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Research Article

Ancient origin of reggie (flotillin), reggie-like, and other lipid-raft proteins: convergent evolution of the SPFH domain

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Abstract. Reggies (flotillins) are detergent-resistant microdomains involved in the scaffolding of large heteromeric complexes that signal across the plasma membrane. Based on the presence of an evolutionarily widespread motif, reggies/flotillins have been included within the SPFH (stomatin-prohibitin-flotillin-HflC/K) protein superfamily. To better understand the origin and evolution of reggie/flotillin structure and function, we searched databases for reggie/flotillin and SPFH-like proteins in organisms at the base and beyond the animal kingdom, and used the resulting dataset to compare their structural and functional domains. Our analysis shows that the SPFH grouping has little phylogenetic support, probably due to convergent evolution of its members. We also find that reggie/flotillin homologues are highly conserved among metazoans but are absent in plants, fungi and bacteria, where only proteins with 'reggie-like' domains can be found. However, despite their low sequence similarities, reggie/flotillin and 'reggie-like' domains appear to subserve related functions, suggesting that their basic biological role was acquired independently during evolution.

Key words. Reggie; flotillin; lipid raft; evolution; oligomerization; SPFH.

Reggie-1 and -2 are highly conserved proteins [1], first characterized in goldfish and rats as plasma membrane-associated proteins which are upregulated during axon regeneration of retinal ganglion cells upon optic nerve transection [2, 3]. The independent identification of mice and fruitfly homologues led to the alternative names flotillin-1 and -2 (reggie-2 and -1, respectively) [4, 5]. Although they were initially thought to be components of caveolae [4], subsequent biochemical and microscopic characterization revealed that these 48-kDa proteins are distinctive non-caveolar markers of detergent-resistant microdomains (DRMs) [3, 6]. In humans, reggie-1 and reggie-2 polypeptides show similar length, primary sequence composition and biochemical profiles, with a conserved protein architecture at the N terminus containing one myristoylation site (Gly-2, only in reggie-1) and four palmitoylation sites (Cys-5, Cys-19, Cys-20, Cys-38), which are thought to assist in membrane association and lipid raft targeting [7–9]. Reggies/flotillins do not have typical transmembrane domains, but harbor two conserved hydrophobic stretches that allow potential interactions with the inner leaflet of the plasma membrane, and possibly protein-protein interactions [7, 8].

Notably, reggies/flotillins are expressed in most cell types [3, 6], including those devoid of caveolae, such as neurons [3, 5], and lymphocytes [6, 10–12]. Their subcellular distribution under light microscopy (LM) typically consists of a punctate membrane co-localization pattern [3], indicative of small (≤0.1 μm) microdomains, as revealed by electron microscopy (EM) studies [6]. At LM and EM levels, reggies/flotillins co-cluster with various specific surface proteins and intracellular signal transduction components like the cellular prion protein (PrP^C), Thy-1, activated cell adhesion molecules (CAMs), Src family kinases (e.g. lck and

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fyn) and interact with actin cytoskeleton-associated protein (vinexins, CAP/ponsin and ArgBP2) [6, 9, 10, 13–16]. The induction of signal cascades is demonstrated by the elevation of intracellular Ca^{2+} and MAP kinase phosphorylation during PrP^{C} capping in T cells [10], by actin cytoskeletal changes [9, 13, 15, 16] and by relocation of glucose transporter 4 to the plasma membrane in adipocytes [9]. Additional lines of evidence such as co-immunoprecipitation, cross-linking and microscopy confirm the idea that reggie/flotillin microdomains play an important role in cell-cell recognition, adhesion and signaling events [reviewed in refs. 17, 18].

Reggies/flotillins are highly expressed in virtually all growing structures of the developing zebrafish, particularly in tissues actively undergoing rapid proliferation and differentiation [19]. Moreover, morpholino knockdown in zebrafish embryos [Málaga-Trillo E., Rivera-Milla E. and Stuermer C. A. O., unpublished data], as well as overexpression experiments in fruitflies [20] bring about striking morphological phenotypes that include cytoskeleton rearrangements, indicating an important requirement for reggies/flotillins during early signaling pathways. Interestingly, abnormal upregulation of reggies/flotillins has also been associated with neuropathological disorders such as Parkinson's disease [21] and Alzheimer's disease [22, 23], as well as with progression of melanoma cell lines *in vitro* [24], and the selective uptake of *Plasmodium falciparum* [25]. While the molecular mechanisms involved remain to be clarified, these preliminary findings emphasize a basic and important cellular function of reggie/flotillin microdomains.

In addition to the well-characterized vertebrate and invertebrate reggies/flotillins [1–5, 26, 27], reggie-like homologues have been proposed in distantly related organisms such as bacteria (yuaG) and plants (NOD53b) [28]. Furthermore, reggies/flotillins have been tentatively assigned to the SPFH (stomatin, prohibitin, flotillin and HflC/K) protein superfamily, based on the sharing of an ~200-amino acid (aa) motif of apparently ancient origin, as judged by its presence in archeal and prokaryotic proteins [29]. However, these efforts at protein classification across kingdoms are based mainly on poor sequence homology matches. Hence, evolutionary relationships supported by additional homology criteria are urgently required to arrive at sound conclusions regarding the origin and conservation of a basic ancestral cellular function of reggie/flotillin proteins in living organisms. Indeed, the strong sequence conservation observed among animal reggies/flotillins appears to support such an ancient role. To characterize the early functional evolution of reggies/flotillins, we undertook a comprehensive update of reggie/flotillin and reggie-like homologues across the living kingdoms, and analyzed their evolutionary relationships using structural and functional considerations.

Materials and methods

Search for reggie/flotillin homologues. Optimized BLAST and PSI-BLAST [30] searches were performed against protein and nucleic acid databases using metazoan reggie-1 and -2 sequences as queries. Most sequences were downloaded from GenBank through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), Ensemble (www.ensembl.org) and TIGR (www.tigr.org). Reggie/flotillin homologues were identified as matches with sequence homology values over a threshold of 30% for protein sequence similarity and E values $<e^{-10}$; and containing both protein domains SPFH (Pfam:PF01145) and flotillin (Pfam: PF03149) in consecutive order.

Molecular phylogenetic analysis. Sequence alignments were performed manually and with CLUSTALW (PAM-250 and PAM-45 scoring matrix; www.ebi.ac.uk/clustalw). Phylogenies were reconstructed using maximum parsimony, distance and minimum evolution methods in PAUP v4.0d64 (Sinauer Associates, Sunderland, Mass.) and MEGA [31]. Reliability of tree topologies was assessed by the bootstrap method (1000 replications). Codon-based selection analysis was conducted through the Z-test of synonymous and non-synonymous differences (Jukes Cantor distance), using the Nei-Gojobori and Li-Wu-Luo methods in MEGA, and substitution ratios were compared by Chi-square test (χ^2).

