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Tubulins in the Primate Retina: Evidence that Xanthophylls may be Endogenous Ligands for the Paclitaxel-Binding Site†,‡

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Abstract—The xanthophylls—lutein, zeaxanthin, and meso-zeaxanthin (L&Z)—are found in the central region of the primate retina, which is called the macula lutea (yellow spot). How they are anchored there and what their function is has been debated for over 50 years. Here, we present evidence that they may be bound to the paclitaxel (Taxol) binding site of the β-tubulin subunit of microtubules and that a major function may be to modulate the dynamic instability of microtubules in the macula. Also, we compare nucleic acid and amino acid sequences of tubulins that are in human brain with those we have isolated from human-retina and monkey-macula cDNA libraries. In so doing, we suggest that in primates, class I β-tubulin consists of at least two subtypes (β_{Ia} and β_{Ib}). Alignment analysis of the sequences of the genes for β_{Ia} and β_{Ib} indicates that the corresponding mRNAs may have other functions in addition to that of coding for proteins. Furthermore, we show that there are at least five different types of β-tubulin in the macula lutea of rhesus monkey.

Introduction

The xanthophylls—lutein, zeaxanthin and meso-zeaxanthin (L&Z; Fig. 1)—are the chromophores that give the primate macula lutea its yellow color.[1] They are derived from food and, along with other lipids, are transported in the blood by lipoproteins.[2] Higher serum levels of L&Z are associated with a lower risk of suffering from neovascular age-related macular degeneration.[3] For a recent review on macular degeneration, see ref. 4. Note that xanthophyll refers to oxygenated polyenes whereas carotene refers to polyenes containing only carbon and hydrogen; both xanthophylls and carotenes are carotenoids.[5] How L&Z contribute to the health of the macula is uncertain.[6] Although carotenoids, including L&Z, are ubiquitous in human tissues,[7] β-carotene, a precursor for vitamin A, is the only carotenoid that has a well-defined function in humans. This is also the only carotenoid that is known to have a protein [β-carotene 15,15′-dioxygenase (β-diox) cleaves β-carotene into two molecules of retinal] that specifically acts upon it.[8,9] Proteins usually define the functions of small molecules in biological systems.

While it has been known for 50 years that xanthophylls account for the yellow color of the macula lutea,[10] only recently has there been any interest in isolating proteins that may be associated with them.[11,12] The field of carotenoid proteins has progressed at a slow pace. For example, β-diox activity was reported in 1965,[13] but the enzyme was only recently cloned and purified.[9,14] Furthermore, people searched for plant carotenoid proteins for decades, but none were isolated until after they were cloned.[15] Although slow progress has been attributed to carotenoid proteins probably being membrane proteins, β-diox was found only in the soluble fraction.[9]

In the soluble fraction of human macular extracts, the cytoskeletal protein, tubulin, copurifies with L&Z.[16] Furthermore, a very recent study shows that various carotenoids bind to tubulin.[17] Because L&Z are especially hydrophobic, they nonspecifically bind to many different proteins. Up until now, there were no data supportive of tubulin specifically binding L&Z.

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β-Tubulin specifically binds paclitaxel (Taxol) and other microtubule-stabilizing agents (MSA).[18–20] With use of a molecular-modeling technique, template forcing, Ojima et al.[20] showed which regions (pharmacophores) of the MSA molecules most probably account for the specific binding of these compounds to β-tubulin (Fig. 2). Most of these regions contain functional groups (conjugated double bonds, geminal dimethyls, isoprenes and six-membered rings) also present in carotenoids (cf. Fig. 1 with Fig. 2). Thus, also with use of template forcing, we show here that L or Z can be superimposed on the tubulin-binding regions of MSA such that these functional groups are coincident.

Microtubules are composed of cylindrically arranged protofilaments, which are linear polymers of α- and β-tubulin heterodimers. There are four isotype classes (I, II, III, IV) of human β-tubulin.[21] Although the amino acid sequences of the different classes are similar, the last 15 amino acids of the C-terminal regions differ enough to justify using them as a basis for isotype classification. Microtubules are copolymers of the available isotypes in a cell.[22] The effects of paclitaxel vary according to the isotypes of β-tubulin that are present. For example, in test-tube experiments, the dynamics of microtubules assembled from purified $\alpha\beta_{III}$ or $\alpha\beta_{IV}$ are less sensitive to paclitaxel than microtubules made from

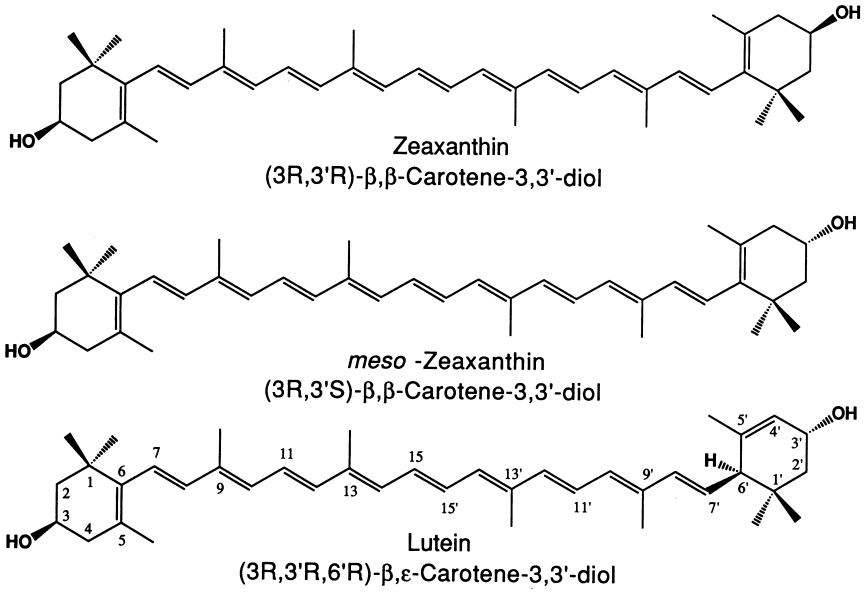

Figure 1. The chemical structures of the three major components of the macular pigment. Taken from ref 1, Fig. 6 (with permission).

