LETTER TO THE EDITORS

Splice Variants of Arrestins

A large number of topologically related G protein-coupled receptors mediate transmembrane signaling by hormones, neurotransmitters, light, odors, and other factors. The signal from the activated receptor is transmitted via trimeric coupling proteins, G proteins, to cellular targets that include membrane ion channels, phosphodiesterases, phospholipases, and adenylylate cyclases (Birnbaumer, 1990; Gilman, 1995). Mutations in genes that encode proteins involved in this type of signal transduction are implicated in many human diseases, including Albright’s hereditary osteodystrophia, thyroid carcinomas, ovarian adenomas, and some forms of retinitis pigmentosa (Schabel and Bohm, 1995). In one of the many mechanisms of desensitization, the receptors are inactivated by phosphorylation in a reaction catalysed by G protein-coupled receptor kinases and recruitment of capping regulatory proteins, the arrestins. Hundreds of G protein-coupled receptors are known; in contrast, only a handful of G protein-receptor kinases (GRKs) (six members; Premont, Inglese and Lefkowitz, 1995) and arrestins have been identified (four genes coding for arrestin cone arrestin, β-arrestin 1, and β-arrestin 2; Palczewski, 1994). Thus, kinases and arrestins must possess broad specificity to account for the stereotypical inactivation of these receptors. Alternatively, a group of homologous but distinct proteins can be generated from a single gene by a mechanism that produces several alternative splice variants of mRNA (e.g. the plasma membrane calcium pump (Stauffer et al., 1994), or proteins could be heterogeneously modified by posttranslational mechanisms (e.g. heterogenous acylation, phosphorylation, or proteolysis) (Johnson et al., 1994; Graves et al., 1994).

Recently, two novel splice variant forms of bovine (p44) and human retinal arrestins have been identified (Smith et al., 1994; Smith, 1996). In this report, extracts of ROS membranes from mice were tested for the presence of different forms of arrestin. When proteins were separated by electrophoresis, electrotransferred to Immobilon, and probed with an anti-arrestin antibody, significant amounts of two lower molecular weight forms of arrestin, M1-Arr and M2-Arr were repeatedly found, in addition to full-length arrestin (Fig. 1). When probed with a panel of anti-arrestin monoclonal antibodies specific to different regions of arrestin, these immunoblots revealed that M1-Arr and M2-Arr are truncated at the c-terminus (data not shown). These data suggest that mouse photoreceptor cells may contain other forms of arrestin derived either from alternative mRNA splicing (Palczewski, 1994) or as a product of proteolysis (Azarian et al., 1995), in addition to full-length arrestin.

To determine whether these forms could be derived from alternatively spliced mRNA, retinal cDNA was screened by PCR using sense primers specific to exons 7, 11, 12, and 14 paired with anti-sense primers specific to exon 16 and the poly(A) tail. Amplification products that showed a size different from that expected for the normal mouse arrestin cDNA were targeted. In the reaction which contained primers against exon 12 and the poly(A) tail, an amplification product that was approximately 50 bp shorter than the expected 400 bp product was observed. This product was cloned and sequenced (Fig. 2). The sequenced cDNA was identical to mouse arrestin up to the splice site between exons 12 and 13 at which point

![Fig. 1. Molecular forms of arrestin in mouse rod outer segment (ROS) membranes visualized by immunoblotting.](image)

Lane a was purified bovine p44; lane b was purified bovine arrestin; and lane c was mouse ROS membranes; all lanes were reacted with C10C10 antibodies. In lane c, the upper immuno-reactive component was native mouse arrestin (it migrated in SDS-PAGE slower than bovine arrestin); the lowest immuno-reactive components (M1-Arr and M2-Arr) were present consistently in all preparations, and their extraction properties and immunoreactivity with different antibodies were similar to bovine p44. The two or three forms of arrestin between M1/M2-Arr and arrestin were likely to be degradation products as they are absent in some preparations, or could be β-arrestins/cone arrestins (Smith et al., 1994).
mArrΔ13-16

...GGGATCTCTTGTGCATACATCATCAAGGTAAGTCTACGCTTGCTCGGTTAAGTGTG 1023
...G I L V S Y H I K V K L T V S G - 338
AGTGTGCCATTCTTCTCTCTCTAGTGACGTCGATGAGGTGAGCAAGTAAAGCCGAGGT 1083
CTACTGATCTCCTGAAGTGGTATAGCCATGGTTCCCTGGTGTGAGCTCTGCAGTTTTGGT 1143
TGGAGGCAATGTATACTGGGAGAAGCAAAAACAAAGTTGTCACCATGAGAACGCTGTAC 1203
GCAAAGTGTTGTCACATCGACACACAGTTGGAGGCTGACAGAGACGGGATTGCATACCGAC 1263
CCCCTTTGTAAGATCAAATTGCTTGTGGGT-poly(A) 1296

mArr

...GGGATCTCTTGTGCATACATCATCAAGGTAAGTCTACGCTTGCTCGGTTTCTAGGA 1023
...G I L V S Y H I K V K L T V S G F L 341

GAGCTCACTCCATCGAAGTGGCTACCAGGAGGTGGCCTGGCTCTCATGACCCCGAGCCT 1083
ELTSSSEVATVEVFPFRLMHPQ 361

GAGGATCCAGAAAGATAAGTGTTCAAGATGAAATTTGTGGTTTGGAGAGTTTGCTCGC 1143
EDPAKESVQDENSENFLVFEAR 381
CAAAATCCTGGAAGATCTGGAGAAGAAACAGAGAAGAAGATGAGATGCTGGCACG 1203
QNLKDTGENTEGKKEDEAGQ 401
GATGAGTGAAGACTTAGCTAGCTAGCTAGAAACTGCTTTGTAGTTCGGGCTCTTGAGG 1263
DE 403
GAAGCCGCGGATAGACTCTCACAAGTTATGCTAGATATCAGATACCGATGACATTCTCCTCCAG 1323
AAATAAAGTCTCCTGGCTCCTCTT-poly(A) 1346

