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Annexin gene structures and molecular evolutionary genetics

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Abstract. Annexins provide an exemplary model for studying the pattern and process of molecular evolution in multigene families. Their related gene structures, broad dispersal in eukaryotic genomes and abundant coding sequences permit a phylogenetic reconstruction of their genetic history. The emerging picture is one of prolific expansion by gene duplication to more than 27 paralogous subfamilies that have undergone steady sequence divergence, speciation and differential selection. Homologous recombination via the common tetrad of internal repeats has, nevertheless, strictly preserved this core structure for over 1200 million years, implying a basic functional role. The existence of multiple annexins with unique 5′ coding and regulatory regions has facilitated their adaptation to the varying ontogenetic and cell-specific needs of diverse organisms. Computational and cladistic sequence analyses have permitted the determination of original gene duplication dates and mutation rates for the ten known vertebrate annexins. Molecular genetic and evolutionary studies of annexins can help to define their structure-function relationships, elucidate their individual physiological roles and ultimately link them to hereditary phenotypes.

Key words. Chromosomal maps; exon-intron splicing; gene structures; gene duplication dates; homologous recombination; molecular evolution; multigene family; phylogenetic analysis.

Abbreviations. *ANX* = human annexin gene locus or DNA, *Anx* = nonhuman gene, ANX = annexin protein, Myr/Mya = million years/ago.

Introduction

The annexin multigene family now comprises over 94 unique members from 45 species in all eukaryotic kingdoms except fungi. Each of the 27 classified subfamilies [1, 2] which contains at least one full-length sequence representative is designated by a Roman numeral after the name, or an Arabic numeral after the gene symbol *ANX* (human) or *Anx* (other species), followed by the genus-species abbreviation. Their eclectic nomenclature includes synonyms such as lipocortins, calpactins, endonexins, synexin, anchorin, calcimedins, calelectrins, calcyclin-associated protein-50, calphobindins, placental or vascular anticoagulant proteins, p35, p36 and p68. Annexin proteins are structurally defined by an ancient conserved domain of four homologous repeats responsible for ion channel activity and calcium-dependent binding to anionic phospholipids, the cytoskeleton and extracellular matrix proteins [3]. The evolutionary conservation of this tetrad structure since the most primitive eukaryotes contrasts with the variable amino-termini of individual subfamilies through which binding and functional properties may also be influenced. The upstream regulatory region of each gene is likewise unique and determines a characteristic expression pattern that is responsive to many forms of cellular stimulation and differentiation. It is this combination of structural similarity and regulatory versatility that must account for the evolutionary success and, by inference, the biological significance of the annexin gene family. Studies have therefore been aimed at elucidating the genetic mechanisms responsible for their creation, the patterns of mutation contributing to their mutual divergence and the biological forces that have selected them for specific functions. Comparative analyses of annexin gene structures, genomic location and molecular phylogeny can provide insight into their genetic history and perspective on the scope of their diversity. Clarification of their structure-function and genotype-phenotype relationships will contribute to the important goal of understanding their true biological roles.

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Annexin gene structures and organization

Evolution of the core tetrad. Annexin gene structure comparisons have served as a useful basis for inferring homology, i.e. common ancestry, among human annexins [4]. Available genomic sequence data from protists, plants, invertebrates and vertebrates now permit a more extended evaluation of the conserved and variable regions in annexin genes. Figure 1 outlines the coding and noncoding regions of structurally characterized genes and also shows the different exon-splicing patterns in annexins from species at progressive stages of evolution. The most striking feature is the strict conservation of a 3′ coding tetrad region in which four internally homologous repeats of 68 to 69 codons each are linked by

lesser conserved segments of four to 15 codons. The only allowed variation in this structure occurs in the length of repeats 3, where more abundant cDNA data show that the 5′ linker segment can have a single codon insertion (annexins II, VIII and X) or deletion (annexin VIb) and that the repeat 3 core region can also accomodate single codon insertions (*Dictyostelium Anx14*, and *Caenorhabditis Anx16* and *Giardia* annexins). A single codon insertion early in the conserved portion of repeat 3 in all known annexins provides evidence that the intact tetrad structure probably originated prior to the divergence of eukaryotic kingdoms. Plant annexins can be distinguished by a separate codon deletion in the middle of repeat 3 that identifies their common progenitor as a structural variant of the primary annexin ancestor. The broad sequence divergence among repeats 3 suggests that it may have been the earliest monomeric repeat, which successively duplicated to form the original tetrad [4, 1], or alternatively, that its relative lack of structural constraint has been an important determinant of selection for subfamily specificity [5, 6]. The ancient appearance of the annexin tetrad in protists over 1200 Mya and its unique, enduring structure point to its basic functional role, consistent with the ion channel model of annexins [7, 8].

