5 DISCUSSION

5.1 Synaptic DRMs as a platform for synaptic vesicle cargo protein sorting and retrieval as an entity by CME

During all coated-vesicle mediated membrane trafficking events, cargo needs to be enriched within the nascent vesicle according to the cellular requirements. Although many cargo proteins or cargo receptors are sorted by virtue of specific sorting signals in their cytosolic tails, some cargos lacking obvious sorting modules can still be incorporated into coated-vesicles. Synaptic vesicle proteins for which no sorting motifs have been identified yet can be recycled via CME. In this system, sorting might be achieved independently of sorting signal peptides or by combining sorting modules and other sorting mechanisms. One possible mechanism to achieve sorting would be the use of PM microdomains.

We have used quantitative nanoLC-MALDI-MS/MS from synaptic DRMs and identified 159 proteins. These are involved in a variety of cellular functions, such as plasmalemmal signaling, synaptic scaffolding, as well as some mitochondrial proteins etc. Since triton extraction in the cold is supposed to generate large heterogeneous microdomain aggregates (Heerklotz 2002), the identification of this diverse set of proteins involved in different cellular functions may reflect the co-purification of different PM microdomains enriched in cholesterol. One surprising finding is that many synaptic vesicle, endocytic and exocytic proteins are identified as components of synaptic DRMs. This implies that synaptic vesicle proteins may be organized in cholesterol-enriched microdomains.

Previous immuno-electron microscropy studies showed that ~25% of synaptobrevin 2 is present at the presynaptic plasmalemma under physiological conditions (Taubenblatt, Dedieu et al. 1999). More recent work by Wienisch and Klingauf using proteolytically cleavable and pH-sensitive synaptobrevin 2- and synaptotagmin 1-GFP constructs revealed that synaptic vesicle proteins mix with stranded vesicle proteins after exocytosis. Furthermore, compensatory CME internalizes previously stranded vesicle proteins (Fernandez-Alfonso, Kwan et al. 2006; Wienisch and Klingauf 2006). Synaptotagmin 1 stranded at the PM after previous rounds of exocytosis was shown to localize in microdomains (Willig, Rizzoli et al. 2006). A general concern of the biochemical isolation of DRMs from any biological sample

is artificial aggregate formation due to treatment with detergent. Our flotation assays using both Triton X-100 and CHAPS for extraction demonstrate that synaptic vesicle proteins Syt 1, synaptophysin and synaptobrevin 2 all localize to synaptic DRMs in a cholesterol-dependent manner. These data suggest that synaptic vesicle proteins are *bona fide* cholesterol-dependent synaptic membrane microdomain components.

If synaptic vesicle proteins partition into DRMs, one question would be whether the synaptic vesicle proteins already formed a cholesterol-dependent complex before vesicle exocytosis or following integration into the PM. Many early data demonstrate that synaptic vesicle proteins are associated in protein complexes (Becher, Drenckhahn et al. 1999; Mitter, Reisinger et al. 2003; Reisinger, Yelamanchili et al. 2004). For example, a large CHAPS-resistant complex including SV2, synaptotagmin 1, Rab3A, synaptophysin and synaptobrevin 2 has been identified by Scheller and colleagues (Bennett, Calakos et al. 1992). Furthermore, synaptophysin binds to synaptobrevin 2 in a cholesterol-dependent manner (Mitter, Reisinger et al. 2003). As both synaptophysin and synaptotagmin 1 have been shown to directly associate with cholesterol (Thiele, Hannah et al. 2000), one can envision that synaptic vesicle proteins may have been present as a cholesterol-dependent complex before vesicle exocytosis, consistent with the high concentration of cholesterol (Deutsch and Kelly 1981; Takamori, Holt et al. 2006). Co-immunoprecipitation experiments using antibodies against synaptotagmin 1 from CHAPS-extracted crude synaptic vesicles show that synaptotagmin 1 forms a cholesterol-dependent complex with synaptophysin, synaptobrevin 2 and SV2. In contrast, another transmembrane protein, the transferrin receptor, does not co-immunoprecipitate any synaptic vesicle transmembrane proteins. Synaptotagmin 1 can bind to the μ-subunit of AP-2 through a basic stretch in its C2B domain, and this interaction is strongly enhanced by co-recognization of tyrosine-based endocytic motifs derived from the cytoplasmic tail of another cargo, such as SV2 (Haucke and De Camilli 1999). One can imagine that after exocytosis, synaptic vesicle proteins retain concentrated or re-clustered in microdomains enriched in cholesterol. According to this model cholesterol may act as a permission scaffold to keep synaptotagmin 1 and at least some synaptic vesicle proteins lacking sorting signals co-clustered. During the following round of CME, the synaptotagmin 1-AP-2 interaction and the binding of the tyrosine-based motif in SV2 to the exposed cargo binding pocket of AP-2 μ subunit will foster SV cargo enrichment within clathrin-coated pits. At presynapses, additional adaptor proteins like stonin 2 may act together with AP-2 to achieve internalization and retrieval of synaptotagmin 1 (Diril, Wienisch et al. 2006; Walther, Diril et al. 2004). Overexpression of synaptotagmin 1 in fibroblasts lacking stonin 2 results in

its PM localization. Co-expression of stoning 2 in this system has been shown to be sufficient to restore clathrin/AP-2-dependent synaptotagmin 1 endocytosis.