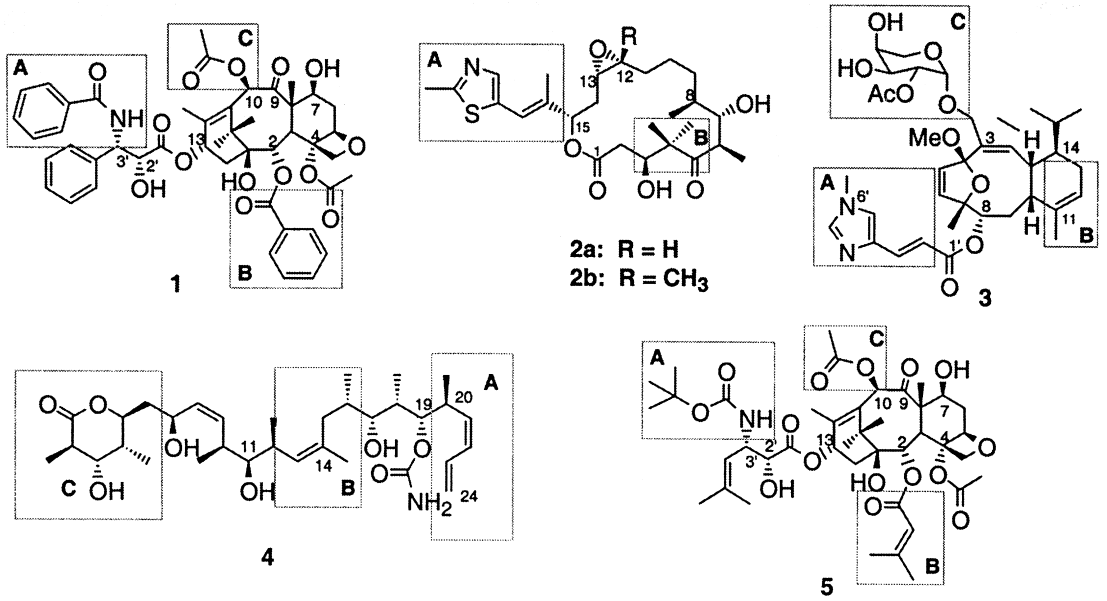

Figure 2. Structures of microtubule-stabilizing agents. Boxed regions labeled A and B contain the pharmacophore regions. Boxed regions labeled C indicate regions that do not contribute to binding to tubulin. (1) Paclitaxel; (2) epothilones A and B (2a and 2b); (3) eleutherobin; (4) discodermolide; (5) nonataxel. Taken from ref 20, Fig. 1 (with permission).

unfractionated tubulin.[23] Consistent with this, human-prostate carcinoma cells that become resistant to paclitaxel show an increase in β_{III}-tubulin.[24] However, similar studies on other cell lines, from various species, show different changes of expression of tubulin isotypes.[25] Hence, the exact mechanism for the effects of paclitaxel or other MSAs is uncertain.

Paclitaxel is used to treat breast and ovarian cancer.[26] The pharmacological mechanism is that paclitaxel's binding to a well-defined pocket in the β-tubulin subunit of microtubules reduces their dynamic instability and hence interferes with cell division.[27] No endogenous ligands have been reported.[28] That L&Z have the same pharmacophores as the MSA is evidence for L&Z potentially being the endogenous ligands of the paclitaxel-binding site of β-tubulin in the macula. We infer from this that a major function may be to modulate the stability of microtubules in the primate macula.

Very recently, it has been shown that L&Z are associated with rod outer segments.[29,30] We do not rule out the possibility that β-tubulin also retains L&Z there, but this work does not consider these studies.

Although the modulation of microtubule stability is not well understood, both α- and β-tubulin contribute to this property.[28] Hence, we sequenced cDNAs of both α- and β-tubulins with use of cDNA libraries constructed from human retina and monkey macula. Furthermore, we show that rhesus-monkey macula contains at least five different types of β-tubulin.

Results

Template forcing

By means of template forcing, subsequent relaxation via local minimization, and then superimposition, the functional groups common to the MSA and the macular-pigment carotenoids were coincidentally superimposed (Fig. 3). Because the exact conformation of the ligands at the binding site of tubulin is not known, the template forcing was done only on the pharmacophore regions and on the regions of the molecules in between the pharmacophore regions.

With the Insight II/Discover program, when a molecular structure with a varying conformation (the mover) is forced onto a structure with a constant conformation (the template), subsequent superimposition yields an exact fit. Hence, the difference between the final and initial values of the conformational energy of the mover can be unreasonably large. All four template-forcing procedures initially yielded unreasonable energy differences. Hence, a local minimization of the conformational energy of the mover to within 10 ± 1 kcal of its initial value was performed. This minimization procedure could change the conformation of the mover such that subsequent superimposition of it onto the template could not be achieved. That did not occur for the superimpositions shown here (Fig. 3).