Exon-intron splicing patterns. The number, splice location and phase of introns all show interesting and informative variations in annexin genes from species within and between different phyla. Intron numbers range from none in protists, to several in plants and invertebrates, and up to 12 or 13 in vertebrate annexins. The lack of introns, absence of 5′ and 3′ terminal coding regions and short untranslated regions in three *Giardia lamblia* genes [9, 10, 11], subsequently identified and classified as tetrad annexins *Anx19*, *Anx20* and *Anx21* [12, 1, 2], represent the current historical starting point in our knowledge of the structural evolution of annexins. Fragmentary genomic data are available on plant annexins from *Anx24* of *Lycopersicon esculentum* (tomato p34) [13, 2], *Anx25* from *Armoracia rusticana* (horseradish) [14, 2] and the unclassified annexin from *Malus domesticus* (apple) [15]. They indicate either the absence or displacement of exon splice sites relative to vertebrate annexins. The unique splicing pattern and rare occurrence of phase 2 introns (i.e. after the second base codon position) in the 5′ coding region of the *Dictyostelium* annexin [16] were among the many features that warranted its reclassification to *Anx14* [1] from the original designation as annexin VII. Invertebrate *Anx15*, *Anx16* and *Anx17 from C. elegans* [17] possess variant intron numbers and locations generally distinct from higher metazoa and the phase 2 intron in the proposed *Anx15* tetrad is particularly unusual. The only overlap with vertebrate annexins occurs in strongly conserved repeat 4 where one splice site in *Anx15* and two in *Drosophila Anx10* [18] coincide in phase and location with splice sites of annexin II.

Ten annexin subfamilies that predominate in vertebrates have human representatives and six of these genes have been structurally characterized in several species. Although 5′ coding and untranslated regions remain unique for each subfamily, the intron-exon splicing pattern within the tetrad region is congruent with the annexin II prototype for all subfamilies except annexin VII (fig. 1). This suggests that vertebrate annexins derived from at least two related but distinct progenitors and that most are likely to be mutual duplication products. Human [19, 20] and mouse [21, 22] annexin II thus show greatest sequence divergence from their annexin VII counterparts [23, 24] but have core gene structures virtually identical to the five annexin I genes [25–28], human annexin III [29], human [30, 31], chick [32, 33] and rat [34] annexin V and both halves of the human annexin VI octad [35]. Fragmentary data for human annexin IV [36] and bovine XI [37] do not currently permit their association with either the annexin II or VII archetypes, but for phylogenetic reasons (see later) they and annexin VIII are expected to resemble annexin II. Vertebrate annexin tetrads contain only three symmetrical exons (fig. 1) that are flanked by introns in phase 0 (i.e. between codons) while a fourth is flanked by introns in phase 1 (after the first base position). The alternating phases and differing locations of intron insertions in annexins from separate kingdoms favour the theory of 'late' intron insertion throughout eukaryotic speciation. This would be expected to obviate ancient exon shuffling and negate any correlation between exons and elements of protein function, as seems to be the case for annexins. The phase 2 splice between annexin VI tetrads is especially noteworthy. Intron phase considerations also seriously undermine earlier hypotheses such as the interconversion of exon 5 (114 bp, phase 0/0) and exons $10 + 11$ ($96 + 59$ bp, phases 1/1/0) [19, 25, 4], and the proposed shuffling of individual repeats in different annexins [23].

Annexin gene promoter structure. The regulation of annexin gene expression is virtually unstudied at the molecular level, with the exception of comparative studies on the two pigeon annexin I genes [28] and c-*fos*-mediated induction of annexins II and V [38]. The lack of apparent homology in 5′ coding and untranslated regions between duplicated annexins from different subfamilies (i.e. paralogous genes) implies their creation by homologous recombination, with subsequent conservation limited to the tetrad portion. This has resulted in a broad range of regulatory control mechanisms evident in the divergent tissue expression patterns of various annexins. Annexins II, V and XI are ubiquitous while annexins III, VIII and XIII have more restricted tissue distribution. Annexin I alone shows variable but significant induction or secretion in response to glucocorticoids [3] while annexin VIII expression can be suppressed by retinoids [39]. General features of an-