Protein structure prediction and domain analysis. Prediction of secondary structure and post-translational modifications were performed using the following software: peptide cleavage sites by SignalP v3.0 (www.cbs.dtu.dk/services/SignalP); N-glycosylation sites by NetNGlyc v1.0 (www.cbs.dtu.dk/services/NetNGlyc); transmembrane helix prediction by HMMTOP (www.enzim.hu/hmmtop); de novo repeat detection in protein sequences by RADAR (www.ebi.ac.uk/Radar); and secondary/tertiary structures with ProModII, as described before [32], based on mouse reggie-1 (flotillin-2) Band-7 domain (1WIN.pdb; www.rcsb.org/pdb/cgi/explore.cgi?pdbId=1win), and human prohibitin-1 (1lu7.pdb; www.rcsb.org/pdb/cgi/modelBrowser.cgi?pdbId=1LU7). Coiled-coil structures were predicted using the servers MATCHER (cis.poly.edu/~jps/matcher.html), Paircoil (paircoil.lcs.mit.edu/cgi-bin/paircoil), Multicoil (multicoil.lcs.mit.edu/cgi-bin/multicoil) and Coils (www.ch.embnet.org/software/COILS_form.html). Detection of functional motifs was performed using the Eukaryotic Linear Motif server (elm.eu.org). Conserved protein domains were analyzed using the NCBI conserved-domain database CDD (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), the protein family Pfam server at the Sanger institute (www.sanger.ac.uk/Software/Pfam/search.shtml) and

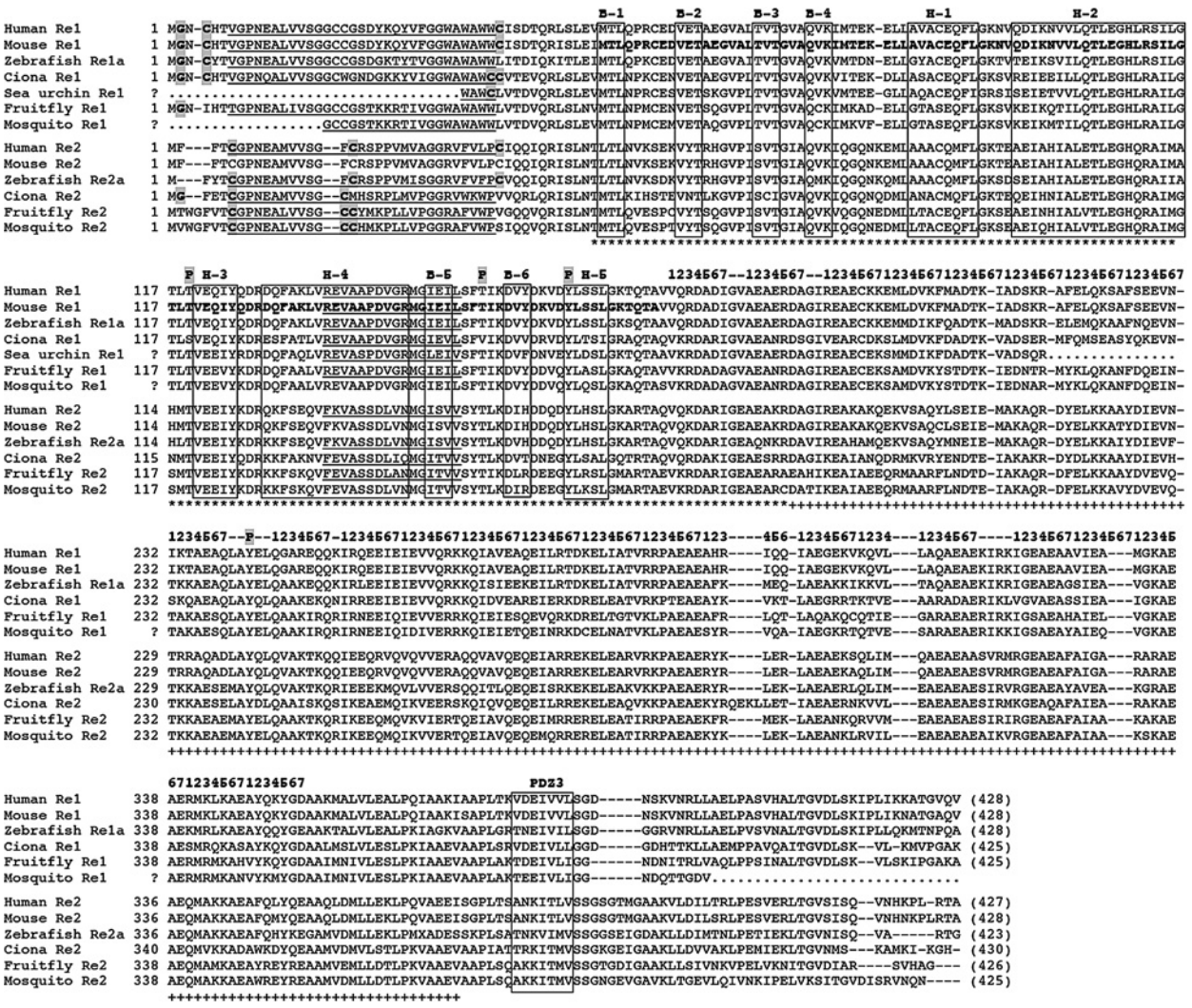

Figure 1. Conservation of amino acid sequences among metazoan *reggie-1* and *-2* genes. Alignment of representative vertebrate and invertebrate *reggie-1* and *-2* genes highlighting the following conserved post-translational modifications: phosphorylation sites (P) in gray columns, myristoylation and putative palmitoylation sites in black boxes. The resolved three-dimensional structure of the mouse reggie-1 SPFH domain (file 1win.pdb, bold sequence) was used to support secondary-structure predictions; six β strand (B-1 to B-6) and five α helices (H-1 to H-5) are accordingly shown in framed boxes. Both conserved N-terminal hydrophobic stretches are underlined. Contiguous blocks of putative coiled-coil heptad motif are designated by top legends '123467'. A putative PDZ3 binding motif (better conserved in reggie-1) is highlighted by a framed box. The symbols * and + below the alignment indicate the spans of the conserved SPFH and flotillin protein domains, respectively. Relative aa positions are specified at the left margin of the alignment. The numbers in parentheses at the end of the alignment indicate total protein length. Gaps are denoted by – and unknown sequences by · .

the simple modular architecture research tool (SMART) server (smart.embl-heidelberg.de).

Supplementary tables are omitted due to space reasons and are available upon request.

Results

Metazoan reggies/flotillins have a unique structural and functional domain composition. Using known reggie-1 and -2 sequences as templates, we searched public databases for novel homologues. From a total output of 656 expressed sequence tag (EST) and 300 genomic sequences, we filtered redundant matches and assembled the longest contiguous consensuses encoding partial or entire open reading frames for 57 reggie/flotillin proteins from 29 animal species (supplementary table 1), including 13 unreported sequences in vertebrate groups and in the urochordate *Ciona intestinalis*. Six novel sequences were also identified in invertebrate taxa such as the sea urchin (*Strongylocentrotus purpuratus*), mosquito, honeybee, domestic silkworm (*Bombyx mori*), and two species of flies (*Drosophila pseudoscura* and *D. yacuba*). Length and sequence conservation are remarkably high in this dataset (fig. 1), with average amino acid similarity values of 89.7% among vertebrate reggies/flotillins and

