Volume 302, number 2, 172–176 FEBS 11014 May 1992

Cloning and sequencing of the 23 kDa mouse photoreceptor cell-specific protein

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Received 26 February 1992; revised version received 17 March 1992

The 23 kDa protein was localized by immunocytochemistry to photoreceptor cells of the mouse retina, and bovine and mouse cDNA clones were isolated and sequenced. The deduced amino acid sequences showed that the mouse 23 kDa protein is 91% identical to the bovine protein, and is the same as S-modulin, the CAR (cancer-associated retinopathy) protein and recoverin, the Ca^{2+}-dependent activator of photoreceptor guanylate cyclase. The amino acid sequence reveals two Ca^{2+} binding sites, no internal repeats, 59% homology to the chicken visinin protein and 40% homology to calmodulin while Northern analysis demonstrated a single 1.0 kb mRNA species in bovine and mouse retina.

Immunocytochemistry; Retina; cDNA library; Bovine; mRNA; S-Modulin; Cancer-associated retinopathy (CAR); Recoverin; Visinin; Calmodulin

1. INTRODUCTION

Photoreceptor cells have the unique function of detecting light, converting it to a signal which is relayed, through synapses with other retinal neurons, eventually to the brain where the result is vision. We previously postulated that the enzymes necessary to catalyze these activities would, by necessity, be structurally unique and could possibly be recognized by comparing the immunologically identified proteins which were present in the retinas of normal adult mice with those in the retinas of mice from which the photoreceptor cells had been removed by genetic dissection [1]. This strategy has proven very successful and has resulted in our cloning the cDNAs for mouse rhodopsin [2], arrestin [3], phosducin/33 kDa [4], and now the 23 kDa protein (hereafter referred to as 23kD).

Using polyclonal, polyspecific antisera against soluble proteins from normal adult mouse retina, in combination with Western transfer analysis, we recently purified 23kD from bovine retina and used it to generate monospecific antisera (unpublished data). The application of these monospecific antibodies has resulted in the immunocytochemical identification of 23kD as a photoreceptor-specific protein, and the isolation of 23kD clones from mouse and bovine retina cDNA expression libraries. The protein, 23kD, is shown to have 59% homology to chicken visinin [5] and about 40% homology with calmodulin, a Ca^{2+}-binding regulator of many enzymes [6]. 23kD is now shown to be identical to the same protein which has been designated the CAR (cancer associated retinopathy) protein [7], recoverin [8] and S-modulin [9].

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2. MATERIALS AND METHODS

2.1. *Immunocytochemistry*

Monospecific anti-bovine 23kD sera were produced in rabbits using purified bovine 23kD and procedures described previously [1]. Immunocytochemical localization was performed on 5 μm thick sections as described previously [10] using a 1/1,000 dilution of antisera.

2.2. *RNA*

RNA was isolated by the method of Chomczynski and Sacchi [11] and Northern analysis was performed by the method of Thomas [13] as described previously [12]. The full-length 1,063 bp mouse cDNA, clone m23A, was labelled with ^{32}P by the random primer method [14] and used to probe Northern transfers.

2.3. *Cloning and sequencing*

Mouse retinal poly(A)$^+$ RNA was extracted and used to construct a cDNA expression library in λgt11 and immunologically screened as described previously [15]. The initial screening resulted in the isolation of a 1,063 bp clone (m23k) containing the entire coding sequence for 23kD. The bovine retinal cDNA library was purchased from Stratagene and was also screened with the monospecific anti-23kD serum at a dilution of 1/8,000. The longest bovine clone, 990 bp, was sequenced (data not presented) and the deduced amino acid sequence compared to that deduced from the mouse cDNA sequence. Amplified DNA was directly sequenced using sequence-specific primers and the double-stranded sequencing technique as described previously [16]. Nucleotic and amino acid sequences were analyzed using PCGENE and Dnasis & Prosis software programs.

