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Analysis of the human, bovine and rat 33-kDa proteins and cDNA in retina and pineal gland

(Monoclonal antibody; recombinant DNA; mRNA; amino acid sequence; rods; thioredoxin; transducin; phospholipase)

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SUMMARY

A monoclonal antibody (mAb) was produced against a bovine retinal 33-kDa protein. Several clones of 33-kDa protein were isolated from each library of cDNA from human, bovine and rat retinas and rat pineal gland by mAb screening and by hybridization with cDNA probes. Each of the four cDNA sequences was determined and amino acid (aa) sequences were deduced from the nucleotide sequences. The latter were nearly identical in rat retina and rat pineal gland (99.6%) and were similar in human, bovine and rat retina (more than 87%). Each of these cDNAs had one long ORF and encoded 245 or 246 aa. The deduced aa sequences in rat retina and rat pineal gland were virtually identical and the sequences in human, bovine and rat retina were highly homologous (more than 88%). The predicted M_r for each of these proteins was 28 246 in the human, 28 176 in bovine, 28 143 in rat retina, and 28 129 in rat pineal gland. Each of the sequences has a putative site for phosphorylation by A kinase; we have confirmed that the putative site is Ser^{73}. These results show that the 33-kDa proteins in the retina and pineal gland have the same sequences and the same phosphorylation site and suggest that the functional role of this protein is the same in the retina and pineal gland.

INTRODUCTION

Phosphorylation is an important mechanism in the regulation of cellular function. There is evidence that in the rod photoreceptor cells of the vertebrate retina, phosphorylation of proteins may participate in the regulation of visual process (see Kuhn, 1984).

The rod photoreceptor cells of the mammalian retina have a major water-soluble phosphoprotein called 33-kDa protein that may participate in the regulation of visual phototransduction or in the integration of photoreceptor metabolism (Lee et al., 1982; 1987). The phosphorylation of 33-kDa protein is catalyzed by A-kinase (Lee et al., 1984). The level of phosphorylated 33-kDa protein is highest in the rod outer segments of dark-adapted retina, and it is dephosphorylated during illumination (Lee et al., 1984). More recently it was reported that the 33-kDa protein binds to the $\beta\gamma$ subunits of transducin complex ($T\beta\gamma$) to form a 33-kDa protein-$T\beta\gamma$ complex (Lee et al., 1987; 1988). Whereas the $T\alpha$ activates phosphodiesterase, the $T\beta\gamma$ stimulates phospholipase A_2 (lipase A_2) activity in rod outer segments (Jelsema and Axelrod, 1987).

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Abbreviations: aa, amino acid(s); A-kinase, cAMP-dependent protein kinase; bp, base pair(s); cAMP, cyclic adenosine 3′,5′-monophosphate; cGMP, cyclic guanosine 3′,5′-monophosphate; $G\beta\gamma$, $\beta\gamma$ subunits of G-binding protein; kb, kilobase(s) or 1000 bp; Kemptide, substrate for A-kinase; mAb, monoclonal antibody; NBRF, National Biomedical Research Foundation; nt, nucleotide(s); ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; S-Ag pept, peptide of retinal S-antigen protein; SDS, sodium dodecyl sulfate; $T\alpha$, α subunit of transducin; $T\beta\gamma$, $\beta\gamma$ subunits of transducin.

