The clathrin and a caveolar coat may assemble similarly

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Running title: Reggie is similar to the MVP

Summary

The predicted structure of the major vault protein (MVP) has been solved and has suggested that one protein only can form a proteinaceous giant particle in the form of a caveola. Caveolae are surface invaginations at the plasma membrane of a distinct size (50 - 60 nm) and are abundant in endothelia, fibroblasts and smooth muscle. As shown here, significant overall structure similarity of Reggie-1/Flotillin-2 can be predicted to MVP with a novel search algorithm HHpred. Our previous results have suggested the quantitative and high abundance of Reggie-1/Flotillin-2 in caveolae. The caveolar vault would thus, if similarity pertains as I propose, contain an outer shell of fibrillar polymers that could in a consertal change affect conformational changes. Clathrin cages assembled by clathrin-adaptor complexes may provide a paradigm for caveolar self-assembly that may implicate cholesterol in surface invagination by caveolin-1 interaction followed in reggie-flotillin vault-like self-assembly.

A major role of coat proteins in vesicular transport

Vesicular transporters are found in the exoplasm releasing immuno-related factors, they are also found in organ growth and development (Fevrier & Raposo, 2004) and they are primarily important in the cytoplasm in delivering cargo from the endoplasmatic reticulum to select intracellular destinations. The cycles of cargo release and internalization have thoroughly been studied and has revealed that coat proteins play a major role (Sollner & Rothman, 1996; Wickner & Schekman, 2008). The coat protein for caveolae, reggie/flotillin, is an abundant intracellular protein and is found in many phyla including species of eukaryotes and procaryotes (Rivera-Milla et al, 2006). The repeat structure is termed the Flotillin domain and is preceded by a domain belonging to the SPFH family (including band 7, stomatin, prohibitin and HflK/C class). It is described to mediate the interaction of flotillin in hetero- and homooligomeric complexes in expressed constructs (Solis et al, 2007; Volonte et al, 1999). The function of reggie/flotillins as coat proteins of an integral membrane protein type has been proposed based on the carbonate resistant relatively tight membrane binding (Bickel et al, 1997) but the exact function has remained unresolved. Expression of the proteins leads to vesicle formation in insect cells which may indicate a function in inducing a membrane bud but the lack of specificity in the assay allowed no further conclusions without membrane reconstitution and quantification (Volonte et al, 1999). In caveolae containing a cage-like surface structure reggie-1/flotillin-2 (48 kDa), strikingly, is the most abundant protein leaving out caveolin-1. This was found on Western blots stained for total protein (21 kDa)(Chatenay-Rivauday et al, 2004). Concerning a transmembrane span, the membrane protein predictions for reggie-1/flotillin-2 were weak (Babuke & Tikkanen, 2007; Bickel et al, 1997) with for example two charges with reggie-1 within residues 26-47 or one charge within the same span 28-45 (TMpred hydrophobicity prediction and other automated procedures). For a monotopic membrane protein, this loop seems short and has now been shown to be non-essential for membrane association since primarily N-terminal myristoylation and palmitoylations provide a stable membrane binding

(Neumann-Giesen et al, 2004). The protein is unlikely to be a multipass membrane protein since no second, hydrophobic stop transfer anchor sequence could be predicted. Also for caveolin-1 questions have been raised about its integral membrane protein nature (Schlegel & Lisanti, 2000). Caveolin-1 can be assigned as the most abundant caveolin in the apical caveolae (Chatenay-Rivauday et al, 2004), yet, reggie proteins belong to the class of proteins that are not only found in caveolae but also in lipid rafts. Lipid rafts are assemblies of microdomains that similar to caveolae require cholesterol for their formation (Anderson & Jacobson, 2002; Simons & Toomre, 2000). They have also been termed a general uptake pathway that could be carried out by vesicle formation from these membranes (Romer et al, 2007) in cycling of vesicles and uptake of glycolipid-bound toxins, and in bacterial uptake. Viral uptake seems to implicate a caveolar route as a possible none-obligatory uptake pathway (Pelkmans et al, 2004). Reggie/flotillin tightly associates to the surface of caveolae as demonstrated by confocal microscopy at high resolution in primary cultures of bovine aortic endothelial cells and in coimmunoprecipitation analysis (Chatenay-Rivauday et al, 2004), and reggie/flotillins may then shape multiple domains and provide their structural stability.