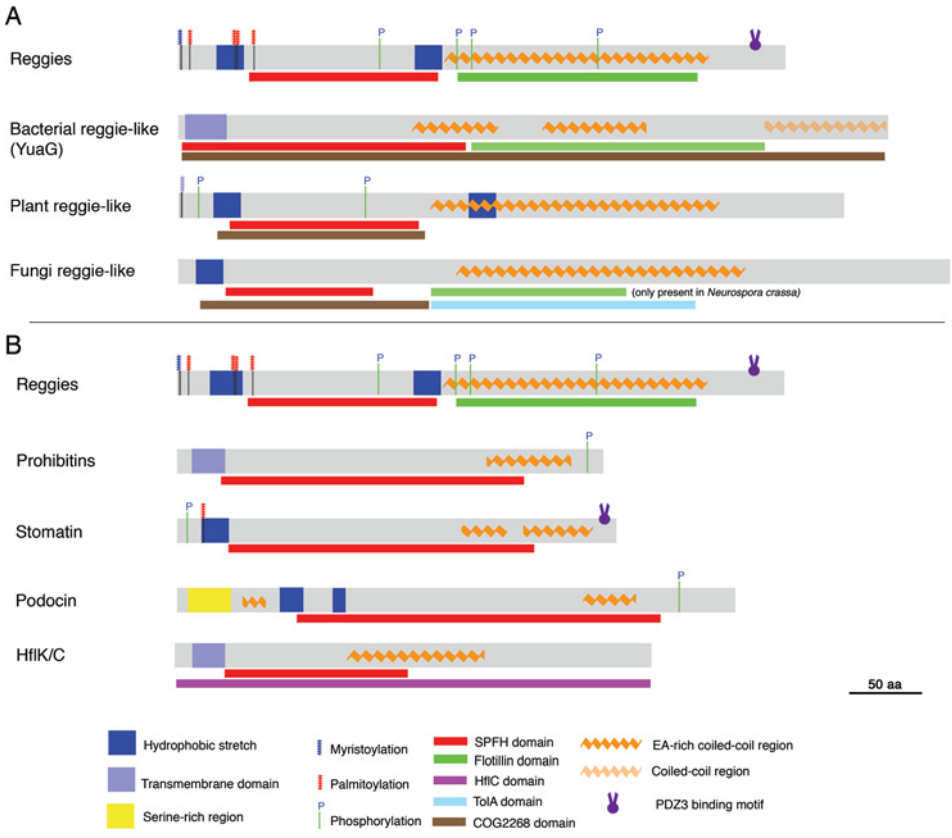

Figure 2. Structural landmarks of reggie/flotillin, reggie-like and SPFH superfamily proteins. Relative location of conserved structural and functional protein domains, according to experimental and prediction data. Gray boxes represent protein backbones; colored boxes contained in each polypeptide represent different protein motifs; horizontal colored bars below protein backbones represent different conserved protein domains. Bar scale, 50 aa. (*A*) Reggie-1 and -2 consensus model versus reggie-like proteins. (*B*) Reggie-1 and -2 consensus model versus other SPFH superfamily members.

63.3% between vertebrate and invertebrate reggies/flotillins, over an ~428-aa-long polypeptide. Beyond metazoan taxa (supplementary table 1), unambiguous assignment of homology was not possible due to the low sequence similarity scores among the matches (below 26%). Therefore, we used the conserved animal dataset to define the characteristic structural and functional domains of metazoan reggies/flotillins (the non-metazoan dataset was re-examined separately using additional criteria; see below).

Mining of public databases revealed two contiguous and slightly overlapping protein domains in all metazoan reggies/flotillins: an N-terminal SPFH domain [29] that covers about 42% of human reggie-1 (residues 7–190) and 48% of human reggie-2 (residues 5–213), and a C-terminal flotillin domain that covers about 40% of both human reggie-1 (residues 193–365) and reggie-2 (residues 190–362; figs. 1, 2). The two domains differ remarkably in their patterns of natural occurrence: while the SPFH domain is present in several other families of membrane proteins, the flotillin domain is found exclusively in reggies/flotillins (fig. 2B; see below). Despite having only about 50% sequence identity, reggie-1 and -2 are predicted to produce very similar secondary structures (fig. 2). Based on the available three-dimensional nuclear magnetic resonance (3D NMR) structure of the mouse reggie-1 SPFH (Band_7) domain (1win.pdb model [33]) (fig. 3A) and using various secondary-structure prediction tools, we consolidated a model that ascribes globular and helical structures to the SPFH and flotillin domains, respectively (fig. 3A), but finds no predictable stable folding for the very N-terminal stretch (residues 5 to 42). Our predictions across a wide range of metazoan phyla agree with the confirmed 3D model of the mouse reggie-1 SPFH domain, which consists of six short antiparallel β sheets and five, partially exposed, α helices covering about 25% of the protein (human reggie-1 residues 43–173), forming an ellipsoidal-like globular domain. Interestingly, the loop sequences are highly conserved as are the α and β structures that they interconnect (fig. 1), suggesting the maintenance of an important function in both types of structural element.

Recent reports suggest that reggies/flotillins interact with F-actin [9], and that small and hydrophobic residues within α helices from actin-binding proteins are likely to interact with actin [34]. We analyzed the α helices within

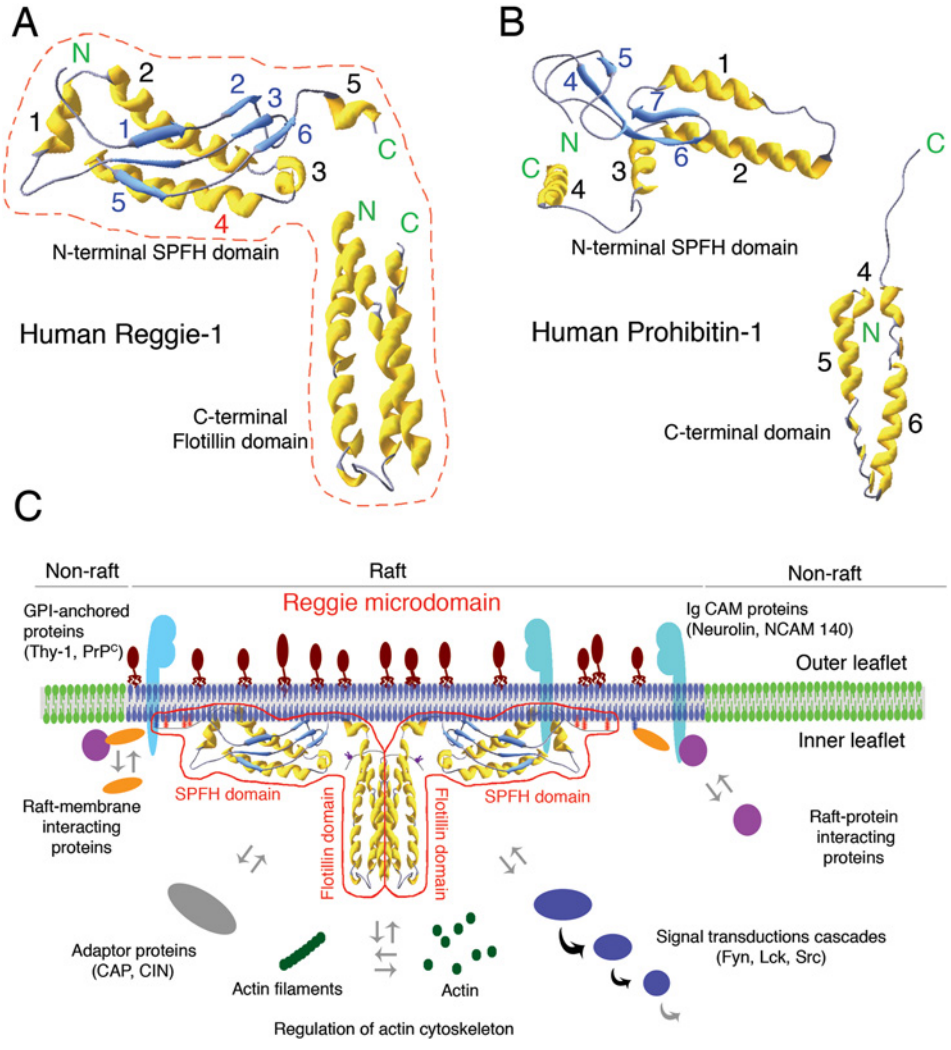

Figure 3. Modeling of reggie/flotillin structural and functional domains. (*A*) Predicted 3D models of human reggie-1 SPFH and flotillin domains, based on 1win.pdb and Swiss-Model server (swissmodel.expasy.org//SWISS-MODEL.html). α helices (in yellow) are numbered in black, with the exception of α helix 4, which is predicted to interact with actin and is numbered in red. β sheets are drawn and numbered in blue, with the exception of human β sheet 4 (located between β-1 and β-5, not shown). The amino (N) and carboxy (C) ends are indicated by green letters and are truncated. (*B*) Ribbon diagram of human prohibitin-1 SPFH and C-terminal domain based on 1lu7.pdb. Numbering of α helices and β sheets as in *A* (β-1 to β-3 are not shown). (*C*) Two dimensional model for the assembly of a reggie/flotillin microdomain, showing the proposed basic interaction between two adjacent reggie/flotillin molecules within a larger hetero-oligomer. Lipid rafts (blue) have distinct lipid compositions that differ from non-raft membrane (green). A hetero-tetrameric cluster of reggie-1 and -2, highlighted in red, is expected to interact with the inner leaflet of the plasma membrane via its SPFH domain and acylation (jagged blue and red features). The flotillin domain coiled-coil structures are assumed to stabilize the tetrameric complex.