Discodermolide. The superimposition of discodermolide onto zeaxanthin yielded an especially good fit (Fig. 3A). Two of three of the methyls and all three C–C double

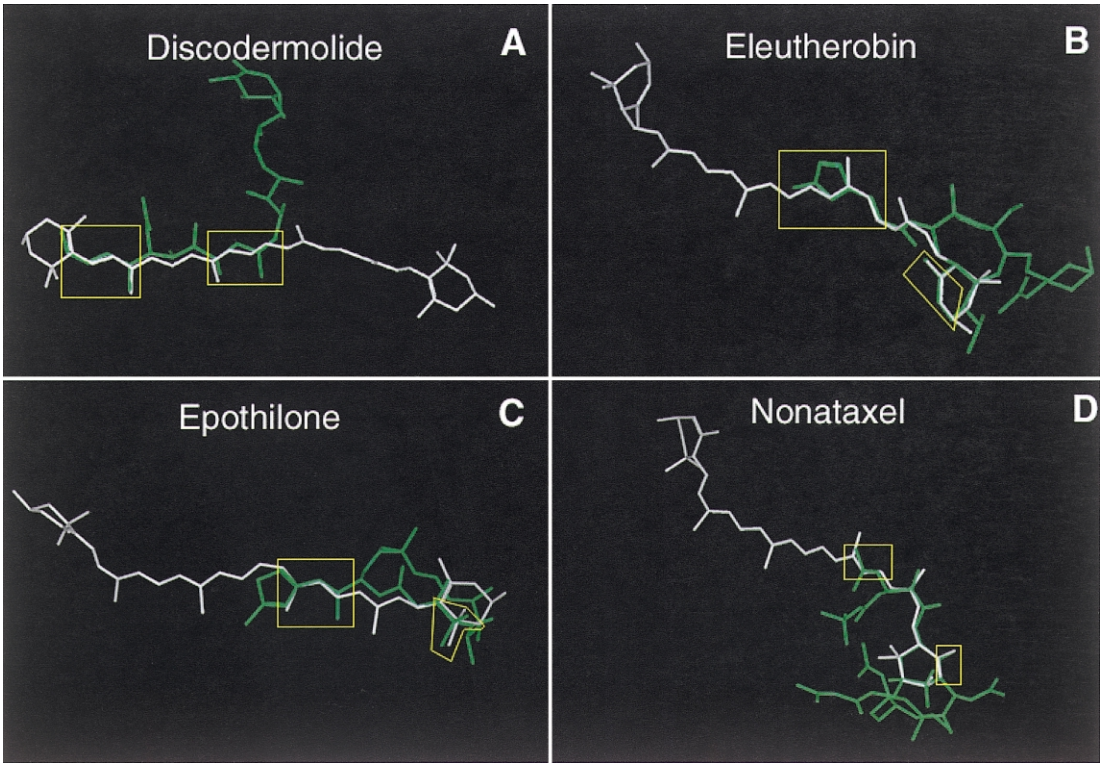

Figure 3. The functional groups of the macular-pigment carotenoids and the pharmacophore regions of microtubule-stabilizing agents (MSA) superimposed. Carotenoid is white; MSA is green; pharmacophore regions within yellow boxes. (A) Discodermolide superimposed onto zeaxanthin; (B) lutein superimposed onto eleutherobin; (C) zeaxanthin superimposed onto epothilone B; (D) lutein superimposed onto nonataxel.

bonds indicated to be in the pharmacophore regions match. Comparison of the structures of lutein, zeaxanthin and meso-zeaxanthin indicates that discodermolide could also have been superimposed onto lutein and meso-zeaxanthin in the same way. Discodermolide is more potent than paclitaxel at inducing tubulin polymerization by a factor of 7.[31]

Eleutherobin. The superimposition of lutein onto eleutherobin yielded a good fit (Fig. 3B). Here, the ring of lutein containing the nonconjugated double bond is superimposed onto the six-membered ring of eleutherobin such that the double bonds and vinyl methyls are coincident. This constraint yields coincident superimposing of the C11′–C12′ and C13′–C14′ double bonds of lutein with the double bond of the spacer arm and one of the double bonds of the imidazole ring, respectively. Eleutherobin is slightly less effective than paclitaxel at inducing tubulin polymerization.[32]

Epothilone B. The superimposition of zeaxanthin onto epothilone B yielded a good fit (Fig. 3C). Here, the geminal dimethyl of the β-ionone ring of zeaxanthin is superimposed onto the C4 geminal dimethyl of epothilone B. This constraint yields coincident superimposing of the C11–C12 and C13–C14 double bonds of zeaxanthin with the double bond of the spacer arm and the C–C double bond of the thiazole ring, respectively. Note that the conformation of epothilone B that was used for the superimposition was similar to the solution conformation.[33] Epothilone B has the same effectiveness as paclitaxel at inducing tubulin polymerization.[34]

Nonataxel. The superimposition of lutein onto nonataxel yielded the poorest fit (Fig. 3D). Here, the ring of lutein containing the nonconjugated double bond is superimposed onto the six-membered ring of nonataxel that also has a double bond, such that the vinyl methyl groups are coincident. This constraint yields coincident superimposing of the C11′–C12′ double bond of lutein with the C4′–C5′ double bond of nonataxel. This superimposition favors assignment of the isoprene of C-3′ as the pharmacophore, rather than the tertiary butyl formate amino group. Support for this assignment comes from 3′-cyclohexyl analogues of paclitaxel exhibiting higher activity than paclitaxel.[35] Also, if either or both of the isoprene groups of nonataxel at C-2 and C-3′ are hydrogenated the subsequent compounds have much reduced cytoxicity.[36] Nonataxel has the same effectiveness as paclitaxel at inducing tubulin polymerization.[20]

Recently, the polyisoprenyl benzophenones, guttiferone E and xanthochymol have been shown to inhibit microtubule disassembly with an activity similar to paclitaxel.[37] Hydrogenating the isoprenyl groups yields a total loss in activity.[37] This provides further evidence that isoprene subunits play a role in the activity of MSAs. We found that zeaxanthin or lutein can also be superimposed onto these compounds such that the isoprene subunits are coincident (data not shown).