Using primary hippocampal neurons, we also showed that synaptotagmin 1, synaptophysin, synaptobrevin 2, AP-2, and clathrin are resistant to extraction with Trition X-100 at 4°C. Functional data using FITC-CTB uptake in hippocampal neurons demonstrated that internalized FITC-CTB co-localizes with synaptotagmin 1 and flotillin, but not the non-DRM protein transferrin receptor. This is consistent with previous studies in which Shaogomori *et al.* showed that cholera toxin was found in detergent-insoluble microdomains of hippocampal neurons but internalized via a raft-independent mechanism (Shogomori and Futerman 2001). In contrast, CTB binds to GM1 on PM surface and is internalized via clathrin-independent, dynamin-dependent pathway in motor neuromuscular junctions (Roux, Colasante et al. 2005). Another virulence factor, tetanus toxin exploits clathrin-independent and clathrin–dependent pathways for its regulated encytosis and targeting in neuromuscular junctions (Deinhardt, Berninghausen et al. 2006). So clathrin-independent and clathrin-dependent routes could overlap to fulfill endocytosis of ligands at least in some model systems. This is further supported by recent observations that anthrax toxin (Abrami, Liu et al. 2003), chemokine receptor 5 (Venkatesan, Rose et al. 2003; Signoret, Hewlett et al. 2005) and prion protein (Sunyach, Jen et al. 2003) can all be internalized by overlapping pathways.

PM microdomains have been indicated to be involved in a variety of cellular functions, like signal transduction, serving as a sorting platform that recruits and excludes certain proteins in PM and T cell receptor signaling (Grzybek, Kozubek et al. 2005; Kusumi and Suzuki 2005; Kabouridis 2006). However, the size and stability of cholesterol-rich microdomains are still a matter of controversy (Stowell, Marks et al. 1999; Sharma, Varma et al. 2004) due to their dynamic character and the limitations of fluorescent microscopy (Heerklotz 2002). The conventional and classical definition of lipid rafts was based on the enrichment of cholesterol, sphingolipids and saturated glycerolipids in membrane microdomains (Simons and Ikonen 1997). Interestingly, later studies in HEp-2G cells showed a reversible disruption of transferrin and EGF internalization upon cholesterol-depletion but not of the general PM marker ricin (Rodal, Skretting et al. 1999). Another unbiased proteomic screen performed to detect lipid raft proteins in Hela cells identified clathrin as a DRM component (Foster, De Hoog et al. 2003). These studies indicate that cholesterol is a common important component of microdomains including both DRMs and clathrin-coated pits in certain cell types.

Cholesterol is not the only lipid needed to regulate synaptic vesicle cycling, others include DAG (diacylglycerol) (Rhee, Betz et al. 2002; Rosenmund, Sigler et al. 2002), sphingolipids (Rohrbough, Rushton et al. 2004) and PI(4,5)P_2 (Di Paolo, Moskowitz et al. 2004; Martin 2000). PI(4,5)P_2 was shown to form sub-micrometer patches at the PM and to regulate the releasable vesicle pool in chromaffin cells for secretion (Milosevic, Sorensen et al. 2005). It also provides binding sites for the α and μ subunits of AP-2 (Gaidarov, Chen et al. 1996; Rohde, Wenzel et al. 2002) and accessory proteins like AP-180 (Ford, Pearse et al. 2001), Dab2 (Yun, Keshvara et al. 2003), dynamin (Zheng, Cahill et al. 1996) and epsin (Ford, Mills et al. 2002) during CME. Lang *et al.* showed that the SNARE protein syntaxin is localized within 200 nm large, cholesterol-dependent microdomains where it co-localizes with another SNARE protein, SNAP-25 (Lang, Bruns et al. 2001). Furthermore, they showed that these cholesterol-dependent microdomains are different from lipid rafts since they are Triton X-100 soluble. Another study by Chamberlain also reported that proteins of the exocytic machinery were present in cholesterol-dependent microdomains and exocytosis were disturbed by cholesterol depletion in PC12 cells (Chamberlain, Burgoyne et al. 2001). The metabolism of PI(4,5)P_2 at the synapse is regulated by both its synthesis and degradation (Wenk and De Camilli 2004; Haucke 2005; Rohrbough and Broadie 2005). At the synapse, the main enzyme responsible for the generation of PI(4,5)P_2 from PI(4)P is PIPKIγ (Wenk, Pellegrini et al. 2001), and its activity is regulated by Arf6 (Krauss, Kinuta et al. 2003) and AP-2 (Krauss, Kukhtina et al. 2006) to facilitate the recruitment of clathrin and AP-2. Synaptic DRM localization of PIPKIγ in our study demonstrates that local generation of PI(4,5)P_2 may also coordinate the cycling of synaptic vesicles. Since Arf6 is not found in synaptic DRMs in our proteomic screen, we do not know whether Arf6 regulates PIPKIγ activity inside or outside synaptic DRMs.