The caveolin-1 membrane assembly likely persists for a long time

The caveolin-1 membrane assembly likely persists for a long time and no exchange of caveolin-1 tagged with green fluorescent proteins could be shown in heterokaryons (Tagawa et al, 2005). Consistence is demonstrated by the existence of stable large 200 kD and 400 kD - 600 kD caveolin-1 complexes (Monier et al, 1995) that may be also similar to the *in vivo* assembly. The complexes can even be discerned on large 2D SDS-PAGE after high urea treatment. Interestingly, tetramers of reggie proteins are outmost resistant to 6 M urea treatment and endogenous reggie complexes can be composed of homo- and heterotetramers of the reggie proteins likely forming by coiled-coil interactions (Solis et al, 2007). It was logic to pursue further the biochemical analysis to pin down more lipid raft factors with endogenous proteins from an animal source. Caveolin-1 can form

homooligomers (e.g. α with β) and based on these interaction studies reggie-1/flotillin-2 stably associates with caveolin-1 α and some is likely bound to non-caveolar membrane domains or dissociates in contrast to the very high quantity observed in the immunoisolate (Chatenay-Rivauday et al, 2004). Naturally, we don't see the quantity in association with caveolin-1 that stably remains attached to a vesicle in the reduced dimensionality of the membrane since more molecules will collide in the plane of the membrane even when loosely attached.

The caveolar membrane coat

Stable caveolae structures are attached to the plasma membrane without a discernible electron dense coat which is observed with coated vesicles in clathrin-mediated endocytosis (Moore et al, 1987). Coatomer-coated vesicles in intra-Golgi traffic also show an electron-dense coat (Schekman & Orci, 1996). But freeze etch technology has only allowed to visualize a striated membrane attached or membrane integral structure similar to a vault with caveolae, which likely contains repeated units (Anderson & Jacobson, 2002; Peters et al, 1985; Rothberg et al, 1992) and may contain a macromolecular complex of homo- or heterooligomers, that are, however, unlikely to be formed out of caveolin coiled-coil α helical chains since none could be predicted with the Lupas prediction for caveolin-1 (below a 10% value) (Lupas et al, 1991). The analysis of the assembly of clathrin triskelia has proven that a shell out of clathrin-AP-2 subunits can form a hollow particle which encloses the clathrin-coated vesicle (Smith et al, 1998).

Caveolar transport and self-assembly

In an attempt to further clarify the role of caveolar coat proteins I have chosen flotillin-2/reggie-1 for structural comparison with the protein database PDB with the novel HHM pred (Hidden-Markov-Model) (Soding et al, 2005) search to analyze structurally similar proteins that would fulfil the stringent criteria. The HHM pred search is implemented with family-wise structural prediction

or PDB DSSP data retrieval, and I stringently assigned the major vault protein (MVP) for a further modelling (Slovak et al, 1995) of a structure with the MODELLER programme (see later). Only a short subdomain of soluble reggie-1 had been analysed in NMR (residues 45-178) which scored at the top of the HHM pred alignment with an overall score of 203.2 (not shown). The SPFH stomatin domain from archaea which is similar to the N-terminal part of reggie-1 scored slightly less, 164.1, and was succeeded by the MVP (Anderson et al, 2007) with a very high score not possible to visualize with simpler database searches including BLAST (Fig. 2). The MVP has a score of 122.0 which is highly significant, with a very likely secondary structure match to reggie-1/flotillin-2 with a domain that is as yet distinct from the major vault protein repeat domain (repeats in residues 99-357). MVP has a predicted coiled-coil region from residues 670-815 which is at the C-terminus of the match. Overall, reggie-1/flotillin-2 matches the second half of the MVP as shown in the alignment (Fig. 2); the probability is 99.4 % of an overall structural match with a highly reliable E-value of 1.8E-17 and single full length alignment P-value with reggie-1 of 9.7E-22. The reggie-1/flotillin-2/ESA, previously named epidermal surface antigen, remarkably aligns with the AEA conserved repeat structure that can be detected with RADAR (Heger & Holm, 2000) against the C-terminal domain strongly predicted to form α helical bent rods in MVB. Conserved repeats of reggie-1/flotillin-2/ESA are indicated in bold font (Fig. 2). Although the C-terminal domain of reggie-1/flotillin-2 likewise has a predicted coiled-coil in this region (residues 225-350) the best match to MVP is proximally in residues 2-124 (obtained with local alignment). This includes part of and extends further into the AEA repeat structure.