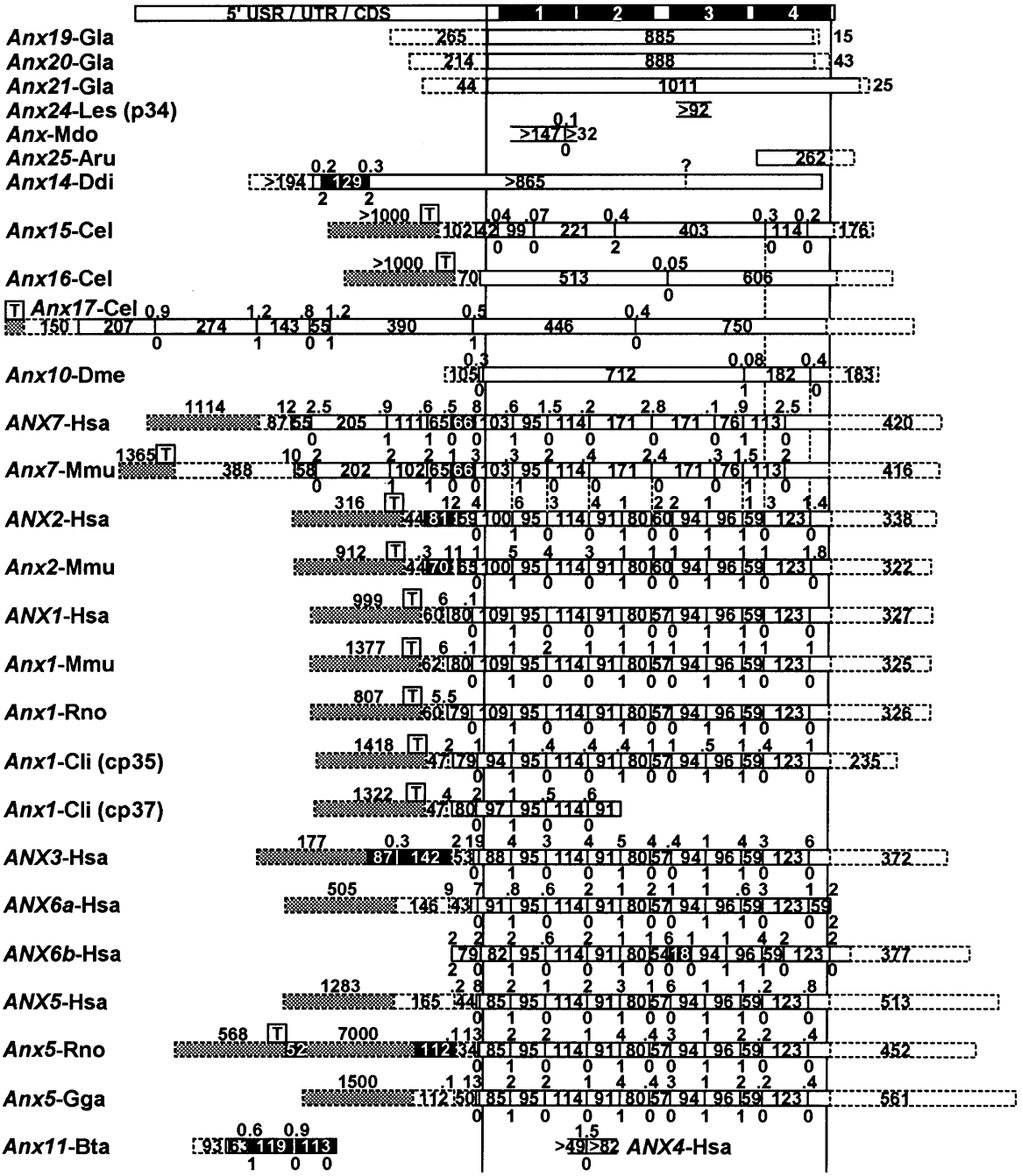

Figure 1. Annexin gene structures. The exon splicing pattern is shown for protein coding and untranslated regions of sequenced genomic DNA. Outlines are displayed in approximate evolutionary order for known annexin genes from protists, plants, *Dictyostelium*, invertebrate and vertebrate animals (see text for genus-species abbreviations). The core tetrad repeat region lies between vertical line boundaries, with coding exons (open boxes), untranslated exons (dashed boxes) and promoter regions (hatched boxes) on the 5′ (left) or 3′ (right) flank. Exons sizes are drawn to scale and specified in bp, while intron sizes in kb and codon phases are shown above and below each splice site, respectively. TATA boxes in certain core promoters (boxed T), alternatively spliced exons (filled boxes) and some exon splice locations coincident with annexin II (vertical dashed lines) are indicated.

