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Deciphering function and mechanism of calcium-binding proteins from their evolutionary imprints

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Abstract

Calcium-binding proteins regulate ion metabolism and vital signalling pathways in all living organisms. Our aim is to rationalize the molecular basis of their function by studying their evolution using computational biology techniques. Phylogenetic analysis is of primary importance for classifying cognate orthologs; profile hidden Markov models (HMM) of individual subfamilies discern functionally relevant sites by conservation probability analysis; and 3-dimensional structures display the integral protein in context. The major classifications of calcium-binding proteins, viz. EF-hand, C2 and ANX, exhibit structural diversity in their HMM fingerprints at the subfamily level, with functional consequences for protein conformation, exposure of receptor interaction sites and/or binding to membrane phospholipids. Calmodulin, S100 and annexin families were characterized in *Petromyzon marinus* (sea lamprey) to document genome duplication and gene creation events during the key evolutionary transition to primitive vertebrates. Novel annexins from diverse organisms revealed calcium-binding domains with accessory structural features that define their unique molecular fingerprints, protein interactivity and functional specificity. These include the first single-domain, bacterial annexin in *Cytophaga hutchinsonii*, the 21 tetrad annexins from the unicellular protist *Giardia intestinalis*, an ancestor to land plant annexins from the green alga *Ostreococcus lucimarinus*, invertebrate octad annexins and a critical polymorphism in human ANXA7. Receptor docking models supported the hypothesis of a potential interaction between annexin and C2 domains as a propitious mechanism for ensuring membrane translocation during signal transduction.

Keywords: Computational biology; Functional determinant; Hidden Markov model (HMM); Membrane-binding mechanism; Molecular evolution; Receptor docking

1. Introduction

Calcium-binding sites can be defined, in the narrowest sense, by the pattern of carboxyl and carbonyl oxygens in acidic and polar residues that coordinate calcium ions. They may be further classified by their loop geometry as EF-hand (helix–loop–helix), annexin (discontiguous pair of helix–loop–helix) or C2 domain (multiple β-strand). Since this binding is often cooperative with respect to membrane phospholipids and/or local pH, it is important to consider the influence of adjacent residues on bond angle flexibility and other protein properties that influence binding kinetics. The literature on EF-hand motifs and C2 domains, and more recently for annexins, amply demonstrates the essential contribution of the surrounding structural environment, not only to the specificity of calcium-binding itself but to its subsequent coupling with other active domains responsible for conformational changes, membrane binding and ensuing receptor interactions [1–4]. Thus, special residues such as cysteine can add sensitivity to the cellular reduction–oxidation state, hydrophobic or bulky aromatic residues such as tryptophan and phenylalanine perturb protein

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flexibility for deeper membrane or receptor penetration, while basic residues can strengthen protein bonds with anionic phospholipids independent of a calcium bridge.

A biological definition of calcium-binding protein function requires additional knowledge of the interrelationships among all other functional domains in the integral protein and the physiological network role that protein fulfills. This objective still remains elusive for the majority of calcium-binding proteins, but it is becoming increasingly viable and reliable to infer functionality from structural information using algorithms of computational biology, particularly those that are based on evolutionary theory [5–8]. The reason is that the functional adaptation of a protein originates in the natural selection of its structural features, and this progression can be examined by the systematic comparison of its cognate orthologs as structural and functional equivalents in different species. The sequence data available from genome sequencing projects now enable the elaboration of refined, evolutionary imprints or molecular profiles of the conservation and divergence pattern for every protein subfamily and this information can be viewed in realistic context by incorporating it into 3-dimensional structures. Such evolutionary models facilitate inferences about function and mechanism and can be further enhanced by accumulated biological knowledge and a guided imagination.

The fact that calcium-binding domains frequently occur in multimers or heteromers within many protein sequences suggests some level of interactive diversity in their function, and alterations of gross structure or occasional ablation of calcium-binding sites also point to supplementary roles or more intricate mechanisms [9–12]. We therefore sought to compare evolutionary aspects of the highly conserved EF-hand protein calmodulin, the more recent and highly diversified vertebrate S100 family, and the ancient, unique superfamily of annexins, with the aims of tracing their distinctive origins and the divergence profiles of their calcium-binding sites. The characterization of new homologs in each group provides insight into the varied mechanisms by which calcium binding and signal transduction are controlled [13–15]. These include the introduction into calcium-binding domains of other residues (esp. Cys, Trp and Lys) capable of modifying calcium- and phospholipid-binding kinetics, rearranging domain architecture, and fostering cross-interactions like annexins with S100 and C2 domains.

2. Materials and methods

Database searches identified potential homologs of calmodulin, S100 and annexin by BLAST (esp. PSI-BLAST) and FASTA sequence comparison of authentic members against protein, cDNA transcript and genomic trace databases at the National Center for Biotechnology Information, USA (http://www.ncbi.nlm.nih.gov/). Protein sequences were retrieved from UniProt (http://www.ebi.ac.uk/uniprot/index.html) and the PFAM database of protein families (http://www.sanger.ac.uk/Software/Pfam/). Original genome sequence data for *Petromyzon marinus* (sea lamprey) originated from Washington University, USA (http://genome.wustl.edu/). Genome sequence data for the bacterium *Cytophaga hutchinsonii*, the green algae *Ostreococcus lucimarinus*, and the annelid worm *Capitella capitata* were from the DOE Joint Genome Institute (http://www.jgi.doe.gov/). The Marine Biological Laboratory (Woods Hole, MA, USA.) was responsible for the *Giardia intestinalis* genome project (http://gmod.mbl.edu/).

Progressively refined sequence alignments of cognate homologs were subjected to phylogenetic analysis to define their evolutionary relationships, transformed into profile hidden Markov models to create sequence logo signatures, and mapped into 3-dimensional structures to reveal site-specific patterns of conservation and divergence for each subfamily. Such representations focus attention on the evolutionarily (i.e. functionally) important sites and reveal how key structural features interact in a physical context. Phylogenetic analysis by MEGA [16] performed neighbor-joining analysis on >1000 bootstrapped alignments and results were confirmed by maximum likelihood or Bayesian analysis of selected clades. Molecular profiles of individual subfamilies were created as hidden Markov models (HMMs) by HMMER [17] and visualized with the sequence Logo-Mat server (http://logos.molgen.mpg.de/). Gene structures and chromosomal linkage maps were deduced by visual inspection and the integration of contig data based on existing models [7,15,18]. Site-specific conservation of multiple alignments and sequence threading into 3D models utilized the CONSURF server [19] and protein structure modelling of evolutionary information employed HHPRED and MODELLER [20,21] for the bacterial annexin, SWISS-MODEL DEEPVIEW [22] for template-based protein models, and MOLMOL for molecular presentation (http://www.mol.biol.ethz.ch/wuthrich/software/molmol/). Molecular docking models of annexins with C2 domains used the CLUSPRO server [23].