Would reggie self-assemble?

The MVB protein is shown to self-assemble and forms vault-like (hence their naming) 41.7- x 41.7- x 67.5-nm^3 cytosolic particles that are proposed to be involved in intracellular drug transport. The vault assembly which pertains to two 48-mers forms a hollow core with an approximately 0.8 nm (α

helical) to 2.4 - 5 nm (globular) thin outer layer (Anderson et al, 2007). The symmetry has allowed to reconstruct the structure at 9 Å EM resolution to match the MVP chain extending full half of the barrel/vault. The double-layered cap extends towards the C-terminal part and has itself reduced 24 fold symmetry. It is striking to observe that the full length reggie-1/flotillin-2/ESA matched to the MVB fold would extend for at least 22 nm (Fig. 1).

The calculated structural model for reggie-1/flotillin-2/ESA (Fig. 1) is at this point truly speculative but the likelihood of a similar molecular fold is given by the full length alignment with the match from shoulder to double-layered cap (see nomenclature of (Anderson et al, 2007)). This includes the domains 9-14 that have been predicted according to the electron density of the vault factor (neglecting the C-terminal residues). ROSETTA *ab initio* prediction had been used in the MVP fold assignment and has allowed to conceptualize the assembled structure (Anderson et al, 2007)(Fig. 3). The MVB fold is now out and allows to conceptualize the self-assembly of the caveolar coat permitted the structural similarity holds true. Following the interaction of caveolin-1 with cholesterol which may induce protein conformational changes (Murata et al, 1995) a caveola may self-assemble with repeated helical units of MVP-like proteins that would form outer layers of the caveolae. Caveolin proteins could provide the attachment for the reggie-flotillin outer shell and membrane stability could be provided by this multi-layered assembly. Considering the self-assembly properties of the clathrin coat it will be interesting to shed light on the similarities and differences to clathrin-adaptor complex self-assembly (Moore et al, 1987; Smith et al, 1998). Possible adaptor functions of caveolin or any other protein for caveolae have not been identified and the possibilities of cargo transport for physiological substrates have not been elucidated to that important point of cargo-mediated clathrin-coated vesicle formation where adaptin-cargo interactions play a crucial role. Moreover, the latency of vesicles in vesicular transport with packaged cargo is also a hallmark of other transport processes which remains to be shown. It may yet be other functions such as mechanotransduction and growth regulation that caveolae may come

across. The mechanical device could shape the membrane to adjust the conformations and activity of a multitude of enzymes and e.g. ion channels at the same time to execute a coupled synchronized signalling. Functions and mechanisms in this direction remain to be explored at a later time.

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Figure 1

The MODELLER (Marti-Renom et al, 2000) output of reggie-1 is presented similar to the overall structural views of cpMVP published in Anderson et al. (Anderson et al, 2007). Reggie-1 from human source was aligned with HHpred to the PDB database. HHpred (Soding, 2005) is a fast performing database search programme based on structural comparison that automatically aligned residues 1-428 in full length of reggie-1/flotillin-2/ESA to the C-terminal half of MVP (the major vault protein, residues 468-873). The last 94 amino acid residues of the MVP have not been modelled and/or crystallized and thus the C-terminal 126 residues of reggie-1/flotillin-2/ESA are also not included in the structure modelled based on the aligned MVP.

Figure 2

HHpred search (Soding et al, 2005) output is shown. For the search parameters: secondary structure score:yes, search:local, realign with MAP:yes, threshold=0.0 in local search mode were chosen with the global realignment. The alignment generated by the procedure lists the secondary structure propensity (E or H) (SS_pred) with a score (SS_conf). Template DSSP values determined from the 3 D structure are indicated. The consensus sequence query (reggie-1/flotillin-2) or template 2qzv (MVP) is indicated (Consensus). This database entry can be retrieved in the PDB websource with the code from http://www.rcsb.org/pdb/. The AEA conserved repeats of reggie-1/flotillin-2/ESA are indicated in bold font. Both proteins have a coiled-coil region predicted with the Lupas programme (Lupas et al, 1991): MVP from residue 670-805, ESA/reggie-1/flotillin-2 from residue 225-350.