3. RESULTS

Purified bovine 23kD was used to generate monospecific antibodies which in turn were used to determine the immunocytochemical localization of 23kD at the

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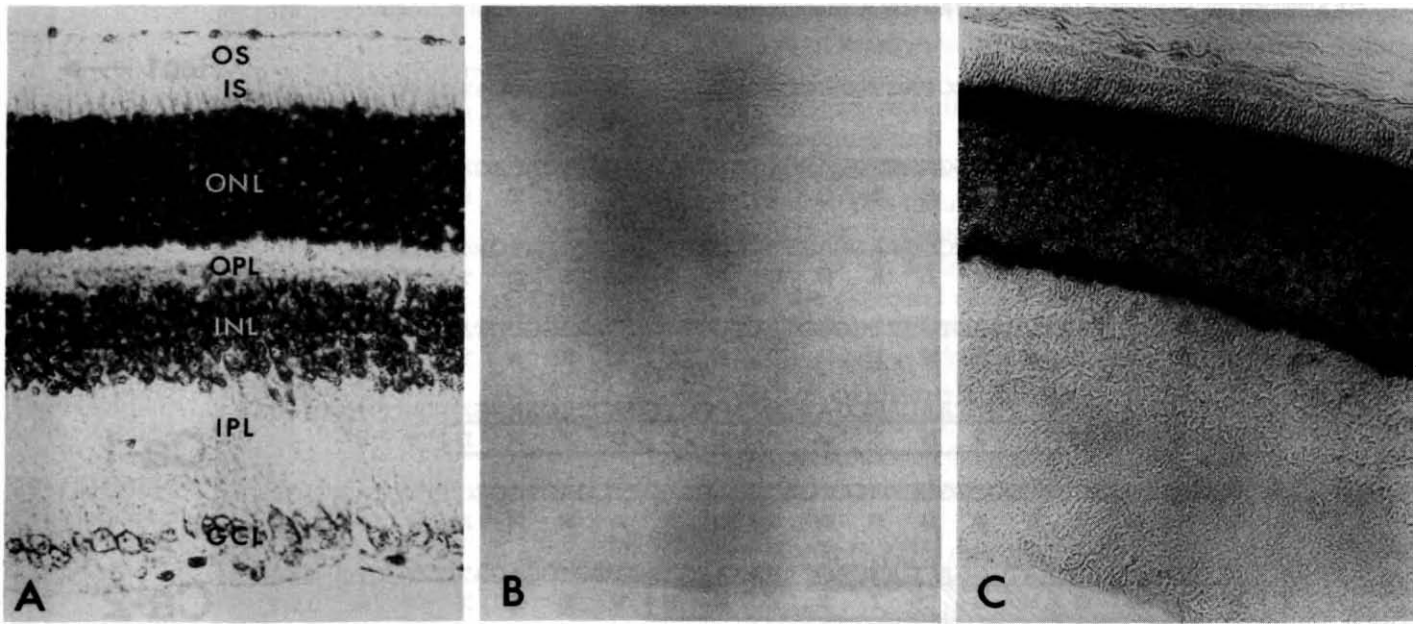

Fig. 1. Immunocytochemical localization of 23kD in mouse retina. The normal cellular architecture of the retina is visible in the Cresyl violet-stained section (A). The retina consists of the photoreceptor cells arranged in ordered columns with their outer segments (OS), inner segments (IS), cell nuclei (outer nuclear layer, ONL), synaptic connections (outer plexiform layer, OPL) to the inner nuclear layer (INL), and the synaptic connections (inner plexiform layer, IPL) between the cells of the INL and the cells of the ganglion cell layer (GCL). Pre-immune serum at 1/1,000 dilution (B) did not stain any cells whereas anti-23kD serum at the same dilution (C) stained only the photoreceptor cells, with the most intense labelling seen in the IS and the OPL.

light microscopic level. The orderly tiered arrangement of neurons with synaptic regions connecting adjacent layers is revealed in a Cresyl violet-stained section of normal mouse retina (Fig. 1A). None of these cells nor synaptic regions stain positive when parallel sections are incubated with pre-immune rabbit serum (Fig. 1B). However, the application of monospecific anti-23kD serum to sections of mouse retina (Fig. 1C) shows positive staining only in the photoreceptor cell layer. The oxidized diamino-benzidine is deposited primarily in the inner segments, the inner nuclear layer and the outer synaptic layer with essentially none detected in the outer segments of the rods. The rodless retinas of adult *rd* mice did not stain with anti-23kD serum.