3. Results and discussion

3.1. Origin and evolution of EF-hand motifs

A survey of some 15,000 recognized EF-hand motifs in PFAM confirmed their presence in the extracellular protein milieu of bacteria and intracellularly in hundreds of eukaryotic protein families, where a significant proportion have acquired conformational sensitivity to calcium and varied modular architecture for participation in calcium signalling mechanisms [5]. The typical EF-hand structure consists of a 12-residue loop flanked by α-helical domains, in which Asp side-chain oxygens of loop residues 1, 3 and 5, other atoms at positions 7 and 9, and a side-chain oxygen from Glu in position 12 participate in calcium coordination. Its representation as a hidden Markov model (HMM) sequence logo (Fig. 1) also reveals a conserved, central Gly and flanking Phe residues that further define what is regarded as a particularly successful variation of the more generalized DxDxDG motif [24]. The latter have been observed in distinct contexts, including the excalibur and thrombospondin domains where vicinal cysteines contribute structural support for calcium ligand presentation. The classical example of an EF-hand protein is the highly conserved calmodulin (Fig. 1), which contrasts with pseudoEF-hand domains of the S100 protein family. The molecular profiles and context of each domain, its response to calcium and target recognition are important determinants of the functional selectivity for diverse EF-hand proteins such as calmodulin and the S100 family [2,12].

Calmodulin presents certain challenges for evolutionary study, because of its uncertain origin in primitive eukaryotes, the strong functional constraint on protein structure conservation, and the divergence in regulatory control of gene expression at 3 distinct loci (encoding 3 identical proteins!) in mammalian genomes [25]. The extreme conservation level of calmodulin

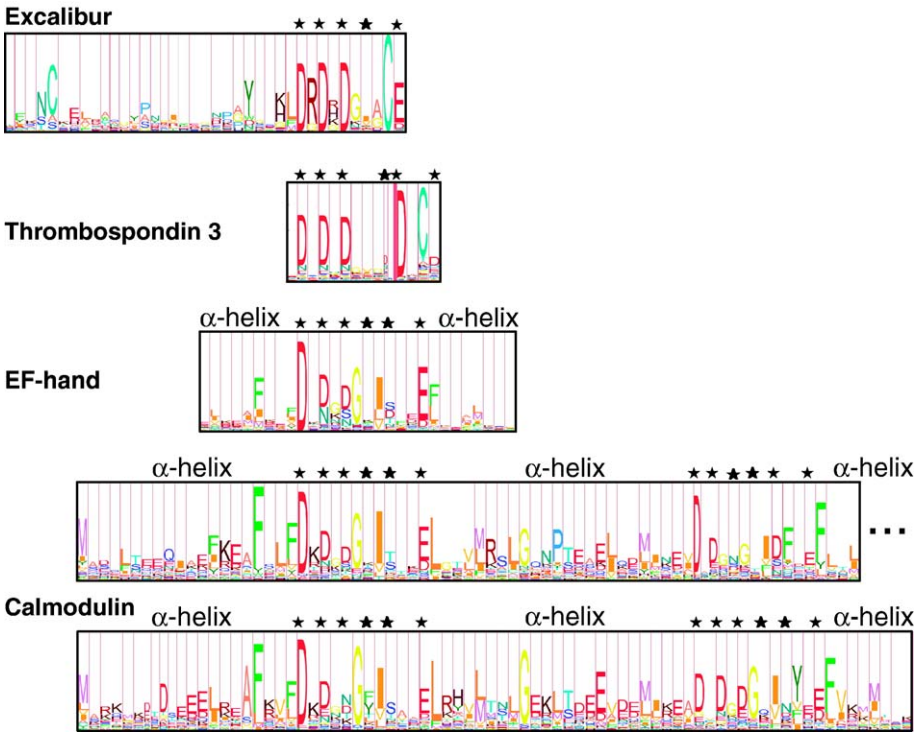

Fig. 1. Profile hidden Markov models (pHMM) of the canonical EF-hand motif (center panel), variants in excalibur and thrombospondin 3 (upper panels) and distribution in the calmodulin protein family (bottom panels). Amino acid sequence alignments were compiled for EF-hand motifs from 1000 diverse proteins, excalibur (38 aa in 10 bacterial proteins), thrombospondin 3 (15 aa in 44 eukaryotic proteins) and full-length calmodulin (149 aa in 300 proteins). Analysis by HMMER and visualization as sequence logos by LOGO-MAT show residue frequencies by their relative height and the site-specific probabilities as total column height, reflecting informative value with respect to conservation and function. Acidic residues implicated in calcium ion coordination are starred (open where poorly conserved) and the prominent conservation of cysteines in excalibur/thrombospondin or methionines and phenylalanines in calmodulin affirm their accessory functional contribution to calcium-binding kinetics, conformational change and hydrophobic receptor interaction.

(especially in vertebrates) required an extensive alignment of 300 full-length eukaryotic proteins to develop a statistical HMM profile that identified the (dis)similarity in its 4 EF-hand motifs and the strategic conservation of Met and Phe residues secondarily involved in the conformational response and hydrophobic target recognition (Fig. 1). It should be noted that more selective profiles of specific subfamilies or functionally related clades can reveal additional features, such as the replacement by Cys at position 27, Leu-72, Leu-86 and Gln-97 in plants, Phe-100 and Lys-144 also common to invertebrates, or Ser-148 unique to invertebrate calmodulins [25]. Nucleotide HMM profiles of noncoding regions, phylogenetic footprints of promoter regions, and genetic linkage maps of calmodulins can effectively identify conserved DNA regulatory elements to distinguish orthologs from paralogs.

HMM statistical representations of sequence conservation are especially informative about functionally important DNA elements and protein residues when confined to individual subfamilies. It is frequently meaningful to view this information in the context of a 3-dimensional model to identify discontiguous segments that interact physically or function cooperatively. The protein sequence alignment used to create the profile HMM was subjected to phylogenetic analysis and amino acid (aa) conservation analysis for incorporation of this information into compatible 3D structures available in Protein DataBank. CONSURF3 was used to create such a functional map of the calmodulin protein structure (Fig. 2), based on site-specific aa conservation variation in 300 calmodulin proteins, using two different conformational models in the absence (pdb:1cfd) and presence of calcium (pdb:1cll). The dynamic, functional role of individual aa have been clarified by experimental models of calmodulin [26], but such evolutionary profiles can offer both insight and corroborative evidence about 3D structure–function relationships in lesser characterized protein families.

3.2. Sea lamprey as a model organism for vertebrate evolution

The emergence of vertebrates from invertebrate stock about 500–600 million years ago has been associated with successive whole genome duplications [27]. We were interested in comparing the gene duplication patterns for calmodulin as a vital regulatory gene, S100 proteins that originated in vertebrates, and annexins that expanded into a distinct vertebrate family. We focused on genome analysis of *Petromyzon marinus* (sea lamprey), a primitive, jawless vertebrate that may represent an intermediate state, having many duplicated genes compared to invertebrates and suggestive of at least one whole genome duplication. The retrieval and assembly of whole genome shotgun traces with high-scoring matches to calmodulin or S100 authentic sequences led to the reconstruction of 3 genes in each family (Fig. 3), apparently