nexin promoter regions show significant variation in the presence of TATA box, in overall G + C content and in the use of alternative splicing as a transcription control mechanism. Annexins I and II, mouse (but not human) annexin VII and the *C. elegans* annexins XV, XVI and XVII all possess a TATA box about 31 bp upstream of their putative transcription start sites. Vertebrate annexins II, III, V, VI and VII have promoters rich in G + C content, in marked contrast to annexin I which also contains a unique BC200-Alu element in its enhancer region [40].

The TATA element of annexin II lies in a region extensively rich in G + C content and its composite core promoter may therefore use both TATA and initiator

(Inr) elements. This ensures transcription initiation for important housekeeping functions and may affect alternative splicing of the recently discovered cassette exon 2 in human [20] and mouse [22] annexins II. Annexin V has also recently been found to possess a composite promoter that is split into a distal TATA-containing promoter and a proximal Inr-containing promoter 7 kb downstream [34]. This apparently allows for tissue-specific alternative splicing of its transcript by the TATA-directed promoter and regulated, constitutive expression by the Inr-directed promoter. Alternative exon splicing is a particularly prominent mechanism of transcriptional regulation, not only for annexins II and V as mentioned above, but also for annexins III [29], VI [35], VII [23, 24], XI [37], XIII [41, 42] and XIV [16]. Since alternative splicing may play a key role as molecular switch in the developmental and tissue-specific expression of annexins, its molecular understanding should yield greater insight into the role(s) of annexins at specific stages of cellular differentiation.

Annexin genetics

Chromosomal mapping and synteny. The physical mapping of all human annexin genes has recently been completed and results (fig. 2) available in the Genome DataBase (The Johns Hopkins University, Baltimore, Maryland) cite multiple studies for each locus consensus. Individual studies have determined map positions for *ANX1, ANX2* and its three pseudogenes [43], *ANX3* [44], *ANX4* [36], *ANX5* [45], *ANX6* [46], *ANX7* [23], *ANX8* [47], *ANX11* and *ANX13* [48]. Cytogenetic maps for mouse annexins are available from the Mouse Genome Database (The Jackson Laboratories, Bar Harbor, Maine) and include *Anx1* [25], *Anx2* [21], *Anx4* [49], *Anx5* [5], *Anx6* [46], *Anx7* [24] and *Anx11* [6]. The pattern of gene duplication by which vertebrate annexins have retained structural congruity might lead one to expect some evidence of a pattern in their genomic locations but this is not the case. Although chromosomal loci neighbouring each human annexin frequently exhibit the corresponding linkage or synteny with the orthologous mouse gene, there is no evidence for the creation of paralogous annexins by intrachromosomal tandem duplication in either species. Even the aggregated loci for annexins VII, VIII and XI on human chromosome 10 and mouse chromosome 14 show segregation [6, 48] that diminishes any likelihood of mutual duplication. The only exception is the annexin VI octad in which a Z-DNA motif between the homologous tetrads [50] and the lack of other cross-hybridizing loci [46] point to it being a probable fusion product of tandem duplication.

Genotype-phenotype relationships. There is no proven functional association between annexins and their neighbouring loci, which include many growth factors and their receptors. Possible direct links to diseases such as cystic fibrosis and familial Mediterranean fever have been discounted on a genetic basis. There is also no established genotype-phenotype relationship for any an-