The monospecific antibodies were also used to isolate bovine and mouse retinal cDNA clones encoding 23kD. Immune screening of the mouse retina cDNA library yielded a number of positive plaques and the size of their inserts was determined using the polymerase chain reaction with λ forward and reverse sequencing primers. The clone with the longest insert, 1,063 bp, was selected and the amplified insert directly sequenced. The insert (Fig. 2) contained a 606 bp open reading frame encoding 202 amino acids, the initiator methionine and a 99 nucleotide 5'- and a 339 nucleotide 3'-non-coding sequence. The AATAAA polyadenylation signal is located at position 1,026, 19 nucleotides from the beginning of the poly A tract. The deduced amino acid sequence of bovine 23kD is 91% identical to mouse 23kD and the amino acid differences are shown in Fig. 2.

From the deduced amino acid sequence of mouse 23kD its calculated molecular mass is 23,406. It has a theoretical pI of 4.83, three potential protein kinase C phosphorylation sites (at amino acid residues 41, 60 and 98), five potential casein kinase II phosphorylation sites (at residues 24, 45, 107, 118, and 168) and one potential tyrosine kinase phosphorylation site (at position 53). There are two EF-hand calcium-binding domains at positions 74–86 and 110–122 and a potential glycosylation site at residue 114. The deduced amino acid sequence for the mouse protein is 91% identical to the bovine 23kD protein.

Northern analysis of 23kD mRNA (Fig. 3) shows a single species of 23kD mRNA in normal mouse and bovine retina of about 1.0 kb, which is not detected in the rodless retina of the adult *rd* mouse.

4. DISCUSSION

The successful localization, cloning, sequencing and identification of 23kD has validated our original strategy [1,15] to recognize photoreceptor-specific proteins on the basis of their immunological detection in the normal mouse retina and absence in the rodless retina of the adult *rd* mouse. The monospecific antisera made against purified bovine 23kD unequivocally demonstrate (Fig. 1) that 23kD is localized exclusively within the photoreceptor cell layer of the retina. Although the inner layer, the outer nuclear layer and the synaptic region of the photoreceptor cells stain intensely, there