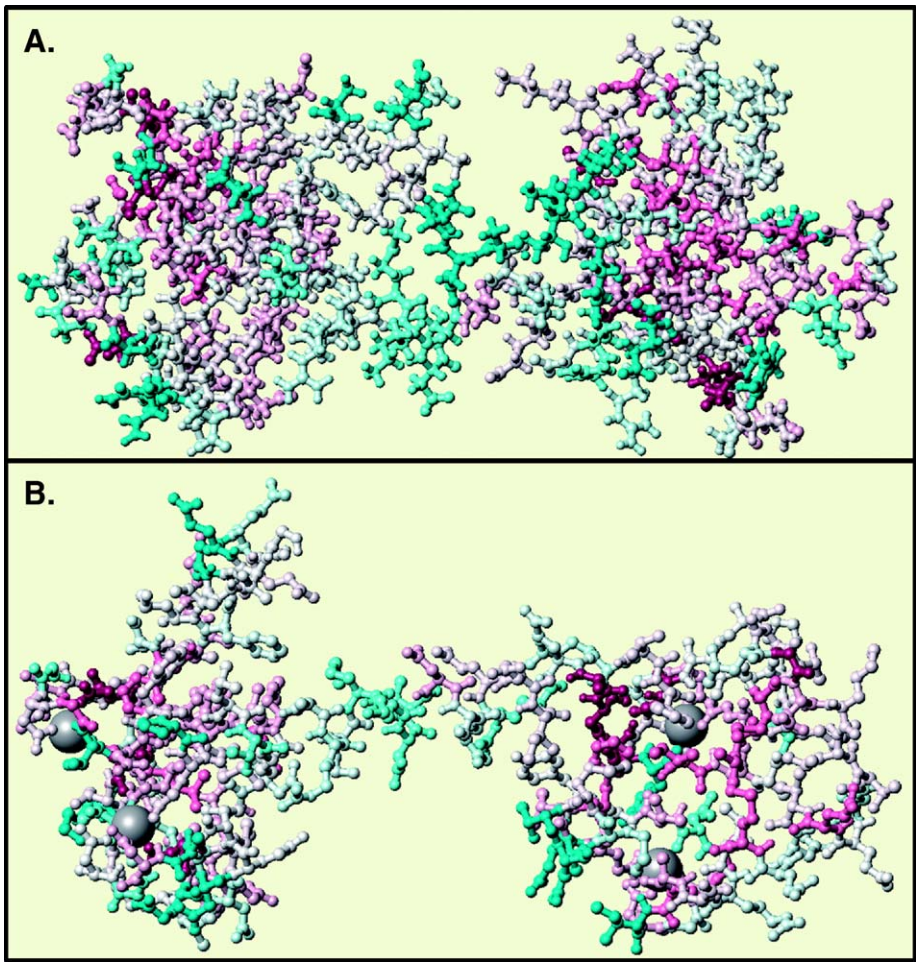

Fig. 2. Structural representation of amino acid evolutionary conservation in calmodulin. Different conformational models in the absence of calcium (pdb:1cfd top) and presence (pdb:1cll bottom) were used to depict the spatial arrangement of conserved (burgundy) and variable (blue) atoms and bonds in calmodulin structures, computed from the sequence alignment by the CONSURF server and modelled by MolMol. Conserved residues directly associated with calcium ion coordination are supplemented by others with accessory roles in maintaining conformational flexibility and hydrophobic receptor interactions (see text).

representing the complete repertoires, given the intermediate stage of genome sequencing. Two of the calmodulins had 99% aa identity with each other and 85% nucleotide identity in coding regions, with 98% aa identity to human calmodulin and identical gene structure with 6 coding exons. A third lamprey calmodulin with 95% aa identity to the others was represented by a single expressed sequence tag without genomic confirmation, but with sequence characteristics (e.g. Phe-100, Lys-144, Ser-148) resembling invertebrate calmodulins [25].

Genomic trace assembly of lamprey annexin sequences has reached a gene number of 12, equivalent to that of mammals, but this so far appears to represent half the expected number of gene families present in duplicate copy. Thus, both the founding member annexins A13, A7 and A11 and annexins A1 through A4 seem to be present as paralogous duplicates, while annexins A5, A6, A8, A9 and A10 remain to be detected. This is interpreted to suggest that not all vertebrate annexin subfamilies may have been created at the divergence time of jawless fish from teleost–tetrapod lineage, and that the lamprey genome itself may have suffered a unique tetraploidization event that duplicated an incomplete set of annexin genes, including annexin A7 (Fig. 3), which exists as a single gene in cartilaginous fish and is absent from all teleost fish genomes. Further comparative genome studies will be required to determine the model organisms most suitable for documenting gene and genome expansion in formative vertebrates.

The three S100 proteins deduced from lamprey genomic traces shared approximately 42% aa pairwise identity between them indicating they are unlikely to be recent duplication products within the lamprey lineage. Phylogenetic analysis (Fig. 4) and HMM models provided clear statistical identification for the known S100 subfamilies and just one of the lamprey proteins was thereby confirmed to be a true S100P ortholog. A second lamprey S100 branched from the base of the S100A2–A6 clade and may therefore have originated from their common ancestor prior to its amplification in higher vertebrates. The third lamprey protein was weakly associated with the S100A10–A11 pair. The pending completion and genome assembly for lamprey and direct comparison with more teleost fish S100 subfamilies should resolve the true genetic relationships of these S100 loci with their mammalian homologs.

CALM_Pma1 (149 aa)
MADQLTEEQI AEFKEAFSLF DKDGDGTITT KELGTVMRSL GQNPTEAELQ
DMINEVDADG NGTIDFPEFL TMMARKMKDT DSEEEIREAF RVFDKDGNGY
ISAAELRHVM TNLGEKLTDE EVDEMIREAD IDGDGQVNYE EFVQMMTAK

CALM_Pma2 (149 aa)
MADQLTEEQI AEFKEAFSLF DKDGDGTITV KELGTVMRSL GQNPTEAELQ
DMINEVDADG NGTIDFPEFL TMMARKMKDT DSEEEIREAF RVFDKDGNGY
ISAAELRHVM TNLGEKLTDE EVDEMIREAD IDGDGQVNYE EFVQMMTAK

CALM_Pma3 (149 aa)
MADQLTEEQI AEFKEAFSLF DKDGDGTITT KELGTVMRSL GQNPTEAELQ
DMINEVDADG NGTIDFPEFL TMMARKMKDT DSEEEILEAF KVFDKDGNGF
ISAAELRHIM TNLGEKLTDE EVDEMIREAD IDGDGQINYE EFVKMMMSK

ANXA7a_Pma (>497 aa)
... PGAGFGAPP SGYPTGYPGA
PGQPSIQNYG GGGGGGGGGA PPGPGPTQPG MPGAPGAYPG PAPPAQPTPS
PAPVPQPNPY PAPAPAPAPA PAPYQPTPPI GGMAGLSLGP QGTVKPIGNF

NVQDDCEMLR KAMKGLGTNE QVLMDLVVNR SNAQRQKIKL TFKTMYGKDL
IRDLRSELSG NFEEIMLALF MPTTYYDAYS LQKAIQGAGT NEKVLVEILC
SRTNQEIRDI VSQYKDEFGR DLEKDIKSDT SGHFKRLLIS MCQGNRDESL
SVDLALALSD AQKLQSAGEG KLGTDESAFN MILAVRSFPQ LQATFQEYIK
LSQRDIINTI DREFSGNVKD GLKAIVQCAK NRPVFFAERL YEAMKGGGTD
DSTLIRIVTS RSEIDLVNVK HAFLEKYNKT LYKMIEGDTS GDYKRMLQAI
VGQN

S100_Pma1 (S100P, 102 aa))
MSVSNLEQVM QDLISVFHQY AGKEGNKYTL SKHELKDLVS HELAGFLKGK
KDPTTVDKLL KDLDADGDGE LDFSEFAAMV ASFTIACNVY FEDYLKTQAA
AK

S100_Pma2 (106 aa)
MSDKYVPTGL ELTIASMMGI FKAYAGKDGD SSRLSNAELK CLLTKELPGY
LQNANDSAAI EKEMKNLDED GDGQVDFKEF IIFIAALTAA CYDAIEAQAS
KSPVKK