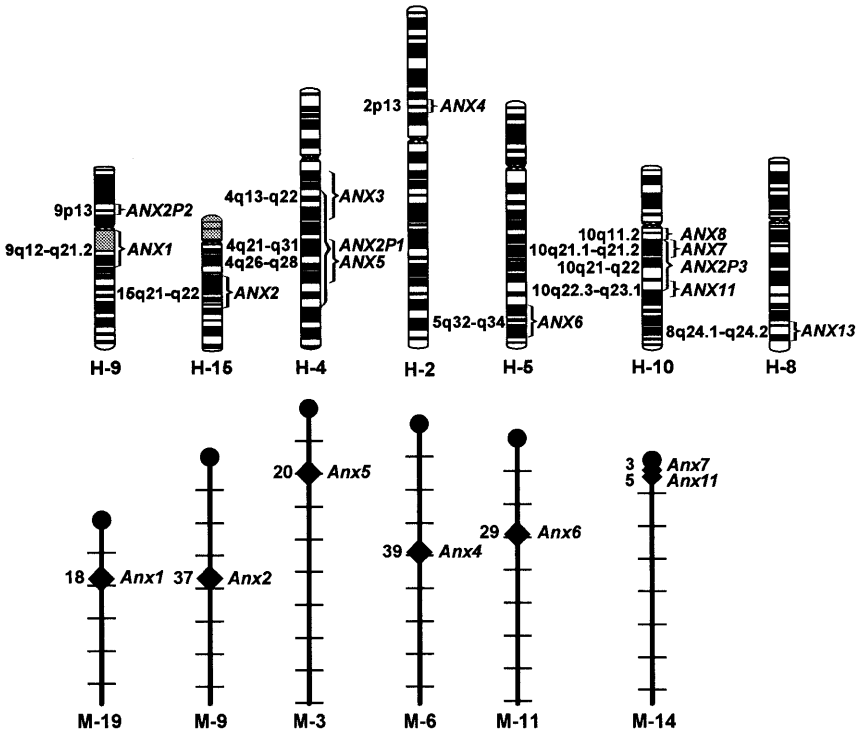

Figure 2. Annexin gene maps. Physical map locations are shown on chromosomal idiograms for all known human annexin genes (above) and cytogenetic map positions are given in centimorgans from the centromere for orthologous mouse loci (below). Compiled data were extracted from the GDB and MGD electronic databases (see text for references).

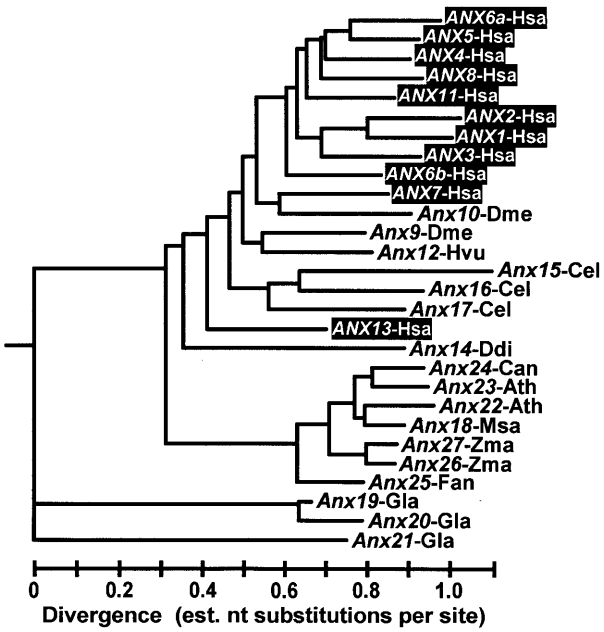

Figure 3. The annexin gene evolutionary tree. Nucleotides in the first and second base codon positions of tetrad cDNA sequences from 27 paralogous annexins were analyzed by DNA maximum likelihood. Branching order was specified by the tree topology derived from bootstrap analysis of protein evolutionary distances [2]. Branch lengths reflect the extent of sequence divergence between taxa and clade formation reflects common heritage. The ancestral root is assumed to antedate the appearance of protists 1200 million years ago and the mutation scale (abscissa) would be proportional to time if constant rates prevailed in all lineages. Human gene names are reverse-shaded.

nexin, although annexin II is overexpressed during cell proliferation [3], annexin V is selectively overexpressed in cystic fibrosis epithelial cells [51] an annexin VI overexpression in heart is associated with changes in ion metabolism and connective tissue [52]. Annexin VIII overexpression is an indirect consequence of the hereditary translocation in acute promyeloyctic leukemia, although it may contribute to the phenotype associated with the retinoic acid receptor alpha defect [39]. A *Dictyostelium Anx14* deletion mutant was observed to have ostensibly normal growth characteristics [53]. The availability of annexin genetic maps should facilitate future efforts in positional cloning and gene knockouts, and they will ultimately be crucial in defining the relationship of annexin gene defects to specific diseases.