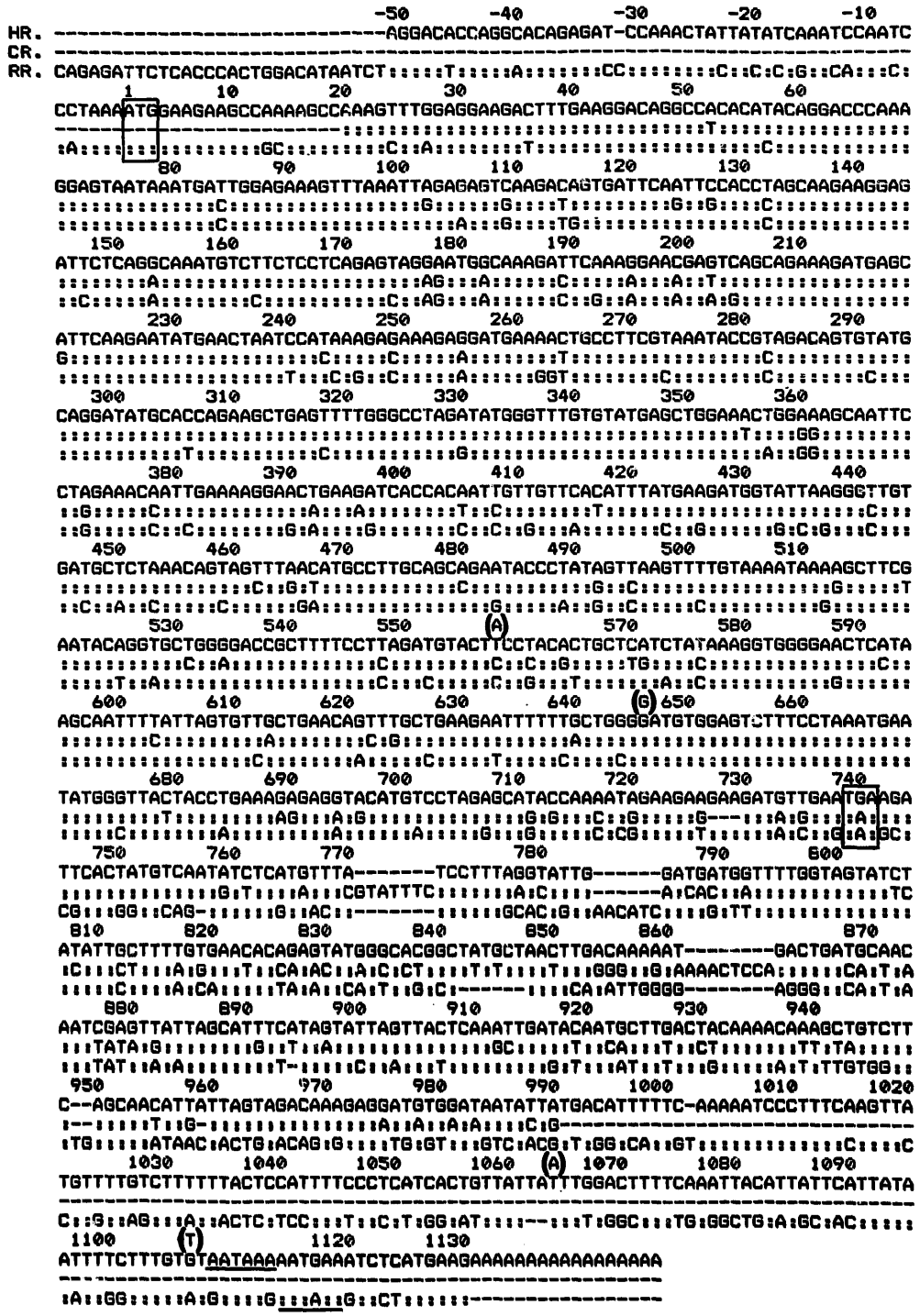

Fig. 1. Nucleotide sequences of the 33-kDa protein cDNA from human retina (HR., HRC-7), bovine retina (CR., CRC-4), rat retina (RR., RRC-11) and rat pineal gland (RP., RPC-6) were aligned for maximum homology. The sequence for RPC-6 is not shown, however, the 4 nt which differ from that of the rat retinal sequence are indicated in parentheses under the rat retinal sequences. Colons indicate the identical nt with the human sequence. Numbers above the nt sequences indicate the nt position starting with the first codon (ATG) of the human sequence. Boxed ATG and TGA (or TAA) indicate the start and stop codons, respectively. Putative polyadenylation signals are underlined. A rat pineal λgt11 cDNA library (gift from Drs. R. McKinnon and D. Klein, NIH, Bethesda, MD), a rat retina λgt10 cDNA library (gift from Drs. G. Liou and F. Gonzalez-Fernandez, Baylor College of Medicine, Houston, TX), a bovine retina λgt11 library and a human retina λgt11 cDNA library (Clontech Laboratories Inc., Palo Alto, CA) were used for screening. The phage (2×10^6) from the rat pineal gland λgt11 library and *Escherichia coli* Y1090 (1×10^9) were plated and incubated at 37°C for 4–8 h. The antibody-screening method is described in Mierendorf et al. (1987). cDNA clones from rat, bovine, and human retinas were isolated by the DNA probe hybridization method described in Maniatis et al. (1982). Each DNA fragment from the clones was treated with *Eco*RI and separated from phage DNA by agarose gel electrophoresis. The cDNA was ligated in the *Eco*RI site of pBluescript plasmid vector (Stratagene, San Diego, CA). The nt sequence determination was performed by the dideoxy chain-termination method (Sanger et al., 1980) with [^{35}S]dATP (3000 Ci/mmol, Amersham, Inc.). The sequencing primers were synthesized using a Biosearch DNA synthesizer (model 7800, Burlington, MA). Last digits of numerals are aligned with corresponding nt.