the reggie/flotillin SPFH domains and, based on the content of small and exposed hydrophobic residues (i.e. Val and Ala), we identified α helix 4 (indicated by a red '4' in fig. 3A) as a potential actin-binding stretch. Recent data showing co-localization of reggies/flotillins and actin filaments in Hela cells support this assumption [Langhorst M. F., Solis G. P. and Stuermer C. A. O., unpublished data]. There are no available experimental 3D data for the flotillin domain but our structural predictions for the C terminus reveal a large tendency to form helical structures (fig. 3A). However, being only marginally significant (all values <0.5), these prediction scores make an α helical prediction rather unlikely. Therefore, we focused our analysis on the content of coiled-coil structures, a class of helical elements with less structural stability. Different predictions methods detected a significant coiled-coil content (overall scores <0.6) over almost the entire human flotillin domain (reggie-1 residues 184–363). Furthermore, we noticed the presence of 25 heptad (i.e. 7 aa) tandem arrays, the typical structural units of coiled-coil structures [35] (figs. 1, 3A), distributed variably along the second half of the protein. As in other coiled-coil proteins, the reggie/flotillin heptad sequences are quite variable across and within species but share the conserved

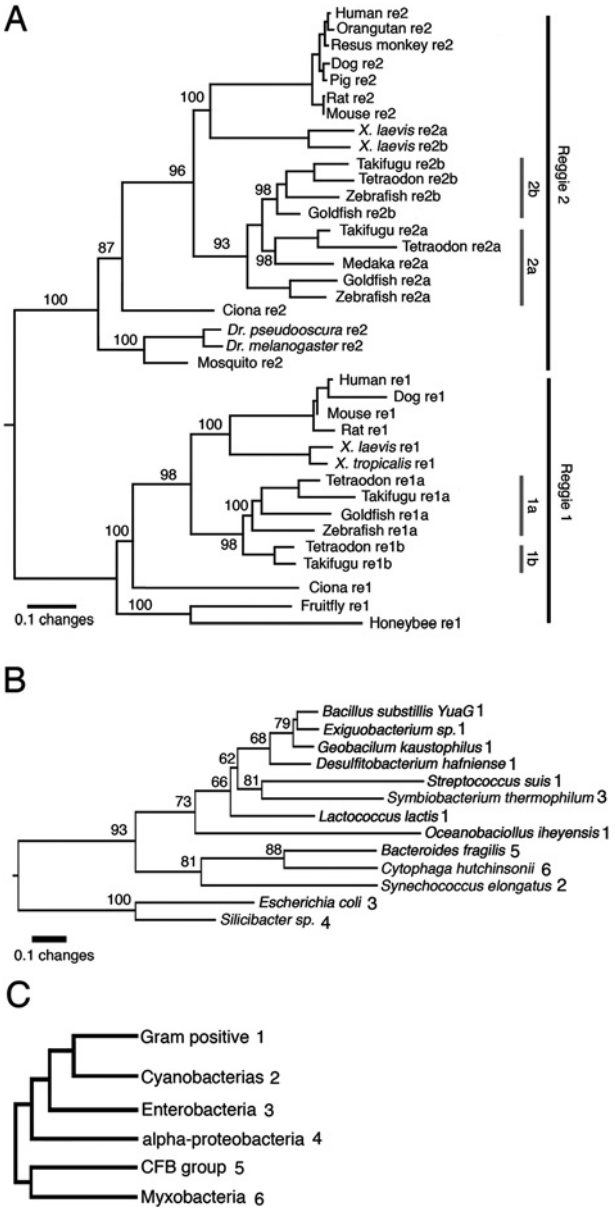

Figure 4. Evolutionary relationships among metazoan reggie/flotillin and reggie-like genes. The consensus tree was generated using maximum parsimony, minimum evolution and distance-based methods based on an amino acid sequence alignment. (*A*) Metazoan *reggie-1* and *reggie-2* genes form monophyletic groups with similar evolutionary dynamics, as reflected in their branching patterns. (*B*) Phylogenetic relationships among yuaG proteins of representative bacterial species do not reflect their expected taxonomic relatedness. Species names are numbered according to the taxonomic groupings shown in *C*. (*C*) Schematic relationships among major bacterial groups (based on Gupta and Griffith [67] and Daubin et al. [68]). Multiple sequence alignments ALIGN_000864 and ds_Align01945 have been deposited with the European Bioinformatics Institute (ftp.ebi.ac.uk/pub/databases/embl/align). Bootstrap support is shown for relevant nodes. Scale bar represents 10% protein sequence divergence. Phylogenetic analysis of plant and yeast reggie-like proteins is not shown due to their poor representation in sequence databases.

presence of charged amino acid groups at positions 2, 3 and 5; the corresponding alignment in figure 1 produces the most structurally parsimonious array of heptads. In addition, we detected a highly conserved PDZ3-binding motif (VDEIVVL) at the C-terminal end of reggie-1 (human residues 378–384; fig. 1); this motif is rather degenerated in reggie-2.

Both reggie-1 and -2 have two conserved hydrophobic stretches at the N-terminal half, corresponding to human reggie-1 residues 14–35 and 134–150, and human reggie-2 residues 10–30 and 134–151 (fig. 2A). These stretches are not hydrophobic enough to form an integral transmembrane domain (below 0.8 standardized units [36]), but are likely to allow interactions with the plasma membrane or plasma membrane-associated proteins. Interestingly, the first hydrophobic stretch of reggie-1 contains more – up to four – apolar tryptophan (W) residues than reggie-2 (fig. 1), which is likely to confer on reggie-1 a more stable association with the membrane than reggie-2. We also examined the distribution of post-translational modifications and found that all metazoan reggies/flotillins share a similar distribution of predicted lipid modification (myristoylation and palmitoylation) and phosphorylation sites (fig. 2). All fatty acid modifications are predicted at the beginning of the N terminus, around the first hydrophobic stretch. While all reggie-1 proteins contain putative myristoylation sites at Gly-2, invertebrate reggie-2 proteins do so at Gly-4, and reggie-2 of *C. intestinalis* is the only vertebrate reggie-2 predicted to undergo this modification at Gly-2 (fig. 1). The pattern of palmitoylation is rather variable, with two experimentally confirmed palmitoylated sites in reggie-1 (human Cys-4 and Cys-38), and three sites in reggie-2 (human Cys-5, Cys-17 and Cys-34) [7, 8]. Four putative phosphorylation sites are conserved in all reggies/flotillins examined [3, 4], with some differences in their location in *Ciona* reggie-1 (fig. 1). According to the 3D resolution of the mouse reggie-1 SPFH domain (1win.pdb), three of these sites are exposed in the interconnecting loops of the SPFH domain (human reggie-1 Thr-119, Thr-153 and Tyr-163; fig. 3A), and the fourth site is located in the middle of the flotillin domain (human reggie-1 Tyr-240).

Having delineated the characteristic set of conserved structural features defining metazoan reggies/flotillins, we set out to identify the patterns of molecular evolution that shaped and maintained these features. The phylogenetic tree of representative metazoan reggies/flotillins in figure 4A recovers the expected reggie-1 and -2 ancient monophyletic groups with internal branching patterns that correspond well with the known taxonomy [1, 37], as well as with the duplication of reggies/flotillins in bony fish [1] and in the amphibian *Xenopus laevis* [38]. Phylogenetic analysis of each separate domain recovers the same tree (not shown). In agreement with the similar tree topologies of reggie-1 and -2, their average ratios of non-synony-

Table 1. Substitution ratio observed in reggie, reggie-like and other SPFH superfamily proteins.