Annexin molecular evolution

Subfamily classification. Gene duplications that generate novel subfamilies represent a significant opportunity for adaptation to new organismal needs. Although the subsequent course, rate and extent of divergence may be dependent on random mutation and selection pressures, such events are an important basis for the classification of gene families. The corresponding nomenclature for annexins has been in some disarray with the identification of ever more divergent molecular species because of the inherent difficulty in distinguishing between duplication (paralogous) and species (orthologous) divergence on the basis of pairwise sequence comparisons alone. Where global sequence similarity may be equivocal and other structural or genetic data are unavailable, molecular phylogenetic analysis offers a more computer-intensive but statistically reliable means of determining evolutionary relationships. The application of such techniques has made it possible to categorize annexins across distant phyla into 27 distinct gene subfamilies [1, 2], each comprising at least one (near) full-length representative. Additional subfamilies can already be predicted on the basis of fragmentary sequence data from plants (apple, celery, rice, tomato) and animals (sea urchin, sooty sea hare, blood fluke, nematode) and others, including human, are likely to be identified by database analyses of genome sequencing projects. The confirmation of distant homologies in prokarya and archaea, like that of yeast annexins in eukarya, may ultimately require examination of evolutionarily better conserved three-dimensional protein structures.

Evolutionary tree for annexin genes. Evolutionary relationships between annexin subfamilies can initially be determined through phylogenetic analysis of protein sequence alignments using bootstrap resampling techniques for statistical determination of the correct branching order. Such a tree has been used to describe the relationships between protein tetrads of 27 distinct annexin subfamilies, including ten human representatives [2]. The determination of paralogy was facilitated by the occurrence of multiple proteins in single species and was otherwise evident from the grouping (i.e. clade formation) and bootstrap branching statistics. The topology of this protein tree has been incorporated here into a DNA maximum likelihood analysis involving the first and second bases of each codon in the homologous tetrads (fig. 3). Nucleotide substitutions at these positions are mostly nonsynonymous (i.e. cause amino acid changes) so that branch lengths reflect the extent of divergence by random mutation between these subfamilies. Assuming a constant, average mutation rate of 1% every 7 million years (Myr), these branch lengths should also be roughly proportional to the time period(s) over which these annexins have been diverging (see later).

The branching order in the annexin gene tree (fig. 3) shows earliest separation of the three protist annexins, followed by a cluster of seven plant annexins, the *Dictyostelium* annexin, and subsequent formation of six invertebrate and 11 vertebrate animal subfamilies (counting both tetrads of annexin VI). Some human annexin subfamilies branch with earlier diverging invertebrates (*ANX13* and *ANX7*) while others associate with either *ANX2* or *ANX5* on bifurcating branches of separate clades. The correspondence between annexin subfamilies and the evolutionary order of eukaryotic

kingdoms suggests that monophyletic clades formed from distinct progenitors available at or prior to the successive speciation times. Such a pattern of clade formation implies frequent gene duplication during the course of eukaryotic speciation with fixation of distinct annexins in the genomes of disparate organisms. Selection would depend on the evolutionary status of prevailing progenotes at the time of speciation and on the regulatory and functional suitability of individual genes to each organism. It is clearly important to distinguish separate genes when considering their hereditary or phenotypic expression, and the extent of their divergence as well as clade association should also be regarded as significant determinants for evaluating functional relatedness.

The origin and divergence of human annexins. The biological significance of human annexins can be assessed in earlier diverging species if we are sure that the corresponding orthologous genes represent structurally or functionally equivalent proteins. It is therefore helpful to have at least an approximate idea of the ages and mutation rates of individual human annexins. These estimates have recently been computed [48] by analysis of nucleotide substitutions between annexin tetrads from species pairs with known separation times. A constant mutation rate in different species of annexin II was determined by the 'relative rate test' and sequence divergence between the human and *Xenopus* tetrads was calibrated to their assumed separation time 325 Mya. The estimated results for other annexins (fig. 4) indicated an average age of about 425 Myr (range 250–850 Myr) and a nonsynonymous nucleotide substitution rate in the first two codon positions of 1% every 7 Myr between human and rodent sequences. This compares to an average amino acid replacement rate of 1% every 4 Myr [1, 5] when calibrated to annexin II species, although there is actually a four fold variation in the mutation rates of different human annexins (fig. 4).