Figure 3

The barrel of the vault with predicted matched polypeptide chains is shown. The vault consists of 96 mers of the 95.8 kD major vault protein (MVP), the other minor vault proteins are not required for self-assembly and include approx. 3 copies of telomerase associated protein TEP1, 12 copies of poly(ADP-ribose)-polymerase and small untranslated RNA. The high resolution structure determined at 9 Å resolution allows to fix the polypeptides to the provided geometry. The view corresponds to the shortened C-terminal vault. Since 120 amino acids have not been included in expression of cpMVP the structure will include an extension of unknown fold in the centre. The function of the vault has not been established but may include intracellular transport in drug shuttling or resistance to bacterial infection and clearance of pathogens (Mossink et al, 2003).

Figure 1

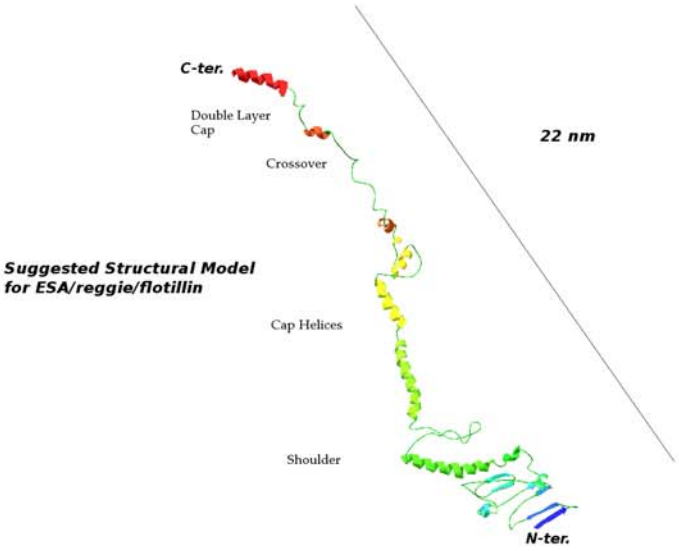

C-ter.
Double Layer Cap
Crossover
22 nm
Suggested Structural Model for ESA/reggie/flotillin
Cap Helices
Shoulder
N-ter.