Group	dN/dS per site	Protein	dN/dS per site
Metazoan		Stomatin	0.759***
Reggie-1	0.823**	SPFH	0.771***
Reggie-2	0.843***		
Whole re-1 and -2	0.848***	Prohibitin-1	0.810***
SPFH re-1 and -2	0.786***	SPFH	0.845***
Flotillin re-1 and -2	0.896**		
		HflC	0.911**
Bacteria		SPFH	0.987*
yuaG reggie-like	1.121***		
SFPH	1.083**	HflK	0.897**
Flotillin	1.070**	SPFH	1.002*
Plant			
Rreggie-like	1.148***		
SPFH	1.031**		
Fungi			
Reggie-like	1.194***		
SPFH	1.143***		

Estimated pairwise average rates of non-synonymous/synonymous substitutions (dN/dS) per site among reggie, reggie-like and SPFH superfamily member genes, based on Jukes-Cantor distance values. Estimations were based on the whole and partial SPFH and flotillin protein domain sequences. Statistic confidence of H_0: dN/dS = 1 is shown as follow: *p < 0.1; **p < 0.05; ***p < 0.001.

mous/synonymous substitution per site (dN/dS) are comparable (0.823 and 0.843, respectively, $\chi^2 = 14.2$, $p < 0.001$; table 1) and indicate negative selection against amino acid replacements resulting in a higher predominance of synonymous changes, therefore explaining the high levels of sequence conservation observed. To learn more about the functional importance of each structural domain (see above) and whether different selection constraints act upon them, we separately analyzed the rates of substitutions at the SPFH and flotillin domains. Although the SPFH domain shows a lower dN/dS ratio (0.786) than the flotillin domain (0.896), this difference is not statistically significant ($\chi^2 = 10.3$, $p < 0.01$; table 1), suggesting that the functions of both domains are largely sequence dependent and of high biological importance, making these molecules refractory to major amino acid replacements.

Reggie-like proteins in bacteria. Our initial searches for reggie/flotillin homologues in non-metazoan organisms yielded distant matches in bacteria, fungi and plants. However, their low similarity scores made it difficult to assess their evolutionary relationships to metazoan reggies/flotillins based on primary sequence comparisons. The structural criteria established above provided us with analytical tools to refine our search. Thus, we analyzed the bacterial, fungal and plant matches for their content of reggie/flotillin structural features, such as the combined presence of SPFH and flotillin domains with the distinctive secondary structure and post-translational modification patterns described above.

Among bacteria, 78 proteins from 44 different species (supplementary table 1) encode homologues of *Bacillus subtilis* yuaG, the bacterial locus with the highest sequence similarity to metazoan reggies/flotillins (<36%, E values $\leq e^{-14}$). This gene is part of a '*yuaF–I*' operon activated by the regulon σ^w, and encodes a 60-kDa protein which may be involved in detoxification and the production of antimicrobial compounds [39]. The sequence variation found among yuaG homologues is somewhat higher than the one observed among metazoans, with an average similarity value of 46% (ranging from 26% to 98%). The phylogeny of representative *yuaG* homologues (fig. 4B) does not correspond to the known relationships between the main bacterial groups (fig. 4C); in some cases, genetic variation can be observed even within bacterial strains (i.e. 6% and 8% among *Bacteroides fragilis* and *Escherichia coli* strains, respectively). yuaG proteins are generally larger than metazoan reggies/flotillins (fig. 2A), ranging from 414 aa in *Synechococcus elongatus* to 562 aa in *Silicibacter sp*. (supplementary table 1). Domain and structural analyses of *B. subtillis* yuaG reveals the presence of a large and uncharacterized bacterial domain named COG2268 that spans almost the full open reading frame (ORF) (E value $< e^{-47}$). In addition to this conserved domain, we identified several reggie-related features, such as an N-terminal SPFH domain (E values $< e^{-23}$) and a C-terminal flotillin domain (E values $< e^{-20}$) covering about 38% and 34% of the protein, respectively (fig. 2A). While the former (residues 38–137) yields structural predictions containing various globular arrangements of helical and strand structures, the latter appears to be helical in nature and also contains multiple reggie-like Glu-Ala (EA) motifs between residues 120 and 394. Three coiled-coil stretches can be identified at

positions 120–172, 204–408 and 456–509, with the potential to support the formation of oligomers (fig. 2A), as suggested by the prediction programs COILS and MULTICOIL [35, 40]. In contrast to metazoan reggies/flotillins, we found bacterial yuaG to contain only one predicted transmembrane stretch between residues 6 and 40 (fig. 2A).

Reggie-like proteins in plants and fungi. In green plants, we identified 17 reggie-like proteins in ten species (supplementary table 1), which match human reggie/flotillin sequences with low similarity scores (reggie-1 <20%, E values $\leq e^{-08}$). All these plant reggie-like proteins show high sequence similarity with the soybean reggie-like protein (accession number AAC72337, <61%, E values $\leq e^{-76}$). Ranging in length from 417 to 485 aa, plant reggie-like proteins are slightly larger than reggies/flotillins (fig. 2A). In *Arabidopsis thaliana*, we found reggie-like homologues encoded by three loci on chromosome 5. Interestingly, two of these loci (At5g25250 and At5g25260, here named reggie-like-1a and -1b) seem to have arisen by recent tandem duplication, since they are contiguous and encode 95% identical proteins. The third locus (At5g64870, here named reggie-like-2) encodes an 85% identical protein and is located 17.2 Mega base pairs downstream of the first two, which makes it likely the result of an older tandem duplication. These reggie-like proteins contain an N-terminal domain with high similarity to the SPFH domain (38% of the protein; E values $<e^{-3}$) and to the bacterial domain COG2268 (45% of the protein; E values $<e^{-6}$; fig. 2A). However, no domains can be clearly identified at the C terminus, despite the presence of several EA motifs between residues 171 and 365 in the soybean reggie-like protein (AAC72337), which are reminiscent of those forming the reggie/flotillin coiled-coil structures; accordingly, we were able to detect up to 18 heptads in a region that covers about 40% of the protein, with the predicted ability to oligomerize. In addition, plant soybean reggie-like proteins contain two N-terminal hydrophobic stretches (residues 15–41 and 249–264), and two conserved phosphorylation sites at Tyr-10 and Tyr-145 (fig. 2A). No consistent evidence of myristoylation or palmitoylation was found, except for the presence of a putative myristoylation site at Gly-2 in rice (AAP54307).

Finally, we found 11 reggie-like proteins in seven species of fungi, displaying low levels of similarity with metazoan reggies/flotillins (<22%, E values $\leq e^{-07}$); no significant matches could be found in the model yeast organism *Saccharomyces cerevisiae*. With an average length of 493 aa, the uncharacterized fungi reggie-like proteins are longer than metazoan reggies/flotillins (supplementary table 1) and vary considerably in length, ranging in ascomycetes from 423 aa (*Aspergillus nidulans*) to 526 aa (*Giberella zaes*) (fig. 2A). The length differences are mainly the result of species-specific indels that appear to be randomly distributed along the protein and can span up to 25 aa (fig. 2A). Variation between ascomycete polypeptide sequences is high, with similarity values ranging between 50–70%. *Neurospora crassa* reggie-like proteins possess two conserved domains: the N-terminal one, which matches better with the uncharacterized bacterial domain COG2268 (58% of the protein; E values $<e^{-12}$; fig. 2A) than with the SPFH domain (<20% of the protein; E values $<e^{-3}$); and the C-terminal one, which matches with equally low scores to flotillin (31% of the protein, E values $<e^{-2}$) and the TolA domain (Pfam PF06519; 36% of the protein, E values $<e^{-4}$). The EA motif-rich region of fungi reggie-like proteins covers about 37% of the protein (*A. nidulans* residues 199–354), and contains 17 heptad units that are also predicted to form oligomers. There is only a single and weak N-terminal hydrophobic stretch at *A. nidulans* residues 26–35, which occurs before a short and highly variable motif covering residues 81–93. We did not find significant evidence for conserved post-translational modifications in fungi reggie-like proteins.