Carotenoid and MSA sizes differ. Cursory inspection of the four superimpositions indicates that the carotenoids are longer than the MSAs (Fig. 3). For eleutherobin and epothilone B, approximately half of the carotenoid molecule extends beyond the imidazole and thiazole rings, respectively. Although discodermolide is much closer in length to L&Z, its lactone ring and linker methylene do not contribute to tubulin binding (Fig. 2). Furthermore, inspection of the β-tubulin paclitaxel-binding site[28] indicates that the carotenoid molecules would extend out from the binding pocket by 10- 13 carbon bond lengths. This is exactly the correct size to fit into a recently discovered hydrophobic binding pocket adjacent to the paclitaxel-binding site.[38] The length of discodermolide is approximately the same as that of L&Z. Hence, this hydrophobic pocket may account for discodermolide being more potent at inducing tubulin polymerization than other MSAs.

Tubulin types in retina

Human-retina nucleic acid sequences of α-tubulin and β-tubulin were obtained by classical library screening of a retina cDNA library. Monkey-macula nucleic acid sequences were obtained by sequencing overlapping fragments of cDNA that were generated by using a monkey-macula cDNA library as a template in PCR.

Sequences of human-retina α-tubulin. The oligonucleotide probe 5′-TGAGACGGGGGCTGGCAAGCA-3′ was used to isolate the clone, hum-a-tub2 (AN: AF141347), from a human-retina cDNA library. Hum-a-tub2 was found to have the same sequence as the coding region and some of the noncoding regions of human-brain-specific α-tubulin [ANs: NM_006009 (mRNA), X01703 (gene)]. Only one base of 1356 bases in the coding region (position 2484 of the gene) differed and it did not change the amino acid sequence (brain: CTC, retina: CTT). This indicates the same gene (TUBA3) is expressed in brain and retina.

With the oligonucleotide probe 5′-TCCAAGCTG-GAGTTCTCCAT-3′, the clone hum-a-tub1 (AN: AF141348) was isolated from the retina cDNA library. This clone was sequenced and found to have 1197 bp of the 3′ end of the same isotype of α-tubulin as TUBA3. A fragment of similar size and sequence is in fetal brain (AN: K00557). Both fragments contain an especially long open reading frame (897 bp) that, except for two differences [position 137: (brain) D–E (retina); position 155: (brain) G–R (retina)], codes for the same sequence of amino acids. This may be the complete amino acid sequence for a new isotype of α-tubulin. If so, it would consist of the 299 C-terminal amino acids of brain-specific α-tubulin. The 153 amino acids not included encompass much of the GTPase region and many of the residues directly involved in nucleotide binding.[39]

Sequence of monkey-macular α-tubulin. With use of overlapping fragments of cDNA obtained by PCR, we constructed almost all of the coding region and some of the 3′ untranslated region (UTR) of an isotype of α-tubulin (AN: AF141923). Comparison of this nucleic acid sequence with the α-tubulin sequence from human retina (AN: AF141347) indicated that the nucleic acid

sequences differed at 26 sites, but the amino acid sequences were identical and hence identical to human brain-specific α-tubulin (see caveat in next paragraph). We were not able to obtain any of the 5′ UTR nor the first 27 bases of the coding region even though we attempted to do so with 5 different primer pairs designed using the human gene (AN: X01703). It may be that the monkey-mRNA 5′ UTR is very different from that in human.

The nucleic acid sequence for human brain-specific α-tubulin indicated in ref 40 differs from that in GenBank entries NM_006009 and X01703 in three positions (mRNA: 391, 922, 1312; gene: 2903, 3434, 3824). These changes yield three amino acid differences. Because both the human-retina and monkey-macular amino acid sequences agree with the sequence in ref 40 it is probable that the GenBank entry is wrong.

The number of nucleic acid differences between human and monkey α-tubulin was slightly higher than expected. The expected number (mean ± SE) of differences per 100 sites at 2- and 4-fold degenerate sites is 3.4 ± 0.3 and 6.3 ± 0.5, respectively.[41,42] The observed values for α-tubulin were 4.5 (2-fold) and 7.0 (4-fold) differences per 100 sites. Changes in 2- and 4-fold degenerate sites depend mostly on time; the expected number of differences at nondegenerate sites depends on both time and the considered protein.[44] The average value for nondegenerate sites for human versus monkey is 1.4 differences per 100 sites.[41,42] The observed value of zero indicates that the amino acid sequence of this isotype of α-tubulin changes less than that of the average protein. Although amino acid sequences differ somewhat among tubulin isotypes found in a particular organism, any particular isotype varies little among different organisms.[43]

Sequence of human-retina β-tubulin. The oligonucleotide probe 5′-TGAGGGAAATCGTGCACATCC-3′ was used to obtain a clone (AN: AF141349) that had the same nucleic acid sequence as an isotype of β-tubulin that is in human brain (AN: AF070600). This isotype can be classified as a variant of β_I [ANs: V00599 (mRNA), J00314 (gene)] for the following reasons: The C-terminal, isotype-defining amino acids are identical. The two variants differ by only five amino acids. Four of five of the differences are conservative. All five of them are in the intermediate domain where drug binding occurs.[44] These differences cannot be attributed to sequencing errors because both sequences have been independently verified. The sequence for β_I was obtained from two different clones. Clone Dβ-1 was obtained from a cDNA library prepared from human brain tissue[45] and clone M40 was obtained from a genomic library.[46] Because tubulins are classified based on their C-terminal region, we suggest that β_I now be referred to as β_{Ia} and that the variant we report here in monkey retina and previously reported (AN: AF070600) in human brain be called β_{Ib}.