Fig. 2. 3' portion of the splice variant of mouse arrestin cDNA (above) and full-length mouse arrestin cDNA (below). Nucleotide numbering begins with the deoxyadenosine in the initiating 'ATG'; amino acid numbering begins with the initiating methionine. Arrows and numbers above the sequence indicate the 3' boundaries of each known exon (Tsuda et al., 1991). An alternative exon not used in mouse arrestin is used in mARRΔ13–16 to complete the 3'-untranslated region of this variant. Poly(A)+ RNA was isolated from mice retinas (Balb-c) (QuickPrep Micro mRNA Purification kit, Pharmacia). cDNA was synthesized from 250 ng of the poly(A)+ RNA, priming synthesis with oligo(dT) (First-stand cDNA Synthesis kit, Pharmacia). This cDNA was used for PCR amplification, using sense primers specific to exon 7, exon 11, exon 12, and exon 14 paired with anti-sense primers specific to exon 16, and the poly(A) tail. A typical reaction contained 50 mM KCl, 10 mM Tris/HCl buffer, pH 8.3, 0.2% Triton X-100, 1.5 mM MgCl2, 0.2 mM dNTPs, 15–35 pmol oligonucleotide primer, and 2 U Tbr polymerase (PrimeZyme, Biometra). Reactions were cycled 35 times through 94°C for 45 sec, 50°C for 45 sec, and 72°C for 2.5 min. Amplified products were cloned into pT7-Blue (Novagen), and sequenced by the dideoxy chain termination method (Sanger et al., 1977) in the presence of 32P-dATP.

the cDNAs diverged. For this cDNA, the 3'-splice site acceptor nucleotide was followed by deoxyguanosine, which completed the Gly codon followed by a 'TAA' stop codon. In total, six clones were sequenced from independent PCR amplifications that contained this alternative sequence. This variant of arrestin, most likely M2-Arr (Fig. 1), thus represents a 65 amino acid truncation compared to the normal arrestin polypeptide. Atypical mobility during SDS-PAGE generates significant uncertainty in terms of the actual size of arrestin.

The arrestin family of regulatory proteins involved in the regulation of the activity of G protein-coupled receptors are encoded by three genes enlarged by alternative splicing of the mRNAs (Fig. 3). This observation supports the hypothesis that the C-terminal fragment of arrestins contains a regulatory element (Palczewski, 1994). More must be learned about the differences between function, specificity, and localization of these arrestins, as exemplified in studies related to bovine p44 (Smith et al., 1994). However, in this letter we would like to emphasize that: (i) a significant portion of arrestin heterogeneity (Craft, Whitmore, and Donoso, 1990) could result from alternative mRNA splicing, thus expanding the combination of known arrestin molecules; (ii) in studies on inactivation of the arrestin gene ('gene knockout'), one has to consider that not just one protein but several molecular forms of arrestin may be removed; and (iii) mutations in the arrestin gene that result in nonfunctional or defective arrestin, as found in Oguchi disease (Fuchs et al., 1995), could obliterate several forms of arrestin, thus potentially producing many separate physiological defects.

Preparation of Mouse ROS Membranes

To ensure binding of arrestins to phosphorylated photolyzed rhodopsin ROS membranes were purified under room light as follows: 500 mouse eyes (Balb-c)
were dissected, homogenized using a 20 ml glass-glass homogenizer with 45% sucrose solution in 75 mM NaP<sub>i</sub> buffer, pH 7-5 (ten eyes ml<sup>-1</sup>) to a fine suspension, and vortexed for 20 min. The suspension was filtered through three layers of cotton gauze, the supernatant was collected and centrifuged for 6 min at 3900 g. The supernatant was transferred to a new tube and centrifuged for 6 min at 3900 g again, while the pellet containing large amounts of pigmented granules was discarded. The supernatant was diluted (1:1) with 75 mM NaP<sub>i</sub> buffer, pH 7-5, and the membranes were collected by centrifugation at 15000 g for 10 min. The pellet contained ROS membranes and was still heavily contaminated by pigmented granules. Therefore, the pellet was resuspended in 4 ml of 75 mM NaP<sub>i</sub> buffer, pH 7-5, and loaded onto two-layers of discontinuous sucrose gradient (45% and of density 1:1). The gradient was centrifuged using a swinging bucket rotor for 20 min at 26000 g. ROS membranes, still heavily contaminated by pigmented granules, were collected on the interphase between 45% and 1:1 sucrose solutions, diluted twice with the phosphate buffer and centrifuged for 10 min at 15000 g. The pellet was resuspended in 4 ml of the phosphate buffer and loaded on a two-layer gradient made out of sucrose solutions of 1:13 and 1:11 densities. The gradient was centrifuged using swinging bucket rotor for 20 min at 26000 g. ROS (~ 2–4 mg of rhodopsin)
mostly free of pigmented granules, were collected on the interphase between 1-13 and 1-11 sucrose solutions, diluted twice with 75 mM NaPi, buffer, pH 7.5, and centrifuged for 10 min at 15,000 g. SDS extracts were prepared from these ROS, and proteins were separated by SDS-PAGE. Following electrotransfer to Immobilon membrane, the blot was probed with anti-arrestin antibody C10C10, a gift from Larry Donoso (Jefferson University).

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References


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