In addition to the three taxonomic groups mentioned above, no matches with similarities >20% were found in other distant organisms, such as viruses, archaea or basal eukaryotes. We also analyzed the effect of selection on the bacterial, plant and fungi reggie-like proteins and found that their global and per domain values of dN/dS ratio were statistically higher than those observed in metazoan reggies/flotillins, with slightly higher values in fungi (average 1.194; $\chi^2=25.2$, $p<0.001$) compared to plant (average 1.148; $\chi^2=18.9$, $p<0.001$) and (average 1.121; $\chi^2=21.6$, $p<0.001$) bacterial reggie-like proteins (table 1). This result indicates that reggie-like proteins underwent stronger positive selection than metazoan reggies/flotillins. Interestingly, the separate dN/dS values for the SPFH and flotillin domains alone correspond to those observed for the whole protein.

Structural and functional diversity among SPFH proteins. Because their N-termini display sequence similarity to the SPFH domain, reggies/flotillins have been classified as members of the SPFH protein superfamily, which includes a wide variety of other protein families such as stomatin, prohibitin, and HflK/C. Likewise, the N termini of reggie-like proteins in bacteria, plants and fungi (see above) also show SPFH sequence similarities. The widespread distribution of the 'conserved' SPFH domain across life kingdoms has been taken as an indication of its ancient origin, suggesting the common ancestry and functional homology of all SPFH proteins [29]. However, the structural, functional and evolutionary criteria to support this view have not been clearly established. Careful alignment of different SPFH members within a species reveals that their sequence similarity is very low and restricted to only a few amino acids (e.g. 19% between hu-

Figure 5. Sequence variation among human SPFH protein members. Optimized alignment of human reggie/flotillin (Re-1, Re-2), prohibitin (Phb-1, Phb-2), stomatin (Sto) and podocin (Pod) proteins. As a reference, the canonical SPFH domain sequence of each protein is shown as a framed box. Relative aa positions are indicated at the left margin of the alignment. The numbers in parentheses at the end of the alignment denote total protein length and gaps are denoted by – .

man prohibitin-1 and stomatin or 37% for human prohibitin-1 and reggie-2; fig. 5), in agreement with the differences observed for their 3D structures (fig. 3A, B). Contrary to the SPFH superfamily definition, these few sequence similarities are largely found at the C-terminal regions, where multiple EA motifs are conspicuously present. Similar patterns are observed for SPFH superfamily members of *Caenorhabditis elegans* and *D. melanogaster* (not shown). Thus, while domain databases recognize all these protein families as members of the same superfamily, their SPFH domains are so variable that it is virtually impossible to obtain a consensus sequence, as shown in figure 5. Under these circumstances, it became necessary to accurately assess the relationships among all SPFH members.

We searched protein domain databases and identified 742 different SPFH proteins in 309 species across all kingdoms, with the following family distribution: 331 stomatin and stomatin-like proteins, 217 prohibitins, 126 HflK/C, 53 reggie/flotillins and 15 unknown. As expected, sequence similarity is not evident among the various groups; moreover, the relative position of the SPFH domain within the polypeptide varies among different SPFH members (fig. 5). Thus, phylogenetic analysis can only be attempted when forcing an alignment of the predefined SPFH domains (see legend to figure 6). Such analysis does not provide evidence for orthology (or homology) within the SPFH superfamily, but rather general sequence affinities (large genetic distances with low bootstrap support) between some of its members (fig. 6). For example, overall sequence similarity scores place podocins and stomatins as the closest superfamily relatives with ~48% similarity, followed by reggies/flotillins and prohibitins (~37% similarity). Thus, the SPFH superfamily concept only indicates general functional relatedness between convergently evolved proteins. In the absence of definitive phylogenetic criteria to support these relationships, we analyzed each group separately.

The most representative SPFH superfamily protein, stomatin (also known as band 7.2b protein), is a major integral plasma membrane protein of human erythrocytes [41]. It is about 141 aa residues shorter than reggies/flotillins and contains a hydrophilic N-terminal stretch (positions 1–31), followed by a hydrophobic transmembrane domain (postions 32–51), and the SPFH domain, which spans about 55% of the polypeptide (human positions 52–211; fig. 2B). At the end of the SPFH domain, there is a short EA rich-like motif in partial overlap with the putative coiled-coil region at the C terminus (positions 125–235, 38% of the protein), which contains up to 16 heptads and yields helical predictions. As in reggie-1, a conserved PDZ3-binding motif can be recognized toward the C-terminal end. Two conserved palmitoylation sites (position 29 and 86) and a phosphorylation site (Ser-10) are observed as well (fig. 2B).

Prohibitin-1 (Phb-1) and -2 (Phb-2) are two ubiquitous, abundant and highly conserved proteins that play important roles as chaperones during the assembly of mitochondrial respiratory chain complexes [42, 43]. They are 130 to 157 aa shorter than reggies/flotillins and consist of

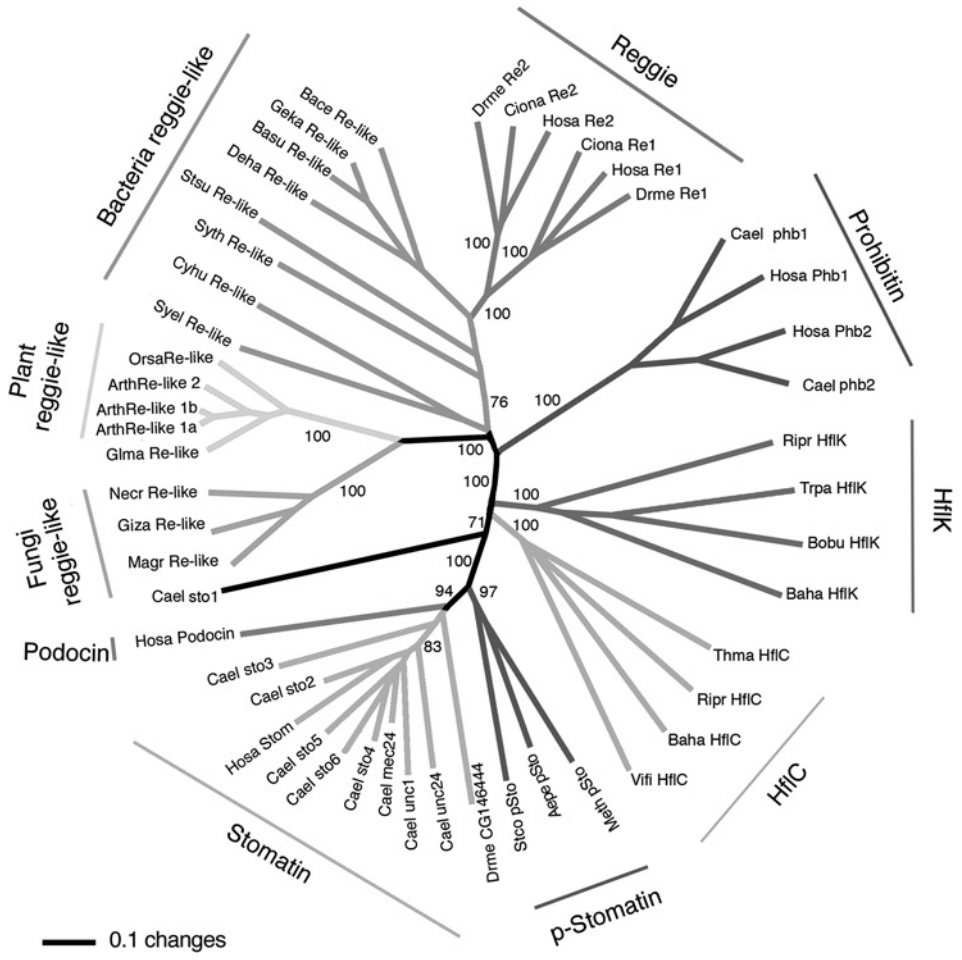

Figure 6. Distance based relationships between SPFH superfamily proteins. Unrooted neighbor-joining dendrogram depicting the relationships among 49 representatives of the SPFH superfamily and reggie-like proteins. The analysis is based on the addition of new sequences to the original dataset from Tavernarakis et al. [29]. Protein clusters are shown by legends above each branch. Supporting bootstrap values out of 1000 replicates are indicated in important nodes. A multiple-sequence alignment ALIGN_000865 has been deposited with the European Bioinformatics Institute. Scale bar represents 10% protein sequence divergence. Sequence ID are given in supplementary table 2.