The human gene for β_{Ib}, which contains four exons and three introns, is in a region of chromosome 6 that contains the HLA class I genes.[47] Alignment analysis of the gene for human β_{Ia} (AN: J00314), which also contains four exons and three introns, with that for β_{Ib} (AN: AB023051) may indicate that they were formed via gene duplication. Subsequent to that one of them appears to have undergone a form of gene conversion. (For a review on gene conversion, see ref 48). Evidence for gene conversion is that in exon 4 there is a cluster of four base differences that all yield differences in amino acids. One other base difference in exon 4 also yields an amino acid change. The remainder of the mRNA has remained unchanged as shown by the following four observations: (1) Among the other three exons, there is only one other base difference, and it does not yield an amino acid difference. (2) Within the 5′ UTRs, there are no differences. (3) Comparison of the 3′ UTRs indicates that they differ only by one 8-bp deletion in β_{Ib} and two 1-bp deletions in β_{Ia}. (The 3′ UTR of β_{Ia} is 180 bp and that of β_{Ib} is 186 bp.) (4) All three introns are in identical positions relative to the exons in each gene although intron 1 differs in size by 94 bases. Contrary to this conservation within the exons, not counting insertions or deletions, the three introns averaged 3.1 differences per 100 bases. The differences within the introns imply that there should be a commensurate number of differences among the 2- and 4-fold degenerate sites within the exons.

Sequences of monkey-macular β-tubulin. With use of overlapping fragments of cDNA obtained by PCR, we constructed the entire coding region and some of the noncoding regions of an isotype of β-tubulin (AN: AF147880). Comparison of this nucleic acid sequence with the full-length β-tubulin sequence from human retina indicated that the nucleic acid sequences differed at 25 sites, but the amino acid sequences were identical.

Similar to the situation for α-tubulin, the number of nucleic acid differences for β-tubulin between human retina and monkey macula was slightly higher than expected. The observed values for β-tubulin were 4.3 (2-fold) and 7.0 (4-fold) differences per 100 sites. As with α-tubulin, there were no differences among the nondegenerate sites. The amino acid sequence of any particular isotype of β-tubulin varies only slightly among vertebrates.[43] A BLAST search of GenBank yielded four other β-tubulins with exactly the same amino acid sequence as this one. The type of tissues in which they were expressed varied. That found in chicken (AN: P09244) and mouse is expressed in most tissues. In rat (AN: BAA32736) it is expressed in brain, but may be expressed in other tissues. Also, it has been reported in Chinese hamster ovary (CHO) cells (AN: AAB18929). Hence, there is no reason to believe this isotype is specific to neural tissues.

Primer pairs designed by Ranganathan et al.[49] to test for the presence of different isotypes of β-tubulin (β_I, β_{II}, β_{III}, β_{IVa} and β_{IVb}) were used in conjunction with the monkey macula library to generate PCR products (Fig. 4). Their sizes were in good agreement with expected values. They were subsequently sequenced and found to have corresponding amino acid sequences in

agreement with their human counterparts. The only difference was in the β_{IVa} fragment [glutamine-432 (human), glutamate (monkey)]. Among vertebrate β-tubulins, these two amino acids are the most predominant at this position.[50] Because the amino acid sequences of β_{Ia} and β_{Ib} are the same in the region of the primer pair, it is uncertain which cDNA was amplified. Because there were no double peaks in the sequencing chromatogram, only one type of cDNA was amplified and subsequently sequenced.

One might expect that constructing full-length sequences of α- and β-tubulins with overlapping fragments of cDNA might yield hybrid sequences containing different isotypes of α- and β-tubulin cDNA. However, the amino acid sequences of the monkey-macula tubulins exactly agreed with those found in human brain and retina; this indicates the sequences are not hybrids.

Discussion

Molecular modeling

With the use of molecular modeling, we have superimposed the functional groups of the macular-pigment carotenoids onto the pharmacophore regions of several MSAs (Fig. 3). This is supportive of L&Z binding to β-tubulin at the paclitaxel-binding site. Also, this provides a reason why the macular-pigment carotenoids