The pineal gland of mammals is believed to have lost its photoreceptor function as it evolved from the third eye into a secretory organ, but both tissues have some proteins in common, such as interphotoreceptor retinoid-binding protein, S-antigen and 33-kDa protein. In contrast, neither opsin nor Tα has been detected in the pineal gland (Vigh and Vigh-Teichmann, 1981; Van Veen et al., 1986).

The aim of the present study was to develop mAb against retinal 33-kDa protein in the bovine, and to use it for isolating cDNAs encoding 33-kDa protein from different animals. The structural analysis of 33-kDa phosphoprotein in retina and pineal gland will be discussed.

RESULTS AND DISCUSSION

(a) Construction and characterization of a mAb

Retinal proteins isolated from bovine eyes were used to produce mAbs using conventional mAb techniques. One of the mAbs (TS-SC-6) bound to a 33-kDa protein which was present in rat retina and pineal gland but not in liver, kidney, colon and brain as determined by Western blot analysis. Immunohistochemical study cryo sections of retina from rat, rabbit, mouse and bovine localized the protein in the outer and inner segments of the rod cells (data not shown) and these results were consistent with those published by others (Lee et al., 1988; Kuo et al., 1989). In contrast to the retina, the pinealocytes on the entire pineal gland were stained uniformly.

(b) Isolation and characterization of cDNAs

Six cDNA clones were isolated from a rat pineal gland λgt11 library with the mAb probe. One of the largest clones (RPC-6) was used for the hybridization probe to screen additional cDNAs from libraries of human, bovine and rat retinas. Ten cDNA clones from a human retinal λgt11 library, nine from a rat λgt10 library and ten from a cow retinal λgt11 library were isolated by the hybridization method using the 5′ region of the RPC-6 probe (200 nt from the 5′ end). The largest cDNA fragments from the rat (RRC-11), human (HRC-7), bovine (CRC-4) retina and RPC-6 were sequenced and the sequences are summarized in Fig. 2. As we expected, these cDNAs had highly homologous sequences: approx. 87% coding sequence similarity

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                10        20        30        40        50        60
HR   MEEAKSQSLEEDFEGQATHTGPKGVINDWRKFKLESQDSDSIPPSKKEILRQMSSPQSRN
CR   ::K:::::::::::::S::::::::::::::::::E::::VA:::::::::::::::::D
RR   ::::A::::::::::::::::::::::::::::::::E:G::::::::::::::::::::D
RP   ::::A::::::::::::::::::::::::::::::::E:G::::::::::::::::::::D

                70        80        90        100       110       120
     GKDSKERVSRKMSIQEYELIHKEKEDENCLRKYRRQCMQDMHQKLSFGPRYGFVYELETG
     D::::::F:::::V::::::::D::::::::::::::::::::::::::::::::::::S:
     D::::::M:::::::::::::QD::::G::::::::::::::::::::::::::::::::
     D::::::M:::::::::::::QD::::G::::::::::::::::::::::::::::::::

                130       140       150       160       170       180
     KQFLETIEKELKITTIVRTYEDGIKGCDALNSSLTCLAAEYPIVKFCKIKASNTGAGDR
     E:::::::::Q:::::::::::::::::::::::::I:::::::M:::::::::::::::
     E:::::::::Q:V:::::N:::::VR:::::::::E:::::::M::::::R:::::::::
     E:::::::::Q:V:::::N:::::VR:::::::::E:::::::M::::::R:::::::::