a single SPFH domain located at their N terminus (human Phb-1 residues 26–187; fig. 3B), covering about 54–60% of the polypeptide (fig. 2B). Prohibitins have an N-terminal transmembrane stretch (Phb-1 residues 11–23) preceding the SPFH domain that is cleaved off in the mature peptide, and a putative phosphorylation site at Tyr-259 [43]. Available 3D structures for human Phb-1 (1lu7.pdb model) indicate that this protein folds very differently to the SPFH domain of reggies/flotillins (fig. 3B). It is composed of six α helices and seven β sheets, the β sheets being arranged as two antiparallel clusters containing strands 1, 2, 4, 5 and 3, 6, 7, respectively, and with helices 1, 2, 3 forming a cluster perpendicular to helices 4, 5 and 6. It is surprising that, despite having bona fide SPFH domains, the 3D models of reggie/flotillin and prohibitin turn out to be so different (fig. 3A, B). Interestingly, our prediction reveals a single structural affinity between reggies/flotillins and prohibitins: the presence of coiled-coil structures (multiple EA motifs) between prohibitin helices 4 and 5, which agrees with the higher flexibility observed for these stretches.

HflK/C are two bacterial transmembrane proteins of approximately 310 aa (*Bacillus halodurans* C-125), encoded by the *HflA* (high-frequency lysogenization) operon that regulates the lysogenic decision during bacteriophage λ infection [44]. Both proteins contain a short transmembrane domain (positions 9–26 in HflK) and two larger overlapping domains: an SPFH domain (HflK positions 25–201) covering roughly 50% of the protein, and an HflC domain (positions 10–300), associated with membrane protease activity in bacteria (fig. 2B). A putative coiled-coil region spanning residues 191–258 can also be recognized, with up to eight putative heptads and the potential to form dimers (fig. 2B). While their SPFH domains are predicted to be globular, the C termini seem more likely to be unfolded and lack multiple EA motifs.

Finally, podocin is an integral membrane protein exclusively expressed in podocytes of the kidney, and plays a key role in the stability of glomerular slit diaphragm [45]. Podocin contains an SPFH domain covering about 40% of the polypeptide (human position 123–279) and a transmembrane domain (positions 102–128). The C terminus seems to lack defined structures and no coiled-coil domain can be predicted. A conserved palmitoylation site is present at human Cys-101, and a confirmed phosphorylation site (human Tyr-332) is located toward the C-terminal end [46]. We also compared the amino acid substitution ratios in reggies/flotillins and other SPFH members. In general, the overall intragroup substitution ratios in stomatins, prohibitins and bacterial HflK/C proteins follow the same trend as in reggies/flotillins, with dN/dS values <1 (table 1), indicating that their intragroup sequence conservation can be explained through negative selection acting throughout the protein, and particularly at the SPFH domain.

Discussion

Distinct origin of reggie (flotillin) and reggie-like proteins. Reggie-1 and reggie-2 subserve functions in the assembly of specific surface proteins and intracellular signal transduction components, leading to signaling across the plasma membrane and regulation of the actin cytoskeleton [17, 18]. The broad evolutionary distribution of reggie/flotillin and reggie-like proteins across distant phyla suggest the requirement of an ancient basic function. Our results suggest the absence of *bona fide* reggie/flotillin proteins outside the animal kingdom, indicating that reggie-like proteins with similar cellular functions may have evolved independently in bacteria, plants and fungi. The discovery of novel reggies/flotillins homologues in the urochordate *C. intestinalis* and the echinoderm *S. purpuratus* expand the taxonomic representation of reggies/flotillins to two of the three major deuterostome groups, chordates and echinoderms. These data confirm the ancient origin and extraordinary sequence conservation of reggie-1 and -2 proteins [1]. Phylogenetic analyses of the metazoan dataset recover reggie-1 and reggie-2 as monophyletic clades. Contrary to the wide distribution of *reggie/flotillin* genes among deuterostomes (e.g. echinoderms and chordates), protostomes appear to have undergone the lineage-specific loss of *reggie/flotillin* genes. Within the ecdysozoan clade (that includes arthropods and nematodes) reggie/flotillin proteins have been found in insects (e.g. *Drosophila* and *Anopheles*) but not in nematodes (e.g. *Caenorhabditis sp.*). Nevertheless, this could also be explained by the selective gene retention-loss known to have occurred in the genome of *C. elegans* [47]. Further characterization of *reggie/flotillin* genes in protostomes like molluscs and annelids (lophotrochozoan) or in hemichordates (acorn worms; Deuterostomata) will be necessary to clarify such aspects.

Beyond metazoan taxa, the low sequence similarity scores of reggie-like proteins made it necessary to use additional structural and functional criteria to identify them (see below). The previously reported bacterial and plant proteins [28], as well as a new unknown fungal protein, met these criteria and were therefore named 'reggie-like' proteins. Given the absence of reggie-like proteins in lower plants (e.g. algae) or lower eukaryotes, their presence in fungi and higher plants is surprising, and raises the scenario of an early symbiotic gene transfer between these two taxa [48].

The absence of viral, archaeal or lower eukaryotic *reggie-like* genes contrasts with the situation for other SPFH superfamily members (stomatin or prohibitin), which have been recorded in a wider range of organisms [43, 49], and in some cases greatly expanded by gene duplication, such as the six *stomatin* genes of the nematode *C. elegans* (fig. 6). In contrast with the phylogenetic 'robustness' of metazoan reggie/flotillin groupings, the low levels of similarity among non-metazoan reggie-like proteins dramatically decreases the phylogenetic support of the analysis. For example, the discrepancy between species and gene trees in bacterial reggie-like proteins (fig. 4B, C) strongly questions the relatedness of this group to metazoan reggies/flotillins and makes it difficult to assess the existence of a common ancestor between the two clades. While such an ancestor cannot be formally ruled out, the large genetic distance between reggie/flotillin and reggie-like proteins argues rather for their independent origin. This view is supported by the strikingly wide taxonomic gap where distant homologues fail to be detected, which goes beyond the existence of gaps in the databases and the masking of these ancestral gene forms through the accumulation of ancient genome rearrangements [50, 51].

We believe that the most plausible scenario for the appearance of reggies/flotillins during evolution is the independent origin of a proto-*reggie* gene in early metazoans, which underwent a gene-genome duplication event to give rise to reggie-1 and -2 [1]. The emergence of reggie-like proteins in distantly related taxa might represent the independent answer of these organisms to the parallel need to fulfill a similar functional niche. The fact that reggie/flotillin and reggie-like proteins display similar domain composition in the absence of homology might suggest that they were assembled using different but functionally equivalent protein modules (domains).