Figure 2

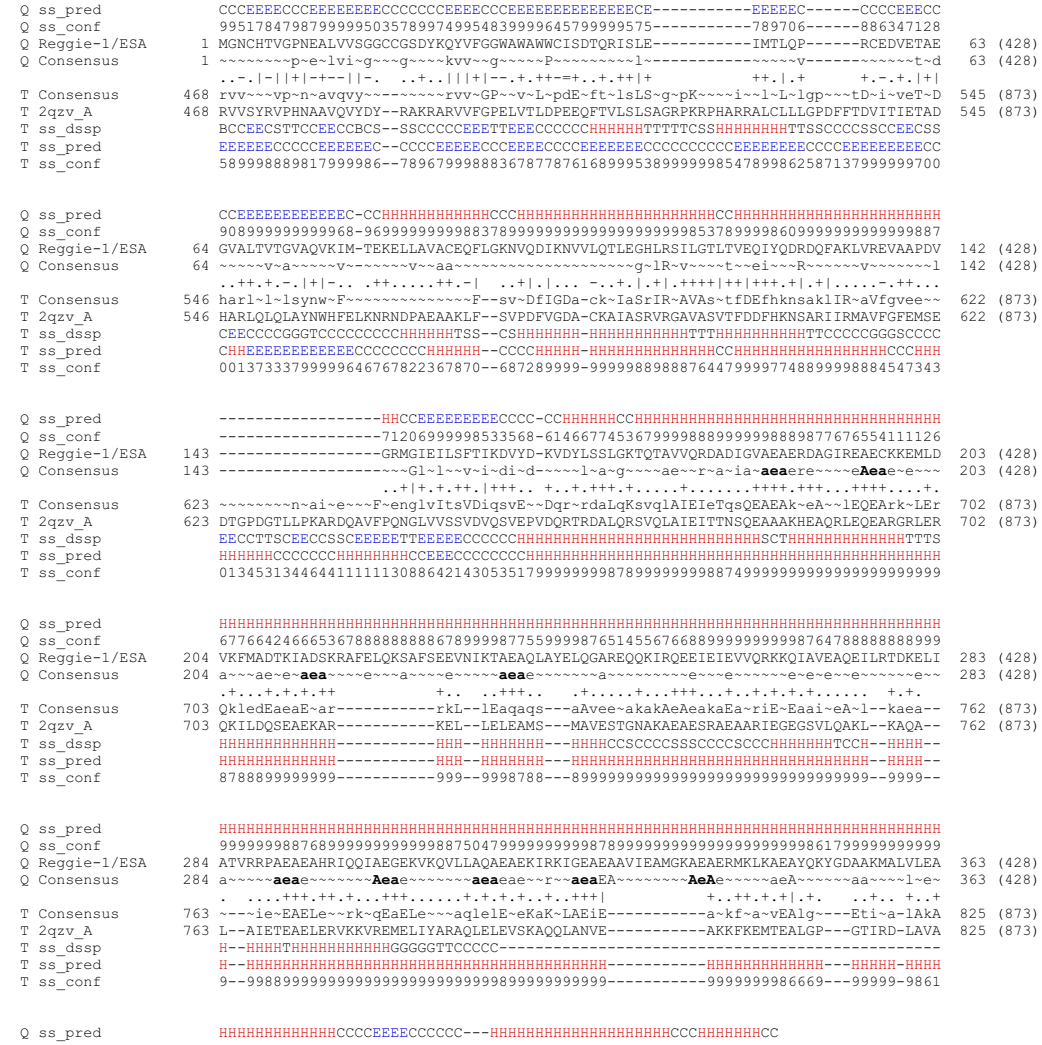
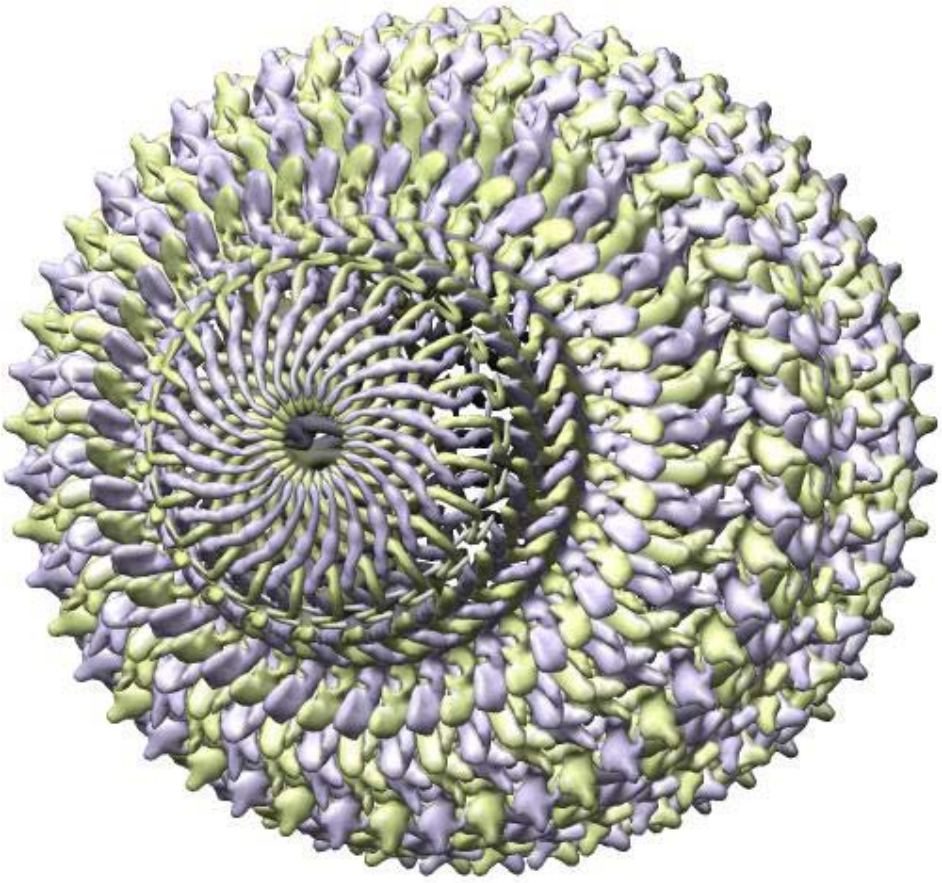


Figure 3



http://www.rcsb.org/pdb/explore/explore.do?structureId=2QZV