                190       200       210       220       230       240
     FSLDVLPTLLIYKGGELISNFISVAEQFAEEFFAGDVESFLNEYGLLPEREVHVLEHTKI
     ::S:::::::V::::::L::::::T::L:::::T:::::::::::::::K:M::::Q:NM
     ::S:::::::V:::::::::::::::::::D:::A:::::::::::::::::I:D:GQ:NT
     ::S::::::::::::::::::::::::::::::A:::::::::::::::::I:D:GQ:NT

        246
     EEEDVE   (246)
     :-::M:   (245)
     :D::I:   (246)
     :D::I:   (246)
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Fig. 2. The deduced aa sequences of 33 kDa HR, CR, RR and RP protein are aligned for maximum homology (for abbreviations, see Fig. 1). The putative phosphorylation and putative carbohydrate-attaching sites are underlined. The numbers above the sequence indicate the aa residues from the N terminal. The aa residues identical with those of human are marked by colons. The numbers in parentheses indicate the total numbers of aa residues. A dash indicates no aa residue. The bovine retina cDNA (CRC-4) lacks 19 nt from the ATG start codon, the corresponding 7 aa were taken from Kuo et al. (1989). Sequence analyses were performed using the IDEAS program written by M. Kaneshisa (NIH-Frederic Cancer Research Facility 1986). Last digits of numerals are aligned with corresponding aa.

was observed between human and rat retinas, 90% between human and bovine retinas and 86% between bovine and rat retinas.

In the rat, sequences of 33-kDa protein from retina and pineal gland were virtually identical, they differed in only 4 nt, which are indicated in parentheses (Fig. 1). The distance from the translation stop codon to the polyadenylation signal is approx. 250 nt and the consensus distance from the polyadenylation signal to the poly(A) tail in these cDNAs was approx. 20–40 nt (Renan, 1987).

The Northern-blot analysis indicated that the corresponding mRNAs from rat retina and pineal gland were approx. 1300 nt in length, which indicates that the sizes of these mRNAs in the retina and pineal gland are also similar.

We believe that the ATG at nt 1 must be the Met start codon, since no other ATG is present in the 5′-noncoding region of nearly full-size cDNA (PRC-11), and the sequence surrounding the ATG is in good agreement with Kozak's (1978) consensus (ccAccATGG).

(c) Amino acid sequence

The ORF of the cDNA is initiated by ATG at nt 1–3 and terminated by TGA or TAA at nt 739–741 and encodes 246 aa residues in man and rat and 245 aa residues in bovine (Figs. 1 and 2). The predicted M_r of the 33-kDa protein in 28246 in human retina, 28176 in bovine retina, 28143 in rat retina, and 28129 in rat pineal gland.

The predicted aa sequences were also similar, approx. 88% sequence similarity was found between human and rat retinas, 88% between human and bovine retinas and 87% between rat and bovine retinas.

In rat, the aa sequences in the retina and pineal gland were virtually identical (Fig. 2), the difference is only one conserved change at aa 191 (Ile in the retina and Val in the pineal gland). The 33-kDa proteins present in retina and pineal gland probably derive from the same gene and the differences in the sequence are perhaps due to DNA polymorphism.

Computer analysis of these proteins using an IDEAS program indicate that they are hydrophilic and have 60–65% α-helical conformation.

The predicted sequence has a putative phosphorylation site at Ser^{73} (Arg-Lys-Met-Ser) residue (Kemp et al., 1977). In addition, there is a putative *N*-linked glycosylation site (Asn-Ser-Ser) at aa positions 152–154 (Wagh and Bahl, 1981).

There are no aa sequence similarities with other known proteins including the Tα nor with any other phosphoproteins in the National Biomedical Research Foundation (NBRF) data bank. However, a statistically significant sequence similarity between the 33-kDa protein and thioredoxin was found. Although the relevance of the similarity is unknown, a mammalian analogue could have some role in day/night-dependent reactions in light-sensitive organs such as the retina and pineal gland, since thioredoxin is involved in coordinating the light and dark reactions of photosynthesis (Holmgren, 1985).

