present may, therefore, employ similar mechanisms for binding to and release from different states of the visual pigment via arrestin phosphorylation as shown for Dm Arr2. In *Drosophila* it has been shown that binding of arrestin is important for stabilizing the M conformation in vitro, although this interaction may not be essential for the formation of a long-lived M form in vivo. Similarly, Am Arr1 binding might also be a factor contributing to the high stability of the M formed from UV-P of *Ascalaphus*.

In summary, we have cloned a novel member of the Arr1 subfamily of invertebrate visual arrestins. Am Arr1 is expressed in the dorsal, UV-sensitive retina of the owlfly Am. This arrestin shows homology to Arr1 isoforms of antennal arrestins as well as the visual Arr1 isoforms of the fly compound eyes. Most importantly, we show that Arr1 isoforms undergo a light-dependent binding cycle to photoreceptor membranes, which was to date only reported for Arr2. This suggests that both Arr1 and Arr2 isoforms function in a desensitization mechanism that is common to GPCRs of visual and antennal insect sensory cells. Evidence for the existence of a second arrestin in *Ascalaphus* photoreceptors, which might correspond to invertebrate photoreceptor Arr2, could not be obtained in any of our binding experiments. This implies that a strict correlation of distinct arrestin isoforms to distinct functions is not a general principle realized in invertebrate photoreceptors.

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