S100_Pma3 (115 aa)
MSKLECAMEQ LIKLFHSYAR EDNDKYTLSK AELKKLITAE LPTLCPQGAQ
KVDSLDKTMK DLDTNGDQQL DFQEFVIMIA GLAINCNDFF VGHQDPPKVK
PRAKSSRPRR SRLRG

ANXA7b_Pma (512 aa) MSEPSAPP
GYYGTNQPGY PPPYQPYPNP GQGPPGGYPP SYGGQPGGYP PSGGYPPSGG
YPPAGGYPPP GGQPAGGYPG ENPPPNASQP QRGGTAPPPF MMPTIPLHPS
FGGVGPSPGG PGPAAGGFGG GYSAPPGGPG YQAAPQPGPQ GGMYQNPPTS
YAPSAPPTQQ PSGASYAQPA YQQSHSTPAQ VSYPSAGGVA RGTIHTASNF

NAQDDAEVLR RAMKGLGTDE RALIDIIVNR SNDQRQKIKL AFKTMYGKDL
IRDLRSELSG NFEEIILALF MPTTYYDAYS LMKAIKGAGT DEKVLIEIMC
TRTNQEIKEI VRVYREEFNR TLEKDIRSDT SGHFKRLLIS MCQGNRDESQ
TVDMSKAQSD AQRLYTAGEG KLGTDESTFN MILACRSFPQ LQATFNEYTR
MAQRDIVNTI DREFSGDIRD GMRAIVQCVR SRPAYFAERL FHSMKGLGTD
DSGLIRVIVS RSEIDLQDIK QAFLQQYQKT LYTMIQGDTS GDYKRILVAI
VGMN

Fig. 3. Novel sequences of calcium-binding proteins from sea lamprey. Sequence searches and concatenation of contigs from the genome of *Petromyzon marinus* permitted the reconstruction of gene organization and prediction coding regions for putative lamprey calmodulins, S100 proteins and annexins. Calmodulins designated Pma1 and Pma2 were derived from complete gene structures with splicing patterns identical to their vertebrate homologs, whereas Pma3 derived from the dual read of a single expressed sequence tag (GenBank accession no. EB717185) without genomic evidence and included Phe-100, Lys-144 and Ser-148 characteristic of invertebrate calmodulins (reverse highlight). The three S100 sequences represent the apparently complete and earliest known gene family repertoire in this primitive vertebrate. The detection of paralogous copies of annexin A7 in lamprey suggests that this lineage (Agnathans) may have suffered ancient genome tetraploidy to cause a doubling of its half complement of mammalian annexin subfamilies. This is consistent with the presence of a single ANXA7 copy (with characteristic Trp and Cys in reverse highlight) in later-diverging cartilaginous fishes and the subsequent silencing of this gene altogether in bony fishes (see text).

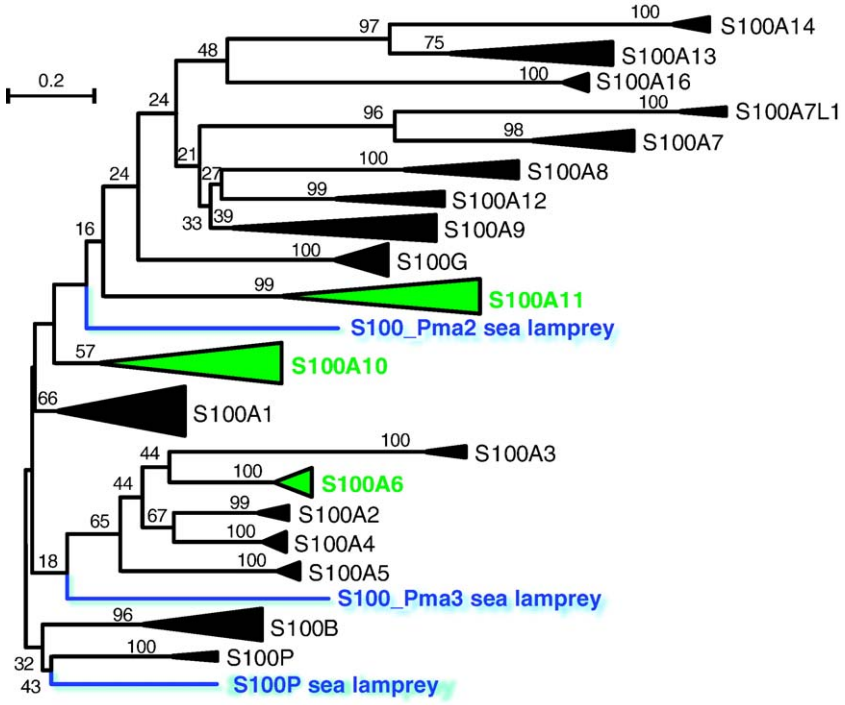

Fig. 4. Phylogeny of sea lamprey S100 proteins. Three novel lamprey S100 proteins were subjected to neighbor-joining analysis of 1000 bootstrap alignments in MEGA together with 150 representatives from known subfamilies to determine their evolutionary relationships. The fanning of branch tips reflects the number of orthologs pertaining to each subfamily and numbers at bifurcations give the branch bootstrap support. S100_Pma1 was thus confirmed to pertain to the S100P subfamily, while Pma2 and Pma3 did not strongly associate with any particular clade for formal classification. Orthologous representatives ($n=12$) determined to belong to each of the S100A6, S100A10 and S100A11 subfamilies of annexin partners were selected for more detailed pHMM analysis.

3.3. S100 subfamily profile HMMs and 3D evolutionary models

Distinctions evident in the profile HMMs of individual S100 subfamilies, such as those involved in annexin binding (Fig. 5), make it imperative to refine all subfamily models with additional representatives from fish, amphibians, reptiles and birds to enable proper classification of more ancient homologs from jawless fishes (lamprey, hagfish) and cartilaginous fishes (sharks, skates and rays). This may also reveal distinctive patterns and time courses of change in the EF-hand modules that have diverged into the separate S100 subfamilies [12,28]. Thus, the S100A10 binding partner of annexin A2 has lost its capacity to bind calcium, along with the displacement of acidic Asp residues and the incorporation of a C-terminal Cys, in common with S100A11. Such subfamily models represent powerful bioinformatic tools to confirm new and more distant homologs, especially after refinement by the continuing addition of confirmed members.

One limitation of sequence profile HMMs is that separate structural domains may not be recognized as participating in the same functional module unless important residues identified by their evolutionary conservation are placed in more suitable 3-dimensional context. To this end, multiple alignments of individual S100 subfamilies were analyzed by CONSURF for phylogenetic relationships and site-specific conservation levels to create functional maps of their key residues in homologous crystal structures (Fig. 6). The extent of sequence conservation in S100A10 is less apparent compared to S100A6 or S100A11, possibly owing to its loss of calcium sensitivity. The specific residue interactions between S100 proteins and their annexin partners can also be studied in detail with molecular modelling programs, and inferences can be made with greater confidence in light of the observed pattern of evolutionary conservation for specific sites in both molecules.