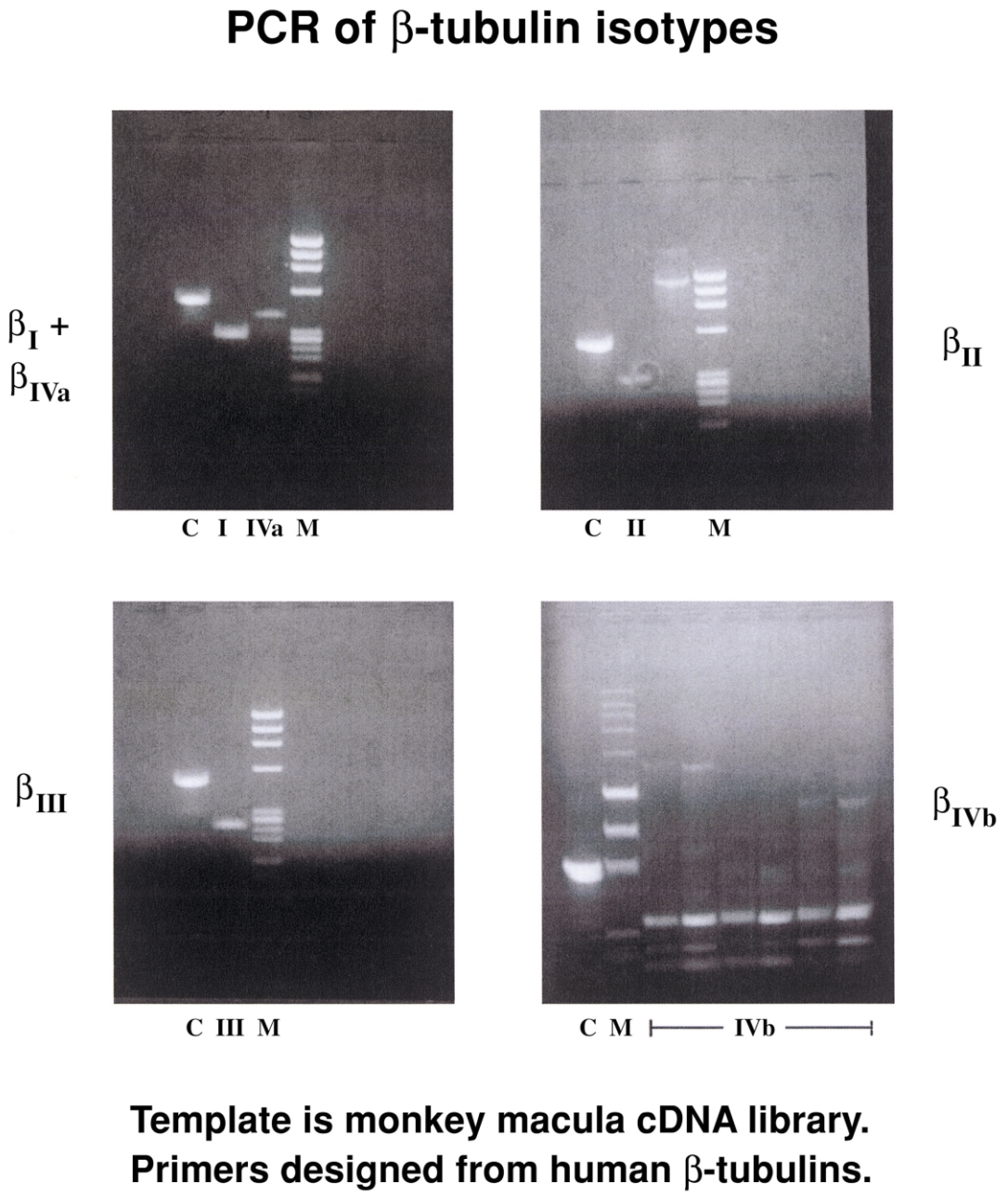

Figure 4. Agarose gels of fragments of β-tubulin types found in monkey macula. The range of the marker (M) is 72–1353 bp. A 500-bp control reaction was made with every run (C). The sizes of the fragments are β_I: 290 bp; β_{II}: 297 bp; β_{III}: 247; β_{IVa}: 388 bp; β_{IVb}: 254 bp.

copurify with tubulin.[16] Furthermore, it is probable that the hydrophobic pocket adjacent to the paclitaxel-binding site[38] also contributes to the binding of L&Z to β-tubulin. Taken together, these data are strong evidence for L&Z possibly being the endogenous ligands for the paclitaxel-binding site of β-tubulin in the primate macula lutea.

Microtubules, which are polymers of α- and β-tubulin heterodimers, are constantly being assembled and disassembled.[51] Different types of cells demonstrate varying amounts of dynamic instability and hence the average half-life of microtubules varies with cell type.[52] In nerve axons, microtubules are much more stable than are microtubules in other cell types.[53] The exact localization of L&Z in the macula is uncertain; however, there is evidence that photoreceptor axons contain high densities of macular pigment.[54] It may be that one of the functions of the macular-pigment carotenoids is to modulate the stability of photoreceptor-axon microtubules.

Paclitaxel is toxic,[55] whereas L&Z are not.[56] If L&Z are endogenous ligands for the paclitaxel-binding site, what accounts for the difference in toxicity? To modulate the dynamic instability of microtubules, such an endogenous ligand would require some means for its removal. For example, when a cell needs a microtubule to be shortened or dismantled, it might direct a microtubule-associated motor protein (MAP) to remove the ligand as the MAP traverses the microtubule. This MAP should be most efficient at removing endogenous ligands. Ligands buried deep in the paclitaxel-binding site, which is on the inside of microtubules,[57] may not be accessible to the MAP; ligands that are accessible may not possess the appropriate binding sites for the motor protein. It is quite possible that the hydrophobic pocket adjacent to the paclitaxel-binding site is there to make the endogenous ligands accessible to a protein that removes them.

This model is consistent with L&Z being responsible for the phenomenon of Haidinger's brushes.[58] If one looks at a white surface illuminated by plane-polarized white light one will see two blue sectors separated by two yellow sectors—the figure called Haidinger's brushes. Because the dichroism responsible for Haidinger's brushes has a spectral distribution almost indistinguishable from the optical density spectrum of the macular pigment, this phenomenon has been attributed to it.[59] Because the long axes of molecules of L&Z contain conjugated double bonds, if L&Z molecules are arranged parallel to one another, they will show greatest absorption of plane-polarized light if the plane of polarization is parallel to their long axes. With use of microdensitometry, when the fiber layers of the fovea, which contain photoreceptor axons, are illuminated with polarized blue light, the greatest optical density occurs when the plane of polarization is perpendicular to the axis of the fibers.[59] Implicit in the hypothesis that L&Z bind to the paclitaxel-binding site is that their orientation in that binding site is consistent among β-tubulin subunits. Because microtubules consist of approximately 13 cylindrically arranged protofilaments, regardless of the orientation of L&Z in the binding site, the L&Z molecular orientation relative to an incident beam of light will be considerably heterogeneous. Haidinger's brushes in the retina can be accounted for if at least 7% of L&Z molecules are preferentially arranged such that their long axes are tangential to circles concentric on the fovea.[58] Because dichroism is a net effect determined by a sum of vectors (here, the long axes of the xanthophylls relative to the plane of polarization), the necessary percentage of preferentially arranged molecules is dependent on the angles between the long axes of the xanthophyll molecules and the plane of polarization.[60] Exact elucidation of these variables will require further investigation.