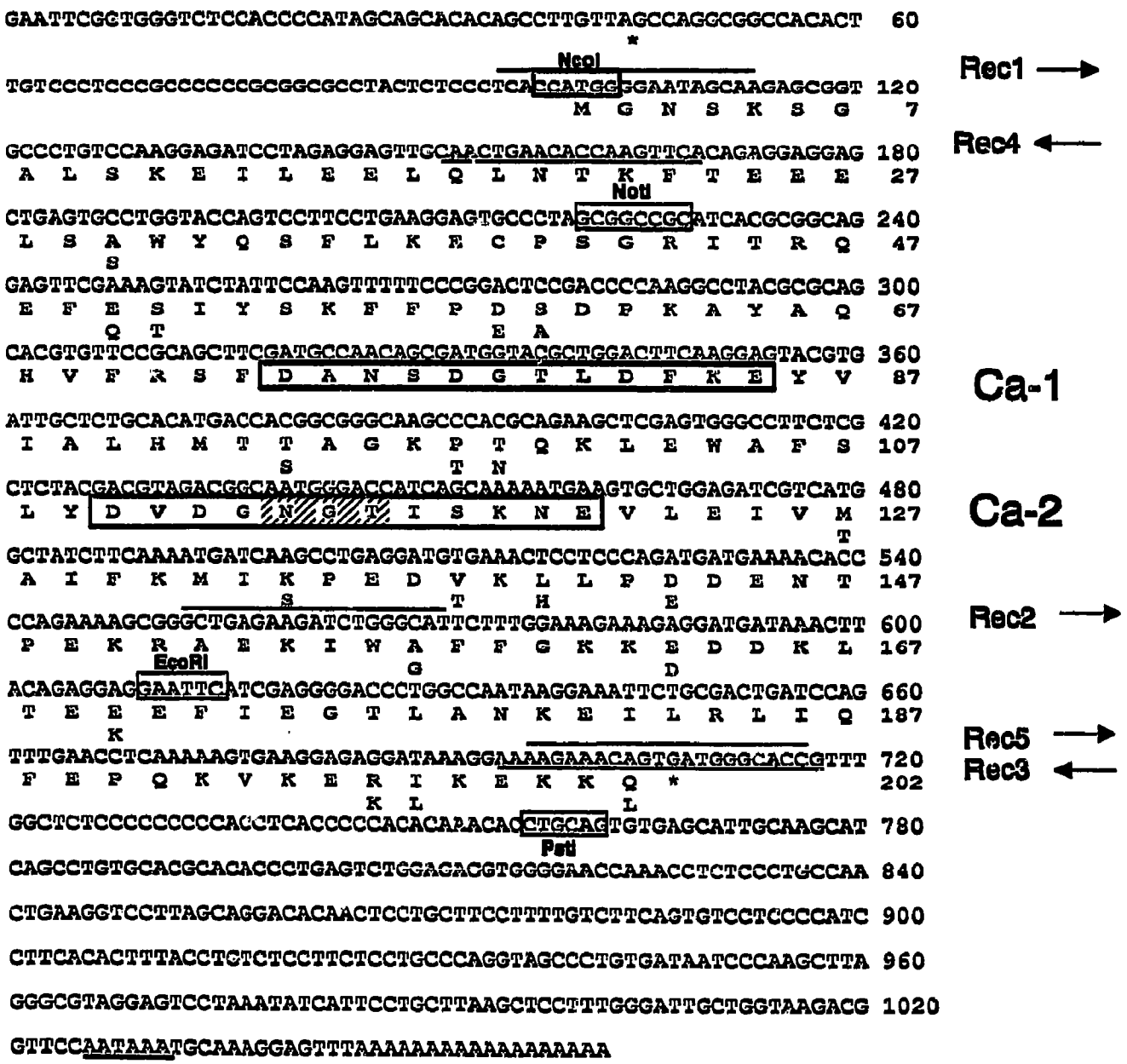

Fig. 2. Nucleotide sequence analysis of mouse and bovine 23kD cDNAs. The sequence shown was obtained by direct sequencing of PCR amplified mouse 1,063 nucleotide DNA insert. The entire deduced amino acid sequence for mouse 23kD is presented whereas only those bovine amino acids which differ from mouse 23kD are shown. Asterisks below the nucleotide sequence depict in-frame stop codons. Rec 1–5 are sequence-specific sense and anti-sense primers. Relevant restriction sites (*Nco*I, *Not*I, *Eco*RI, *Pst*I) are boxed. The predicted EF-hand Ca^{2+}-binding sites are denoted by Ca-1 and Ca-2. A putative glycosylation site (within the Ca-2 domain) is hatched. The polyadenylation signal, AATAAA, in the 3' untranslated region is underlined.

is no stain detected in the outer segments. This is surprising since phototransduction and its cascade of chemical reactions is believed to occur in the outer segments. The absence of detectable stain may be due to the extraction of 23kD from the outer segments during the process of embedding and staining, or to the masking of its antigenic sites, although the outer segments stain heavily for S-antigen. Unlike S-antigen, which moves in a light-dependent manner between the inner and outer segments [10], 23kD remains in the inner segments regardless of the lighting environment [17]. Cones also appear to have 23kD but because of their small number in the mouse retina it is difficult to prove by light microscopy. Evidence in support of the presence of 23kD in the cones is currently being sought by electron and confocal microscopy.