3.4. Structural variations in novel annexins

The discovery of novel and unusual annexins has contributed perspective on what these proteins look like and how they may act [7]. It also offers insight into how nature has adapted their forms and mechanisms of interaction with other cellular components to accomplish what may ultimately be a diverse array of functional purposes. Amidst the complexity of deciphering annexin function in organisms such as humans, which possess up to 12 functionally distinct subfamilies [4,6], it is even more bewildering to realize that the primitive unicellular protist, *Giardia intestinalis*, has up to 21 annexins with divergent structures, varied expression patterns and distinct subcellular locations [29]. We constructed a profile HMM of this entire family (Fig. 7B) to facilitate comparison with the family profile obtained for vertebrate annexins [6]. It revealed a general replacement of the GxGTde...D/E motif in the canonical annexin calcium-binding domain with conserved Asp, most conspicuously in the A–B, B–C and D–E interhelical loop regions. Conserved Trp were located near the end of each repeat domain and in mid-repeats 2 and 3, Cys is common in mid-repeat 4, and a "KGD" motif is conserved in most subfamilies in the A–B calcium-binding loop of repeat 4. Given the predilection of this intestinal parasite for membrane interaction [29] we suggest that the KGD motif and other aspartates, the aromatic, hydrophobic residues and cysteine all foster membrane binding by variant combinations of these same components that are strategically and pervasively conserved in all other annexin families.

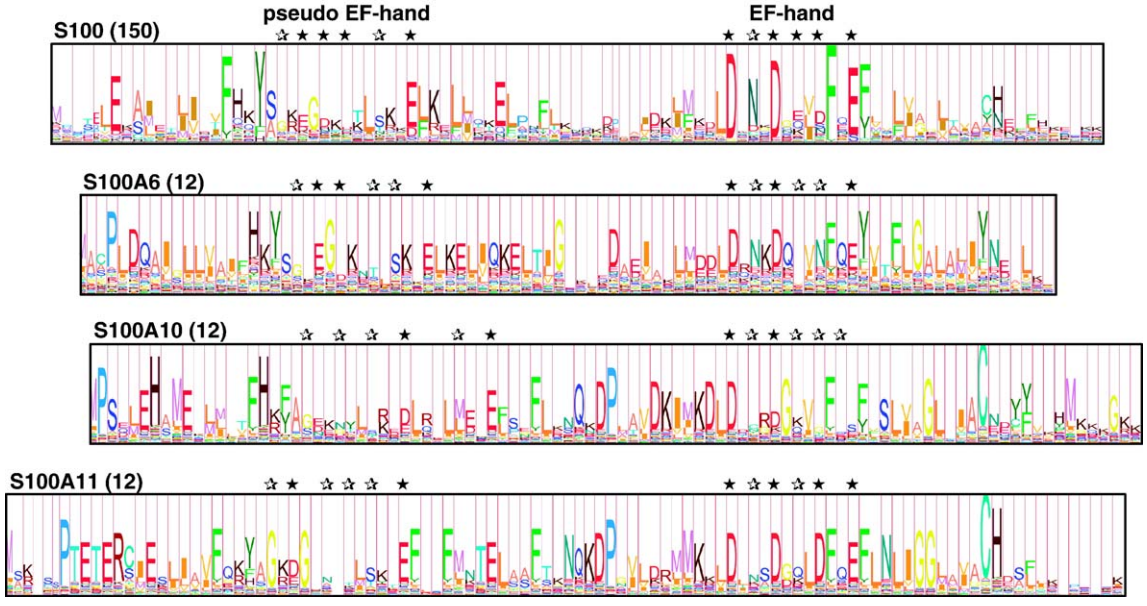

Fig. 5. Profile hidden Markov model sequence logos for S100 family proteins. A general family profile for S100 was obtained by HMMER analysis of an alignment of 150 confirmed members taken randomly from the UniProt database. It depicts the layout of pseudoEF-hand (left) and authentic EF-hand motifs, aligned at the typically conserved glutamate (rightmost starred "E"). Subsets of (12) mammalian representatives were similarly analyzed to create individual subfamily profiles for S100A6, S100A10 and S100A11. These subfamily profiles can be seen to differ in the integrity, spatial arrangement and surrounding environment of the calcium-coordinating residues (starred) with consequent loss of calcium-binding activity in S100A10 members but with an open-fixed conformation conducive to annexin A2 binding via hydrophobic residues.

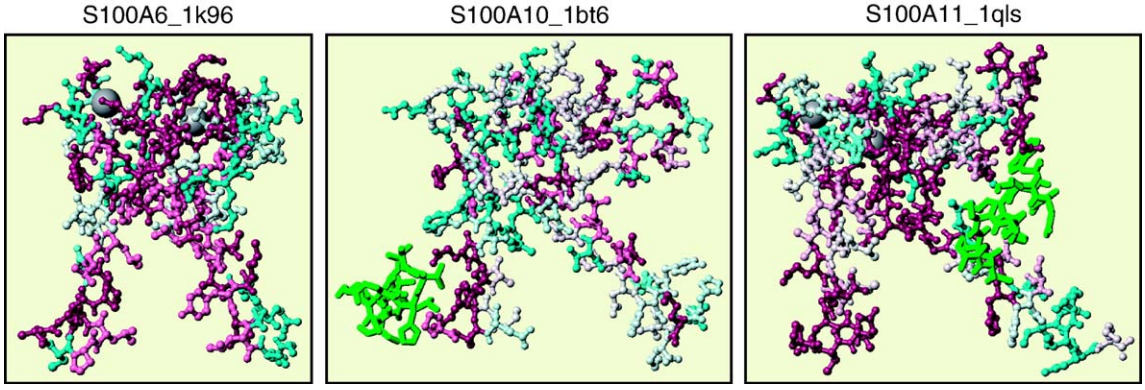

Fig. 6. Evolutionary models of protein structures for S100 subfamily groups. The coloring scheme signifies conservation levels from high (burgundy) to low (cyan) predicted by CONSURF from protein alignments for each subfamily and incorporated into appropriate structural models. The templates for the latter were calcium-bound human S100A6 (pdb:1k96), calcium-free S100A10 bound to annexin A2 terminal peptide (green bonds) (pdb:1bt6), and calcium-bound S100A11 in association with annexin A1 terminal peptide (pdb:1qls). Both the pattern of amino acid conservation (reflected in Fig. 5) and the spatial conformation can be observed to be distinct for each subfamily, with relatively lower primary structure conservation in S100A10.

The emergence of the intact annexin tetrad in *Giardia*, albeit with variant profile, leaves one to ponder whether prokaryotes may have originated an annexin domain, what it looks like and how it functions. Alas, eureka (Fig. 7A). Its sequence (GenBank accession no. CP000383.1, hypothetical protein id ABG58745.1) was detected by pHMM search of the *Cytophaga hutchinsonii* genome (sequenced by DOE-JGI) and its secondary and tertiary structures were modelled by HHPRED and MODELLER [20,21]. The model confirms a 5 α-helical structure with the annexin calcium-binding motif "wfGwGTne...35 residues...EDE" interspersed with bulky, hydrophobic residues that protrude from the primary A–B loop (Fig. 7A). The latter have been shown to be involved in annexin membrane integration in response to calcium for other annexins [30]. The amino-terminal half of the 147 aa predicted open reading frame did not match known domains (e.g. enzyme) in PROSITE or other databases so the biological role of this single-domain annexin in bacteria is unknown.