Tubulin types in retina

We show that there are at least five isotypes of β-tubulin in rhesus monkey macula (I_b, II, III, IV_a, and IV_b), but because β_{Ia} is expressed in human brain,[45] there may be at least six isotypes expressed in macula. β_{Ia}-Tubulin is considered to be constitutive.[21] It is classified constitutive in humans on the basis of its 3′ UTR hybridizing to RNA from a variety of cell lines, including neuroblastoma and retinoblastoma cell lines.[61] Comparison of the 3′ UTRs of β_{Ia} and β_{Ib} indicated that the differences would not be sufficient to be distinguished in RNA blot transfer experiments. Hence, whereas class I β-tubulins may be constitutive, the tissue distributions of β_{Ia} and β_{Ib} are uncertain.

During our screening of the human retina cDNA library, an 1197 bp cDNA was isolated. Human brain contains a similar piece. Both pieces contain an especially long open reading frame (897 bp) that codes for the 299 C-terminal amino acids of brain-specific α-tubulin, which consists of 452 amino acids. Loop T7 and helix H8, which are within this C-terminal region of α-tubulin, play a major role in interdimer interaction.[28] The 153 N-terminal amino acids for which the pieces do not code play a major role in intradimer binding because they make up most of the Rossman fold that binds GTP.[39,44] The binding of this new putative α-tubulin to the plus end of a growing microtubule would halt the addition of heterodimers to protofilaments because they would lack β-tubulin caps. Hence, a function of this putative α-tubulin might be to modulate the size of microtubules. Furthermore, by eliminating the β-tubulin-GTP cap from the plus end of microtubules, this new protein may also modulate the stability of microtubules.[28] Dynamic instability, in the absence of microtubule stabilizing drugs, is thought to be associated with the hydrolysis of GTP, but the mechanism for this is controversial.[62]

Comparison of the human genes for β_{Ia}-tubulin and β_{Ib}-tubulin yielded contradictory results: Although there are 464 degenerate sites among the four exons, at only one of those sites is there a nucleotide difference (in exon 1). This implies that these two genes were the result of a gene duplication in the very recent past. Contrary to this finding, the many differences among the three introns implies that gene duplication occurred

in the distant past. These contradictory results indicate that the mRNAs of both genes have another function(s) in addition to that of coding for proteins. Recently, it has been shown that the RNA components of the ribosome perform the catalytic functions while the protein components provide structural organization.[63] It may be that the β_{Ia}- and β_{Ib}-tubulin mRNAs are also ribozymes.

The spatial distributions of lutein and zeaxanthin as functions of distance from the foveal center in the primate macula have been measured.[64,65] The maximum concentrations of both xanthophylls are in the center of the macula, and both rapidly decline to baseline values within two mm of the center. Although the distributions are similar, for humans and Old-World rhesus monkeys, the concentration of zeaxanthin is much higher in the center than that of lutein. In New-World squirrel monkeys, lutein has a slightly higher concentration in the center. These data imply that there is a means to account for these differences in macular-pigment distributions. Here we suggest two possible mechanisms that are not mutually exclusive: One is that for humans and rhesus monkeys there are different transport proteins (possibly lipocalins) that shuttle lutein and zeaxanthin to different regions of the macula. The other is that, if the β-tubulin subunit of microtubules retains L&Z, different isotypes may bind lutein, zeaxanthin and possibly meso-zeaxanthin differently.

Conclusion

Since the discovery of xanthophylls in the primate macula,[10] various functions have been suggested for them. The suggested functions that attracted the most interest are: reduction of chromatic aberration, absorption of blue light that may cause photochemical damage, and reduction of oxidative species.[66] The evidence presented here in support of microtubules binding the macular-pigment carotenoids is consistent with the first two functions because L&Z would be accessible to incoming light. However, such binding would rule out the third function because L&Z would be buried in the binding site and hence not approachable by oxidative molecules. A recent study[6] has suggested that the macular-pigment carotenoids are not antioxidants, although the possibility that they quench singlet oxygen could not be ruled out. Our new evidence further vitiates the hypothesis that the macular-pigment carotenoids are antioxidants. Furthermore, this new evidence suggests a function not previously considered: A major function of the macular-pigment carotenoids may be to modulate the dynamic instability of microtubules in the primate macula.

Experimental

Molecular modeling

Template forcing and conformational energy minimizations were done with the molecular modeling software, Insight II/Discover 98 (MSI, San Diego, CA, USA). The consistent valence force field (CVFF) was used. Except for nonataxel, the MSA conformations were the same as in Ojima et al.[20] For nonataxel, a gauche bond torsion angle for H2′–C2′–C3′–H3′ of 60° was used. This is energetically reasonable and similar to the conformation of paclitaxel in aprotic solvents[67] and the conformation of docetaxel found in its crystal structure.[68]

Depending on which provided the better fit, either lutein or zeaxanthin was template-forced onto epothilone B, eleutherobin and nonataxel (carotenoid was mover, MSA were template). X-ray crystallographic data for β-carotene indicate that the C–C bonds of the conjugated system alternate in length between that of a single bond and that of a double bond.[69] This implies that L&Z are sufficiently flexible for template forcing. Because discodermolide has many conformations available to it, choosing any one is arbitrary. Consequently, for discodermolide the carotenoid was the template.