The nucleotide sequence data (Fig. 2) and the single band of 23kD mRNA in both mouse and bovine retina (Fig. 3) is consistent with a single protein species of 23kD in mouse and bovine retinas [1]. The slight size

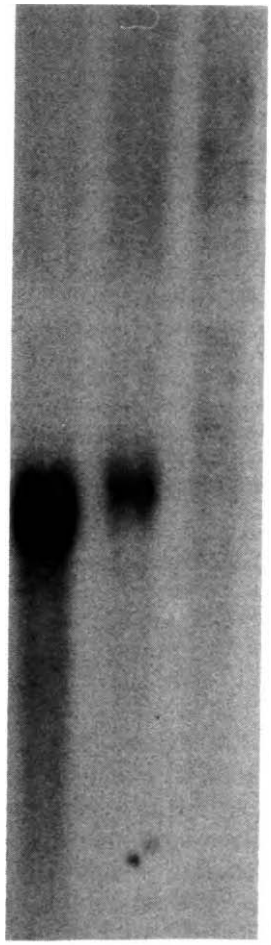

Fig. 3. Northern analysis of retinal 23kD mRNA. Samples of total RNA (20 μg) are from (1) normal Balb/cJ mice, (2) bovine, and (3) photoreceptorless (*rd*) C3H/HeJ mice. The size of the mouse 23kD mRNA is 1.0 kb as determined by comparison with RNA standards.

difference between mouse and bovine mRNA might reflect longer 3' or 5' untranslated regions, a longer poly(A)$^+$ tail on the bovine mRNA or nucleotide sequence-specific mobility effects. The cloning of 23kD enables our biomolecular data [1,15] to be correlated with that which has been obtained with respect to its functional participation in phototransduction and its clinical importance in CAR.

Functionally, in the dark-adapted animal, the cation channels in the photoreceptors are maintained in an open state by the presence of bound cyclic GMP [18]. Exposure to light results in the phosphodiesterase-mediated hydrolysis of cyclic GMP, closure of the channels [19] and a decrease in Ca^{2+}. This is followed by the cooperative activation of guanylate cyclase [20] by recoverin [8] to increase the amount of cyclic GMP, and the re-opening of the channels. The interaction of recoverin and guanylate cyclase is inhibited by the binding of Ca^{2+} to recoverin. Therefore as Ca^{2+} enters the cell through the re-opened channels, recoverin is titrated free of guanylate cyclase and the dark state is restored [8].

It was recently shown [21] that recoverin can be phosphorylated by an endogenous kinase which does not appear to be either protein kinase C or rhodopsin kinase. This data seems to eliminate phosphorylation of 23kD at the potential protein kinase sites on amino acid residues 41, 60 and 98, and suggests that one or more of the potential casein kinase II phosphorylation sites at residues 25, 45, 107, 118, 168 and/or the tyrosine kinase site at 53 may be involved. The existence of a potential glycosylation site at residue 113, in the middle of the second EF-hand sequence, suggests that the ability of 23kD to bind Ca^{2+} and therefore its ability to activate guanylate cyclase might be regulated by glycosylation.

CAR [7,22] is one of a family of degenerative diseases in which the presence of cancer is associated with the death of a population of neurons at a site in the central nervous system remote from the cancer itself. In CAR, the photoreceptor cells degenerate with no detectable loss of any of the other retinal neurons [23,24]. Serum from these patients has been shown to contain antibodies which cross-react with a number of retinal antigens, including one of 23 kDa [25,26]. Polans et al. [27] have recently purified a calcium-binding protein from bovine retina which reacts with CAR patient serum and has an amino acid sequence identical to recoverin and 23kD. No causal relationship between the cancer, the antibodies and the degeneration of the photoreceptor cells has been established.

We are currently studying the structure, position and expression of the 23kD gene in anticipation that such knowledge will be important for the complete determination of the biochemical participation of 23kD in the process of phototransduction and for the resolution of the molecular basis of its involvement in paraneoplastic phenomena in humans.

Acknowledgements: We thank Brian Austin for his excellent technical contributions. This research was supported in part by funds to J.F.M. from the National Eye Institute (EY06085, EY06639) and from Retinitis Pigmentosa International and by funds to WB from the National Eye Institute (EY08123), the National Retinitis Pigmentosa Foundation/Gund Foundation, and the Retina Research Foundation (Houston). W.B. is a Jules and Doris Stein Research to Prevent Blindness Professor.

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