A clue to the evolutionary origin of the 28-member, monophyletic family of land plant annexins comes from the discovery of an annexin in the unicellular green algae *Ostreococcus lucimarinus* (Fig. 7C), encoded by intronless gDNA at bp 545763 in distal chromosome 11 of the DOE-JGI assembly. This 538 aa protein with extended amino- and carboxy-termini contains 3 intact annexin domains complemented by an HGD motif at the amino-terminal junction, a "WkvRGD motif in the calcium-binding D–E loop of repeat 2, flanking Trp and Cys residues in the repeat 4 A–B calcium-binding loop, and another RGD motif in the D–E loop. These embellishments to the basic annexin domains represent a unique mosaic of features now documented in all known annexin families with a potential contributory role in calcium- and membrane-binding kinetics.

Invertebrate annexins exhibit a complex evolutionary history, with variant gene structures, lineage-specific replication of founder annexins in individual phyla, and a broad range of unique protein structures. The characterization of an amphioxus annexin featuring ablation of all calcium-binding sites, incorporation of RGD motifs and an ANXA13-like gene structure [7] was recently determined by comparison with EST transcripts (GenBank accession no. BW887816) to be a single, octad annexin rather than a tandem pair of tetrads (Fig. 7D). Another rare example of an octad annexin equally distinct from vertebrate annexin A6 was found in the annelid worm *Capitella capitata* (Fig. 7E). Although it has intact annexin calcium-binding domains, it incorporates 2 KGD motifs into an ANXA11-like gene structure, and its inter-tetrad linker region (e.g. NCBI trace name BHAZ408981.y2, gi no. 1131458927) has an unusually high content of acidic Glu residues. The at least 9-member annexin gene family of *Capitella* is the first invertebrate to reveal all of the vertebrate-like annexin gene splicing patterns, and the ongoing genome sequencing of its thermophilic cousin the "Pompei worm" and its psychrophilic neighbor the "Glacier worm" are expected to provide intriguing new data about the thermal stability and conformational contortions of calcium-binding proteins.

3.5. Genotype-phenotype detection

Recent advances by the HapMap project (http://www.hapmap.org/index.html) in confirming single nucleotide polymorphisms (SNPs) in human genomes promise to elucidate the molecular bases for many phenotypic variations and disease-causing genes. The functional significance of individual SNPs requires both medical evaluation and molecular rationalization of the consequences for gene regulation and protein function. Our survey of these findings included one particularly interesting example of a nonsynonymous SNP in the penultimate exon of human annexin A7 (codon cGg change to cAg), expected to convert a critical Arg-419 residue to Gln (Fig. 8). A comparable change in human annexin A5 was reported to disrupt a key salt bridge with repeat 2 resulting in loss of the voltage-dependent channel gating and calcium ion selectivity [31]. The predicted allelic frequency of 11% and homozygous A/A genotype of 2% are restricted to the Japanese population and have no known phenotypic manifestation, but clearly merit further investigation. The model in Fig. 8 helps to visualize this and other unique features of the ANXA7 subfamily (i.e. vicinal

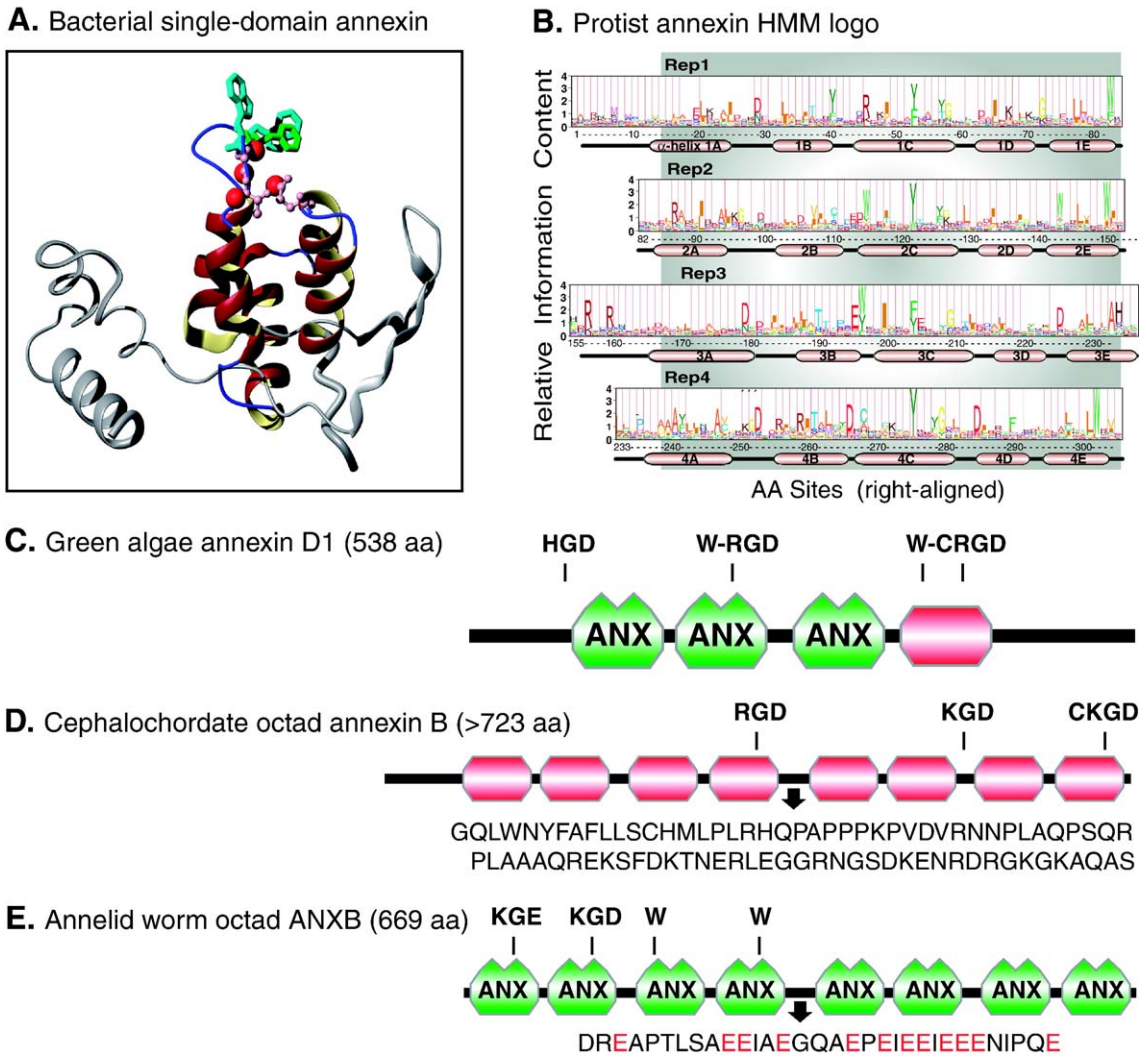

Fig. 7. Novel annexins in primitive organisms contain structural variants common to mammalian subfamilies. (A) The first bacterial, single-domain annexin was identified by pHMM search of the *Cytophaga hutchinsonii* genome sequence. The secondary structure and 3D model were obtained with HHPRED and MODELLER by alignment with homologs and template threading with plant annexins; the results compared favorably with known annexin structures using the DALI server at EBI, UK (http://www.ebi.ac.uk/dali/index.html). The model confirms the five α-helical character of the C-terminal annexin domain (brown colors) with oxygen atoms (red spheres) of a canonical annexin motif (GxGTne…36 residues…E) in discontiguous A–B and D–E inter-loop regions. Two Trp (blue) and one Phe (green) residues are juxtaposed to the putative calcium-binding site. The amino-terminal half had no recognizable sequence homology to known database proteins. (B) pHMM model logo of 21 annexins from the unicellular protist *Giardia intestinalis*. The analysis by HMMER of the protein alignment reveals a general absence of canonical annexin calcium-binding sites but rather highlights the presence of conserved Asp (red) in the vicinity, Trp between repeats and in mid-repeats 2 and 3, and a well-conserved "KGD" motif (starred) in the primary A–B loop of repeat 4. (C) The first annexin from unicellular green algae *Ostreococcus lucimarinus* contains a mutated calcium-binding site in repeat 4 and RGD motifs, Trp and Cys residues in the loops normally harboring calcium-binding sites. (D) Octad annexin from the amphioxus *Branchiostoma floridae* and (E) the annelid worm *Capitella capitatus* are predicted to contain K/RGD motifs, Trp and Cys residues in their calcium-binding loops and the distinctive inter-tetrad linker segments shown.