We also compared our sequences with the bovine 33-kDa protein (MEKA protein) published by Kuo et al. (1989) as this report was in preparation. Kuo's bovine MEKA cDNA sequence lacks one G at nt position 691. We also found five additional differences at nt 84 (C in our sequence, T in Kuo's sequence), 131 (C in our, A in Kuo's), 713 (G in our, C in Kuo's), 714 (C in our, G in Kuo's) and 772 (T in our, C in Kuo's). The two different nt in the triple codons alter the aa sequence in the bovine 33-kDa protein. The aa residue at nt position 44 was Pro in our sequences and His in Kuo's and our sequence between nt positions 231–245 was different from the result published by Kuo et al. (1989) (Fig. 4). In our sequence, there is no additional consensus phosphorylation site such as Lys-Arg-Cys-Met-Ser shown by Kuo et al. (1989). We further confirmed this sequence

TABLE I

Phosphorylation site determination[a]

Synthetic peptides[b]	Sequence of aa[b]	Number of aa
Peptide-A	KERVSRKMSIQEYELIHKEKEDENCLRKYRRQ	32
Peptide-B	SRKMSIQ	7
Peptide-C	KERVSRKMAIQEYELIHKEKEDENCLRKYRRQ	32
Peptide-D	KERVARKMAIQEYELIHKEKEDENCLRKYRRQ	32
Kemptide	LRRASLG	7
S-Ag pept	DTNLASSTIIKEGID	15

[a] Synthetic peptides were used for phosphorylation site determination.

[b] The aa sequences of peptides-A (aa position 65–96) and B (aa position 69–74) are the consensus phosphorylation regions of the rat 33-kDa protein but they differ in length. Peptide-C has an Ala^{73} instead of Ser, and peptide-D has Ala's at aa positions 69 and 73, instead of Ser and the rest of the sequence is the same as that of peptide-A. Kemptide is a well characterized substrate for A-kinase and S-Ag pept was used as a negative control.

was absent in the 33-kDa protein by phosphorylation site analysis (see section **d**).

To verify the predicted aa sequences, an oligopeptide (peptide-A in Table I, from aa 65–96 of the rat aa sequences) was chemically synthesized and was used as antigen to produce an antibody (Anti-32) in two rabbits using the conventional method (Harlow and Lane, 1988). The Anti-32 was bound to only the 33-kDa protein on the immunoblots of the rat retina preparation confirming the predicted aa sequences.

(d) Phosphorylation site determination

To characterize phosphorylation in the 33-kDa protein, the native 33-kDa protein of rats was initially phosphorylated with [γ-^{32}P]ATP and cAMP using a rat retinal extract and bovine-heart A-kinase. The phosphorylated protein was then immunoprecipitated by the mAb (TS-SC-6). The tryptic phospho-peptides of the native protein were found to be identical on autoradiograms after separation on isoelectrofocusing gels (data not shown). The identity of the tryptic peptides indicated that both the kinases from the rat retinal extract and that from bovine heart phosphorylate the same substrate,viz., 33-kDa protein.