Before template forcing, the energy of the mover was minimized to obtain a chemically reasonable conformation. After template forcing, the conformational energy of the mover was subjected to local minimization to return it to within 10 ± 1 kcal of its initial value. Before template forcing of discodermolide onto zeaxanthin, the conformational energy of both molecules was minimized.

cDNA libraries

A human-retina cDNA library made from normal tissue was purchased from CLONTECH (Palo Alto, CA, USA). It had been constructed with a λgt10 cloning vector. The quality control data indicated that the number of independent clones was 1.6×10^6, the average insert size was 1.7 kb, and the insert size range was 0.6–4.0 kb. By plating a serial dilution of the library, we calculated the titer to be 2.8×10^{10} plaque forming units (pfu) per mL.

A rhesus-monkey-macula cDNA library made from normal tissue was provided by Ignacio Rodriguez (National Eye Institute, Bethesda, MD, USA). The age of the monkeys was 4–5 years. The macula was defined by a 5-mm trephine centered on the fovea. There may have been slight retinal-pigmented epithelial cell contamination of the neural macula during the dissection. The library was constructed with an Uni-Zap-XR vector (Stratagene, La Jolla, CA, USA). The number of independent clones was 1.0×10^6. We found the titer to be 1.2×10^{11} pfu/mL.

Classical library screening

The human-retina cDNA library was screened with oligonucleotide probes according to the instructions (Lambda Library Protocol, PT1010-1, Version PR47377) with one modification. To facilitate observation of positive plaques, only 8000 pfu per 150-mm plate were used. Oligonucleotide probes were designed with the software program, Oligo, Ver. 6.0 (Molecular Biology Insights, Plymouth, MN, USA). They were labeled with P-32 by means of a 5′-labeling kit (NEN, Boston, MA, USA). The NENSORB column provided with the

kit was not used because product yields of only 5–20% were obtained with it. Instead, a SELECT-D collection tube (5prime→3prime, Inc., Boulder, CO) was used. With these columns, the product-yield range was 60–90%. Addition of the P-32 labeled probe to the hybridization buffer yielded 8.0 ± 1 μCi/mL.

PCR

Polymerase chain reactions were carried out with a Hybaid PCR Sprint thermal cycler. Although the temperature programs varied with the melting points of the primer pairs, touchdown programs were always used. The primer pairs specified by Ranganathan et al.[49] were used to ascertain the presence of five isotypes of β-tubulin in monkey macula. DyNAzyme EXT DNA polymerase (Finnzymes, US distr. MJ Research, Watertown, MA, USA) and associated reagents were used to achieve amplification of the β_I, β_{II}, β_{III} and β_{IVa} fragments. PfuTurbo DNA polymerase (Stratagene) and associated reagents were used for β_{IVb}. The sources of DNA templates were the cDNA libraries.

Primer pairs used to obtain overlapping cDNA fragments that were subsequently used to construct the entire coding regions of α- and β-tubulins in monkey macula were designed with Oligo, Ver. 6.0 (Molecular Biology Insights, Plymouth, MN, USA) or the PrimerSelect module of Lasergene (DNASTAR, Madison, WI, USA). The five primer pairs used to obtain the α-tubulin fragments were: 5′-AGTGCATCTCCATCCACGTT-3′ and 5′-TTCTTTGCCTGTGATGAGTTG-3′, 5′-TTGGCAAGGAGATCATTGACC-3′ and 5′-CATCTTTGGGAACCACGTCAC-3′, 5-GTGGTTCCCAAAGATGTCAAT-3′ and 5′-GGGTCTGTAACAAAGGCATTC-3′, 5′-ACAATGAGGCCATCTATGACA-3′ and 5′-TGGCATACATCAGGTCAAACT-3′, 5′-CGATATTGAGCGTCCAACCTA-3′ and 5′-AGTGGGAGGCTGGTAGTTGAT-3′. The 6 primer pairs used to obtain the β-tubulin fragments were: 5′-CCAACCTTCCAGCCTGCGACC-3′ and 5′-TCTGCCTCCTTCCGTACCACA-3′, 5′-AGTGTGGCAACCAGATCGGTG-3′ and 5′-CGGAGAGGGTGGCATTGTAGG-3′, 5′-AATGGGCACTCTCCTTATCAG-3′ and 5′-TGCTGGGTGAGTTCCGGCACT-3′, 5′-GACACCGTGGTCGAGCCCTAC-3′ and 5′-TGCCCTCGCCTGTGTACCAGT-3′, 5′-ACCTCACCGTGGCTGCTGTCTTCC-3′ and 5′-AGGTGATGGGGGCTCTGCCTTAGG-3′, 5-TCAAGCGCATCTCGGAGCAGTTCACT-3′ and 5′-GAGGGAGAGGAAAGGGGCAGTTGAGT-3′. The primers that yielded the 500 bp product were 5′-GATGAGTTCGTGTCCGTACAACT-3′ and 5′-GGTTATCGAAATCAGCCACAGCG-3′ and bind to lambda phage DNA.

Agarose gels (2%) of PCR products were run at 65 V and bands were visualized with ethidium bromide. The molecular weight marker (72–1353 bp) was a ϕX174 DNA–Hae III Digest (Finnzymes). If only a single band was observed, the PCR product was purified with a Microcon YM-100 (Millipore, Bedford, MA, USA). If more than one band was present, purification was achieved with a gel extraction kit (Clontech).

Nucleic acid sequencing and sequence analysis

Clones obtained from classical library screening were sequenced by Commonwealth Biotechnologies (Richmond, VA, USA). PCR products were sequenced by the core facility at Tufts Medical School (Boston, MA, USA). The BLAST program at the National Center for Biotechnology Information (NCBI) was used to locate homologous sequences at GenBank via the Internet. All accession numbers can be located at GenBank. Alignments were done at the Genestream Resource Center via the Internet (www2.igh.cnrs.fr/bin/align-guess.cgi) or with the MegAlign module of Lasergene (DNAStar).

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