Trp and Cys residues) that may bear on any ion transport function of this gene [8] and the pathophysiological consequences of these differences with respect to other annexins.

3.6. Annexin docking to C2 domains

We have repeatedly observed the presence of K/R/H–G–D motifs in the calcium-binding loops of annexin subfamilies from all phyla. The simplest interpretation was that the introduction of a basic (usu. Lys) residue might contribute ionic bond strength to anionic membrane phospholipids, but the stable conservation of adjacent GD residues suggested a more complex function. We surmised that the RGD and KGD motifs represent well-known ligands for external transmembrane integrin receptors and recognized that annexins A1 and A5 harboring these motifs have frequently been observed to bind integrins. Apart from the experimental challenge of investigating annexin binding to integrin heterodimers in vitro, and the presumably extracellular nature of this interaction, we were puzzled by the common presence of KGD ligands in annexins of plants, which are reputed not to possess integrins. Recently, an original study began to resolve the mystery of plant RGD/KGE receptor proteins by finding that an RGD in the aspartate proteinase cardosin A bound specifically to the C2 calcium-binding domain

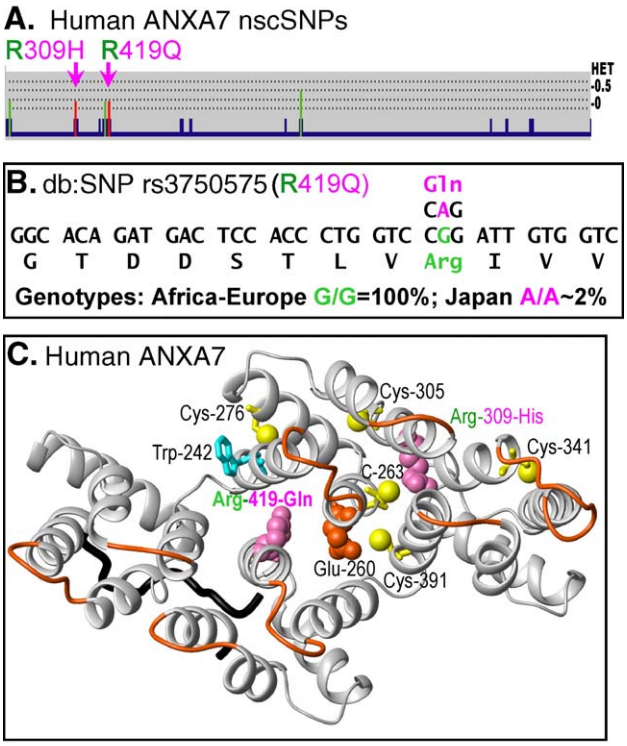

Fig. 8. Unique molecular features of annexin A7. (A) pHMM analysis has previously highlighted the unique conservation of Trp-242, and Cys-276, 305 and 341 within the annexin A7 subfamily [8] and studies of phylogeographic polymorphisms by the human HapMap project have confirmed both synonymous polymorphisms (green bars) and two nonsynonymous coding SNPs (red bars) within the gene region. (B) One of the latter allelic population differences, representing a homozygous genotype frequency of about 2% in Japan, is predicted to change a highly conserved Arg-419-Gln (dbSNP: rs3750575). (C) The strategic location of this polymorphism in the central region of the putative calcium channel selectivity filter of the annexin A7 core tetrad is shown in this structural model by the spherical atoms of Arg-419-Gln (pink) adjacent to Glu-260 (orange-red spheres) with which it normally forms a salt bridge. The other SNP R309H is also displayed as pink spheres, while 5 cysteine sulfur atoms (yellow spheres) are either unique to ANXA7 (Cys-276, Cys-305, Cys-341) or part of a common annexin disulfide bridge (Cys-263, Cys-391). The Trp-242 (cyan stick bonds) is unique to ANXA7 and was also observed in its two lamprey orthologs (Fig. 3).

of plant phospholipase D [32]. Based on their molecular model of this interaction, we investigated by theoretical docking experiments whether annexin KGD motifs might be ligands responsible for certain well-known interactions with other C2-containing proteins involved in signal transduction (Fig. 9). These include the binding and cotransport of ANXA13b with the ubiquitin ligase NEDD4 in apical vesicles [33], the specific binding of annexin A5 and inhibition of protein kinase C [34], the equally specific binding and inhibition of cytosolic phospholipase A2 by annexin A1 [35], and the unique (KGD-independent) binding of the annexin A6 linker region to p120RasGap as a modulator of ras signalling [36,37].

We have now confirmed the existence of compatible splice sites and highly conserved coding sequence for the alternatively-spliced cassette exon 2 in ANXA13 from 25 mammalian genomes (except rodents), and at least two thirds of these preserve the KGD motif in positions 20–23 of the protein amino-terminus. This represents a unique aspect of the ANXA13b isoform that could thus account for its specific, calcium-dependent binding to C2-NEDD4. For annexin A5, the CLUSPRO spatial constraint docking algorithm predicted the highest scoring interaction of external loops in the protein kinase Cδ C2-domain with that region of ANXA5 harboring its unique KGD ligand in the D–E loop of repeat 4 (Fig. 9) (i.e. analogous to other RACKs). We anticipated similar results with the 3 external KGD motifs in annexin A1 [38] binding to the C2 domain of cPLA2, and this was indeed one of the predicted models, but the highest scoring union instead involved the annexin A1 amino-terminal domain. This unexpected result is consonant with the long-standing hypothesis [39] that the amino-terminal tail of annexin A1 is largely responsible for many of the antiinflammatory and immunosuppressive actions of annexin A1, but leaves open the putative function of its characteristic KGD ligands in the core tetrad. These results collectively support the possible interaction of certain annexins with specific C2-domains, explicable by considering their mutual coordination of calcium ions and, where circumstances permit, additional involvement of (intra/extracellular, calcium-dependent) annexin KGD ligands. Such cooperative binding might represent an evolutionary safety mechanism to ensure membrane targeting of key enzymes responsible for signal transduction.

4. Conclusion

The evaluation of what a calcium binding domain (EF-hand, annexin or C2) represents structurally and functionally rests with an appreciation of the full range of variation observed in nature, where diverse structural models have adapted to the functional needs of disparate organisms over hundreds of millions of years. An evolutionary survey of the basic DxDxDG motif [24] reveals broad variability in its detailed molecular profile and in the context of its presentation in calcium-binding proteins. Our initial attempt to define the evolutionary conservation of calmodulin, S100 and annexins, using profile HMM analysis of site-specific protein alignments, provided a means of distinguishing the major groups and even individual subfamilies of these calcium-binding proteins. The unique sequence logos and 3D structures incorporating site-specific conservation patterns revealed characteristic variation, not only in the calcium-coordinating ligands themselves, but in adjacent residues with a known or putative contribution to calcium-binding, conformational responses and receptor interactions. This approach of reading evolutionary imprints to reveal functionally important features confirms much of what has been documented by empirical research on EF-hand proteins, but identifies many novel aspects of the annexin tetrad structure that have yet to been systematically investigated with regard to function. These include potential roles for Cys, Phe and Lys or "KGD" residues that are highly conserved and strategically located in the calcium-binding loops, with a distinct composition and profile for each subfamily, and evident in all phyla including even the most primitive, single-domain bacterial annexin.