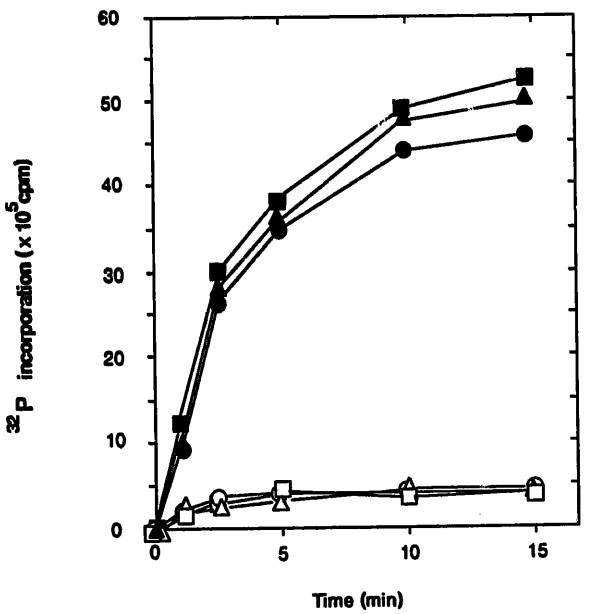

Fig. 3. Phosphorylation of the peptides. The peptides were chemically synthesized using a peptide synthesizer (Bioserch Model 430A, Biosearch, Burlington, MA). After purification by a column 1 x 20 cm (Bio-gel P4; Bio-Rad, Inc., Richmond, CA) with 1% formic acid solution, the peptides were dried and used for the phosphorylation experiments. Two pmol of synthetic peptide A (■), B (●), C (○), D (△), Kemptide (▲), and S-Ag pept (□), were phosphorylated by 10 pg of bovine heart A-kinase catalytic subunits (Sigma Chemical Comp.) with 60 μM [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) as described (Nakabayashi et al., 1987) in 20 μl of solution. The incorporation of ^{32}P into the peptides was analyzed by the phosphocellulose filter paper procedure (Palfery and Mobly, 1987). Each point is the mean of triplicate determinations.

Next we determined a phosphorylation site in the 33-kDa protein. Six oligopeptides were chemically synthesized (Table I). When they were phosphorylated using bovine-heart A-kinase and [γ-^{32}P]ATP, significant ^{32}P incorporation was observed in peptides A, B and Kemptide, but not in peptides C, D and S-Ag pept (Fig. 3). The result indicated that the phosphorylation site is Ser^{73}. Peptide A was also digested with trypsin and the tryptic peptide was analyzed by isoelectrofocusing gel. A band with identical isoelectric points for peptide A and native protein was observed (Fig. 4). These results indicated clearly that the

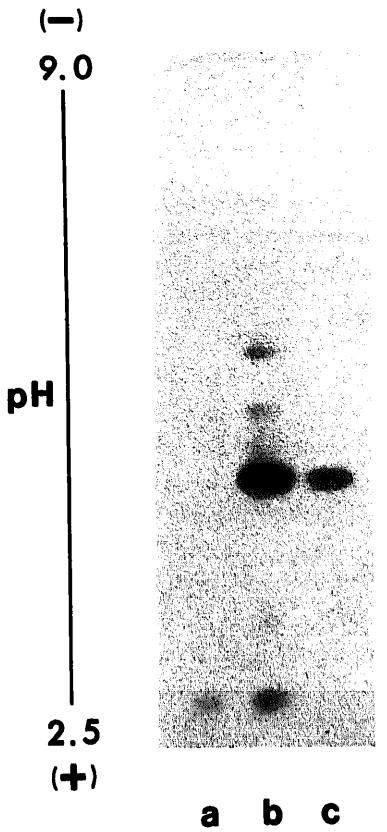

Fig. 4. Isoelectrofocusing of tryptic phospho-peptides of 33-kDa protein (lane **c**) and peptides A (lane **b**) and D (lane **a**). The retinal protein from four dark-adapted rats was phosphorylated with 60 μM [γ-^{32}P]ATP (5000 Ci/mmol, Amersham Inc.,) in 100 μl with and without 10^{-5} M cAMP as described by Lee et al. (1988). Bovine-heart A-kinase (30 μg) was added to some of the reaction mixtures. The phosphorylation was terminated by the addition of 1 μl of 0.5 M EDTA. The 33-kDa protein was immunoprecipitated as described elsewhere (Harlow and Lane, 1988). The proteins in the immunoprecipitates were separated by 0.1% SDS–6% PAGE. The phosphorylated 33-kDa protein band was excised and the protein was incubated with 50 μg/ml of trypsin (type XI-B, Sigma Chemical Co.) for 24 h as described by Palfrey and Mobley (1987). The synthetic peptides were initially released from the phosphocellulose papers by adding 600 μl of 1 M ammonium bicarbonate solution. The peptide solutions were dried and the pellets were dissolved in 500 μl of 50 mM ammonium bicarbonate buffer pH 8.3 containing 50 μg/ml of trypsin as described above. The tryptic peptides (10000 cpm) were applied on the isoelectrofocusing gel (pH range of 2.0–9.5) and isoelectrically focused for 1 h between 30–80 W (Nakabayashi et al., 1987).