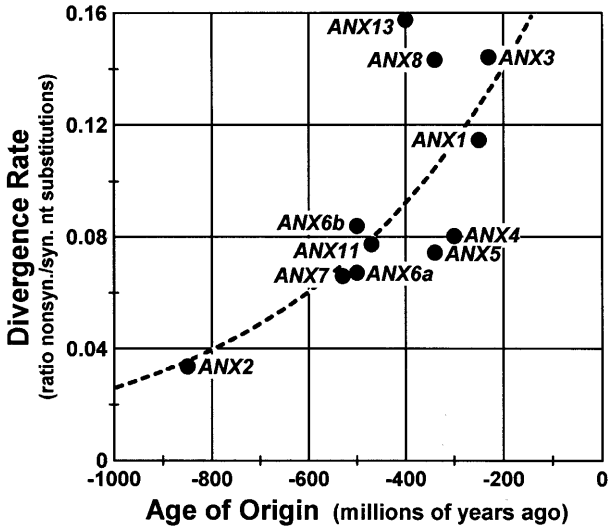

Figure 4. Age and divergence rates of vertebrate annexins. Values for the age in Myr (abscissa) and relative divergence rate (ordinate) of 10 known vertebrate annexins were determined by comparison of nucleotide substitutions between orthologous and paralogous annexin tetrads [48]. Times were calibrated against human and amphibian sequences of annexins II, V and VII and the ratio of nonsynonymous to synonymous nucleotide substitutions between human and rodent sequences was used as a measure of divergence. The exponential regression curve ($r = 0.82$) reflects a logarithmic decline in the mutation rate at nonsynonymous codon positions with respect to gene age.

The corresponding phylogenetic tree (fig. 3) can be used to infer, from the calculated, mutual divergence times of annexin tetrads, the probable order and dates of gene duplications along each lineage. Our own interpretation [48] is that annexins VII and XIII evolved from primitive ancestors closely resembling invertebrate annexins and for this reason their gene structures may both be distinct. The concept of a separate origin for annexin VII has been considered [23], although the timing and mechanism of intron insertion remain to be determined upon identification of its immediate progenitor. Annexin II is probably the oldest known human annexin and most other subfamilies may have been derived by successive gene duplication from structurally related ancestors. We have also hypothesized [48] that the tandem duplication which led to formation of the annexin VI octad about 500 Mya was a key event in annexin evolution because the divergence and subsequent duplication of one of its component tetrads may have initiated a novel clade including annexins V and VIII (fig. 3). The characterization of annexins in early-diverging vertebrates such as fish [54] and amphibians [55] will be especially instructive for accurately dating the origins of older human annexins.

Despite their common origin(s), the molecular evolution of annexins has not been uniform and it is important to remember that conserved residues and protein domains imply an underlying functional basis for their evolutionary selection. Hence, conserved differences between the consensus sequences of protist, plant and animal annexins [2] may signify changes or diversification of structure-function relationships. Similarly, the separate origins and apparent clade formation among human annexins may be an indicator that they are evolving on the basis of distinct, non-redundant functions. The comparatively slow, stable mutation of annexin II indicates significant constraint on changes in structure [7] and its extensive divergence from annexins V and VII (fig. 3) in separate clades may be attended by disparate functions. Differential responses to growth, opposing roles as substrates or inhibitors of protein kinase C, individual protein binding properties, and variations in ion channel activity may characterize such divergence [3]. Two recent cases of specific gene duplication offer interesting examples of how twin genes can acquire novel regulatory properties and diverge by random mutation. The *Columbid* annexin I [27, 28] and *Xenopus* annexin II genes [55] underwent complete duplication

about 20 and 30 Mya respectively. It remains to be determined whether paralogous annexins similarly acquired novel 5′ upstream regions by mutational remodeling or by unequal crossing-over during homologous recombination. Further documentation of novel annexin subfamilies and the sequential ordering of gene duplication events will close gaps in our current knowledge of annexin evolution.

Salient points and future directions

1. Identification of novel annexins from protein, cDNA or gene structures and their classification by phylogenetic analysis will help to characterize the range of structure-function variation within this family.
2. Studies of annexin gene regulation at the molecular level are needed to identify the factors and mechanisms controlling annexin expression and function.
3. The available genetic data on annexins can now be integrated into studies of positional cloning, gene knockouts and hereditary anomalies.
4. Conserved regions in annexin gene or protein structure are most likely to have functional significance, and conserved changes should be regarded as potentially significant indicators of regulatory or functional diversification.
5. Human annexin subfamilies were formed during the vertebrate radiation and are diverging at distinct rates due to differing functional constraints.

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