We also focused on the recently sequenced (but incomplete and unassembled) genome of sea lamprey as the most primitive living vertebrate to identify novel homologs of calmodulin, S100 and annexin as distinct representatives of genes families that are highly conserved, newly originated or extensively dispersed, respectively. Their gene count and phylogenetic classification were expected to reveal evidence of the supposed whole gene duplication event but to fall short of the full mammalian gene complement. The annexin results describe an incomplete gene family but with paralogous duplicates (e.g. two ANXA7), in contrast to the full 12-member, single-copy family in cartilaginous fish (one ANXA7, our unpublished results) and the double-copy, complete family resulting from a confirmed whole genome duplication (associated with gene silencing) restricted to the bony fish lineage (no ANXA7!). We interpret this to suggest that lamprey did experience extensive tetraploidy within its own lineage (Agnathans) rather along the lineage common to other vertebrates. This would be consistent with the calmodulin findings if there was a limited duplication of only the vertebrate-like homolog but not the invertebrate-like representative (or its duplicate was silenced), yielding an equivalent number to mammalian calmodulins but with different origins. The S100 findings provide the first evidence for the origination of this family in vertebrates, while the presence of only 3 representatives most closely related to S100P indicates that the formation and amplification of the mammalian S100A cluster (i.e. in human chromosome 1q21) must have emanated from these founding members in higher vertebrates such cartilaginous or bony fish. Complete, assembled genomes of primitive vertebrates will be required to establish comparative genetic linkage maps.

Our quest to understand calcium binding mechanisms and functional consequences requires a broad yet focused view of the full gamut of accessory features inculcated into these proteins to accomplish their diverse tasks. The emerging paradigm must accommodate even the ostensibly calcium-independent actions of S100 proteins [12], C2 domains [1] and annexins like ANXA9 with its conserved KGD motifs [13] and ANXA10 with its high content of cysteine and aromatic residues [14]. Other nuances of functional variability are evident in annexins like ANXB12 that undergo major conformational responses to conditioning factors like pH [3] and ANXA5 with key Arg residues involved both in oligomerization and membrane-binding [10] or calcium-sensitive conformational changes of ANXA5 affecting adjacent tryptophan insertion into membrane

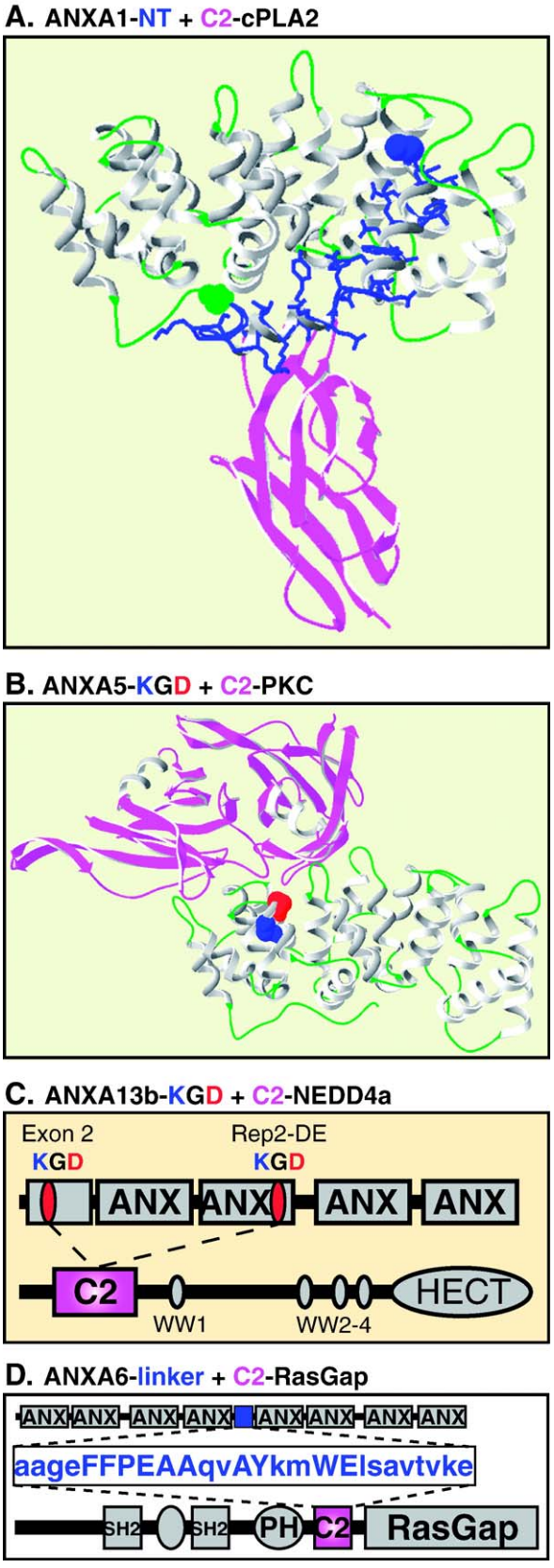

Fig. 9. Proposed docking models for annexins with C2 calcium-binding domains. (A) The specific interaction of annexin A1 (grey/green/blue) with the C2 domain of cytosolic phospholipase A2 (magenta β-strands) [35] is represented by a high-scoring CLUSPRO docking model which utilized known crystal structures for cPLA2 pdb:1bci and ANXA1 pdb:1hm6 (amino terminus starts at blue sphere, tetrad starts at green sphere). (B) The highest-scoring CLUSPRO interaction model for human annexin A5 (pdb:1anx) and the C2 domain of protein kinase Cδ [34] shows the conserved KGD motif (Lys atoms as blue spheres, Asp in red) in the repeat 4 tetrad region of ANXA5 juxtaposed to β-strand loops of the δPKC-C2 domain (pdb:1bdy). (C) A schematic model of the known binding interaction between annexin A13b alternatively spliced segment (from cassette exon 2) and the C2 domain of ubiquitin ligase NEDD4 [33] highlighting a potential receptor interaction with conserved KGD motifs in the amino terminus and repeat 2 of ANXA13b. (D) Schematic model of the known binding interaction between the annexin A6 tetrad linker region (blue sequence) with the C2 domain of human p120 RasGap [36,37].

phospholipids [30]. Such examples emphasize the need to identify all key structural features at the level of individual gene subfamilies (i.e. from precise evolutionary imprints). Their relevance must then be assessed by determining the extent to which alternative and cooperative mechanisms are truly operative throughout the family and in different types of calcium-binding proteins (i.e. from phylogenetic profiles). Finally, evolutionary models from 3D and docking algorithms offer important visual aids to validate known interactions and predict new ones. The eventual comprehension of what evolution has been trying to accomplish by its selective imposition of constraint or relaxation on structural changes in calcium-binding proteins will lead to functional insight through computational analyses.

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