Toward understanding the structural basis of reggie (flotillin) function. Our structural analysis shows a tightly conserved distribution of protein domains in metazoan reggies/flotillins. The analysis of molecular substitution rates confirms that reggies/flotillins have evolved as a highly conserved gene family, and maintained by nega-

tive selection. The expression of reggies/flotillins in so far all tested cell lines [17] supports the notion that reggies/flotillins play a very basic and important biological role. In fact, increasing evidence indicates that the function of reggies/flotillins as scaffolds for multiprotein signaling complexes is crucial for the living cell and the developing embryo [10, 19; Málaga-Trillo E., Rivera-Milla E. and Stuermer C. A. O., unpublished data].

Our structural analysis (fig. 3A) has allowed us to explore the distribution of motifs that could be involved in processes compatible with reggie/flotillin function, such as plasma membrane association, hetero-oligomerization and binding to the actin cytoskeleton. Recent reports show that reggie-1 interacts with apical F-actin in lipid rafts of adipocytes [9]. Based on recently resolved 3D structures of actin/actin-binding protein complexes (e.g. gelsolin, DBP, actin, ciboulot and karibamide C), important interactions have been found to occur in the hydrophobic cleft of actin subunits 1 and 3 [34]. In all these models, the actin cleft is bound by small and hydrophobic residues within an α helix from the actin-binding protein. However, since the α helices known to bind actin share no appreciable sequence similarity, the actin cleft is likely to accommodate interactions with a range of unrelated molecules [34]. Based on its distribution of exposed small and hydrophobic side chain residues, we propose the α helix 4 from reggie-1 (more so than from reggie-2) as a candidate for a reggie/flotillin actin-binding domain (fig. 3A). In fact, experimental evidence from our group using α helix 4 deletion-construct approaches suggests that this region (mainly in reggie-1) is able to bind to actin fibers in Hela cells [M. F. Langhorst, personal communication].

Likewise, ongoing experiments in our group suggest that the reggie/flotillin functional unit is a hetero-tetramer, which can even associate into high-order clusters [G. P. Solís, unpublished data]. Some of the structural features described here support this notion, such as the presence of multiple heptad coiled-coil motifs in all reggie/flotillin proteins, which are known to stabilize the coiling of heptads around each other [52]. In reggie/flotillin heptads, surface-exposed residues at positions 2, 3 and 5 are frequently occupied by EA motifs or Lys (K), Asp (D), Arg (R) or Gln (Q) (fig. 1) residues, which due to their charged side chains are also likely to interact in intermolecular complexes. This reggie/flotillin arrangement fits well with the typical requirements for the formation of coiled-coil-based oligomeric complexes [35], which are probably the most common oligomerization motif found in proteins [53]. Moreover, reggie/flotillin tandem heptad arrays are organized in three clusters disrupted by incomplete heptads, creating the potential for each of them to interact independently with a different partner molecule (fig. 3C). The existence in reggies of a C-terminal PDZ3-binding motif, which in stomatins is known to mediate the assembly of large multiprotein complexes [54], might also support the function of reggies/flotillins as platforms for the assembly of multiprotein complexes.

Increasing evidence shows physiologic differences between reggie-1 and reggie-2 at the cellular and organismic level [reviewed in ref. 17]. The stability of reggie-2 expression has been observed to depend on that of reggie-1 [20; A. Reuter, personal communication]. Moreover, in some cells, reggie-2 is differentially translocated to the nucleus [55]. These differences between the intracellular dynamics of reggies/flotillins may be explained by the irreversible myristoylation observed in reggie-1 [7, 8]. In contrast, the palmitoylation occurring in reggie-1 and -2 seems to be a more reversible modification that provides a more rapid trafficking to and from the membrane [7]. Indeed, we observe that the sites involved in reggie/flotillin fatty acid modification are highly conserved in metazoans, underscoring their functional importance. The lack of analogous myristoylation or palmitoylation sites in reggie-like proteins from bacteria, fungi and plants suggests that these molecules associate to the plasma membrane using different mechanisms than metazoan reggies/flotillins, for example via a transmembrane domain, as in the case of the *B. subtilis* yuaG protein. This observation is in agreement with the obvious differences observed in the lipid compositions and characteristics of prokaryotic and eukaryotic membranes [56]. Notably, like metazoan reggies/flotillins, one plant reggie-like protein (*A. thaliana* At5g25250) has been found in the first exhaustive analysis of plant DRM preparations [57]. In agreement with Liu et al. [9] and Neumann-Giesen et al. [8], in eukaryotes, lipid modifications are important but not necessary to drive reggie/flotillin and reggie-like proteins to membrane lipid raft microdomains.

The SPFH concept revisited. Members of the SPFH protein superfamily are associated with the plasma or mitochondrial membranes, and are involved in cellular processes leading to protein turnover and oligomerization [29]. In most eukaryotic family members, the SPFH domain lies on the cytoplasmic side of the plasma membrane; in prohibitin, it protrudes from the inner mitochondrial membrane into the intermembrane space, and in the bacterial HflK/C family, it is on the periplasmic side of the plasma membrane. In some cases, as in stomatin, aberrant splicing or deletion of the SPFH domain leads to severe pathologies that can be traced back to early developmental stages [58]; mutation can lead to impaired mechanotransduction processes [59] and locomotion co-ordination (unc-24 in the nematode *C. elegans* [60]. Remarkably, most of the mutations that disrupt the gene activities of *C. elegans mec-2* and *unc-1* occur at conserved residues in the SPFH domain, suggesting that this domain is crucial for such functions. Similarly, disruption of prohibitin activities leads to a larval arrest phe-

notype in fruitflies [61]. Finally, mutations in podocin genes result in the disruption of normal podocyte slit diaphragms leading to lethal kidney disease [46].

Although proteins of the SPFH superfamily appear to be mostly microdomain constituents and crucial for many biological processes, the criteria to define this group rely on coarse sequence comparisons that do not provide information about the expected common molecular basis for their functional importance. Our sequence and structural analyses of SPFH members produced only ambiguous relationships among them, particularly at the SPFH domain (fig. 5). Instead, sequence similarities seemed stronger at the C terminus, where EA-rich motifs become obvious by visual inspection. Moreover, phylogenetic analysis fails to provide support for the common ancestry of the SPFH superfamily, and estimation of the overall dN/dS ratios shows clear differences in their patterns of molecular substitution. This result underscores the distinct molecular evolutionary dynamics among SPFH members and suggests their independent origin. Thus, their evolutionary homology seems rather unlikely, although we cannot discard alternative scenarios, given that phylogenetic information vanishes due to the large divergence times between the organisms considered. Interestingly, all known SPFH family members (even those in bacteria [44], are involved in the scaffolding of specific DRMs, and exert their functions by forming platforms for the assembly of a specific set of proteins involved in signal transduction. For example, the three proteins neph, nephrin and podocin form microdomains in the slit diaphragm of the kidney [62]; prion proteins and other GPI-anchored proteins assemble in reggie/flotillin microdomains [6], and mechanosensory ion channels function within stomatin microdomains [63]. Even unrelated proteins such as caveolins perform comparable scaffolding functions in analogous caveolae DRM structures [64]. Interestingly, caveolins present certain structural similarities to reggies/flotillins, such as palmitoylation sites [65] and oligomers in DRM fractions [66].

The large sequence divergence between reggies/flotillins and reggie-like proteins is likely to be a reflection of their independent origin. Remarkably, they have evolved into structures with similar domain composition, which appear to have similar intrinsic functional capabilities. Our cladistic and phylogenetic analysis of the SPFH superfamily indicates that this protein group has little phylogenetic support, and that reggies/flotillins are distinguished by the unique combination of two conserved protein domains (SPFH and flotillins). The structural features uncovered by our work should allow us to better define, experimentally test and confirm important assumptions about the molecular interactions required for reggie/flotillin function, and the mechanism underlying their intrinsic scaffolding properties.

Supplementary material. Tables containing sequence datasets are available as supplementary material online.

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