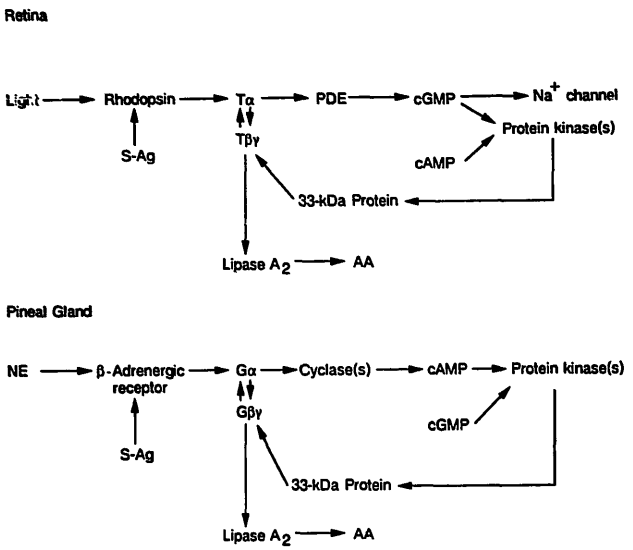

Fig. 5. Comparison of phototransduction cascade in the retina with a hypothetical transduction cascade in the pineal gland. The activation of the phosphodiesterase by light through transducin (T) resembles the activation of adenylate cyclase by hormones such as noradrenaline. AA indicates arachidonic acid. S-Ag indicates S-antigen.

phosphorylation site of 33-kDa protein is unique and at Ser^{73}. The results provide additional confirmation that the predicted aa sequence of the 33-kDa protein is correct.

In the pineal gland, noradrenaline acts to stimulate cAMP accumulation (Vanecek et al., 1985). Noradrenaline binds to the β-adrenergic receptor and stimulates A-kinase activity which phosphorylates 33-kDa protein in rat retinal and pineal tissues (Reig et al., 1990). The 33-kDa protein in the pineal gland, in contrast to the retina, may bind to the Gβγ but not to Tα, since Tα and opsin are not detected in mammalian pineal gland (Vigh and Vigh-Teichmann, 1981; Van Veen et al., 1986).

A secretory organ, the pineal gland, is considered to have evolved from photoreceptor cells common to the retinas of the lateral eyes and of an ancestral third eye. We believe many of proteins utilized in the phototransduction cascade in the photoreceptor rod cells are also utilized in the hormonal signal transduction cascade in the mammalian pineal gland without any significant changes in their structures and functions (Fig. 5). This idea is confirmed by observations by many other investigators. Opsin and β-adrenergic receptor have high aa sequence similarity (Benovic et al., 1986). Both receptors were bound to S antigen, and S antigen quenches the activation of both the visual and the hormonal membrane receptor signaling systems (Benovic et al., 1987). The phototransduction proteins such as S antigen (Abe et al., 1989) and 33-kDa protein in the retina and the pineal gland have the same aa sequence; transducin and G-protein are also highly homologous in both organs (see Gilman, 1987; Stryer, 1986). The members of the G-protein subunit family (Tα, Tβγ and Gα, Gβγ) are interchangeable without losing functional activity (Cerione et al., 1985). The same site on the 33-kDa protein is phosphorylated by A-kinase in both organs. Thus, the similarity between the visual and hormonal cascades is much greater than we expected.

Molecular cloning and nt and aa sequence determination are the first steps in understanding the functional roles as well as the gene regulation and evolution of the 33-kDa protein at the molecular level in the retina and pineal gland.

(e) Conclusions

Several clones of 33-kDa protein were isolated from each cDNA library from human, bovine and rat retina and from rat pineal gland. Their nt sequences were determined and the aa sequences were predicted from them. An antibody produced against the predicted peptide bound to native 33-kDa protein indicates that the predicted aa sequence is correct. The nt and aa sequences of the 33-kDa protein were highly homologous among these species.

The aa sequence had a consensus sequence, Arg-Lys-Met-Ser, which is known to be a phosphorylation site for A-kinase (Kemp et al., 1977). The Ser^{73} in the consensus sequence was indeed a phosphorylation site by A-kinase. The 33-kDa proteins from retina and pineal gland may be derived from a single gene since their nt and aa sequences were identical. Based upon our sequence information and others', we proposed a hypothesis that the proteins utilized in the phototransduction cascade in the photoreceptor rod cells are also utilized in the hormonal signal transduction cascade in the mammalian pineal gland without any significant changes in their structures and functions.

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