Since membrane microdomains are presumably heterogeneous in terms of size and lifetime within the PM, one future challenge is to find out whether our *in vitro* data are of physiological relevance in synaptic vesicle cycling *in vivo*? In STED microscopy, the excitation beam is overlapped with a doughnut-shaped beam that is capable of de-exciting fluorophores by stimulated emission. Co-alignment of the beams ensures that fluorescence is allowed only in the center area of the spot. This allows the separation of point objects which are 45 nm apart in the focal plane. Willig et al. used STED microscopy to visualize the localization of stranded synaptotagmin 1 at the PM after exocytosis and they could show that synaptotagmin 1 remains clustered in isolated patches within presynaptic membrane under both mildly and intensively stimulated conditions. This suggests that at least some synaptic

vesicle proteins stay together at the plasmalemma after exocytosis (Willig, Rizzoli et al. 2006).

Two proteins in the regulation of cholesterol homeostasis have been shown to bind cholesterol, SCAP (SREBP cleavage-activating protein) and HMG-CoA. They both contain transmembrane domains which can bind and sense cholesterol (Brown and Goldstein 1999). Synaptophysin and synaptotagmin 1 also bind cholesterol (Thiele, Hannah et al. 2000). More interestingly, we observed that some synaptic vesicle proteins can form cholesterol-dependent complexes. Therefore, synaptic vesicle proteins may use transmembrane regions to interact specifically in cholesterol-dependent lipid complexes at the PM. These interactions can form long-term lipid shells in the membrane which have high affinities for preexisting membrane microdomains (Anderson and Jacobson 2002).

PM microdomains can also form upon either ligand binding or other triggers from outside or inside of the PM. Sharma *et al.* used fluorescence lifetime imaging microscopy based on homo-FRET (fluorescent resonance electron transfer) between GPI-anchored proteins and folate receptor or GFP-GPI. These authors showed that 20-40% of GFP-GPI proteins localize in cholesterol-dependent clusters smaller than pentamers (<5 nm), while the remaining 60-80% exist as monomers. Monomers and small pentamers may be in a dynamic equilibrium and be able to form larger microdomains in a dynamic process. Taking advantage of the size of giant unilamellar vesicles (GUVs) together with fluorescent microscopy, the effect of cholesterol on partitioning different lipids into liquid-order and liquid-disordered states was studied *in vitro* (Kahya, Scherfeld et al. 2003). Cholesterol was shown to promote lipid segregation between dioleoyl-phosphatidylcholine-enriched, liquid-disordered, and sphingomyelin-enriched, liquid-ordered phases. The lipophilic probe 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-C18) was excluded from sphingomyelin-enriched regions, where the raft marker ganglioside GM1 was localized. If this *in vitro* result holds true also in native membrane, we can envision that fusion of a synaptic vesicle (~40 nm) with the plasmalemma and mixing of the released cholesterol might trigger a local segregation into liquid-ordered microdomains. This domain can be used to keep most, if not all, the vesicle components together and prevent their diffusion into the plasmalemma until the following round of CME for the replenishment of recycling synaptic vesicle pool.

Although this is a very attractive model for cargo sorting at chemical synapses, we can not draw a clear conclusion at this stage due to the highly dynamic process of synaptic vesicle recycling; but a combination of a good fluorescent tracer for cholesterol and high resolution microscopy in the future may finally solve this puzzle.

5.2 Membrane bending by Arfs during clathrin-mediated transport

5.2.1 Arfs and membrane curvature

Arfs have long been known to be anchored at membranes via their amino-terminal helix presumably consecutive actions of membrane-associated guanine nucleotide-exchange factors (GEFs) and N-myristoylation of the amino-terminal helix of Arf at Gly2 (Antonny, Beraud-Dufour et al. 1997; Franco, Chardin et al. 1996; D'Souza-Schorey and Chavrier 2006).

In our study, we show that Arf1 and Arf6 can bind and tubulate liposomes with different profiles *in vitro*. This capacity is attributed to their amino-terminal amphipathic helix, since mutants of the hydrophobic residues abolish tubulation capacity and/or association with membranes. By contrast to the broad binding spectrum of Arf6 to many negatively charged lipids, Arf1 only binds to PI(4,5)P_2 liposomes. Among different lipids distributed throughout the cell, phosphoinositides are compartmentalized as signposts at the cytosolic face of different compartments (Behnia and Munro 2005; McLaughlin and Murray 2005; Di Paolo and De Camilli 2006). PI(4)P is the predominant housekeeping phosphoinositide at the Golgi/TGN (Shin and Nakayama 2004; De Matteis, Di Campli et al. 2005; Donaldson 2005), although regulated PI(4,5)P_2 synthesis has been shown to be important for Arf1-mediated exit of cargo from the TGN (Godi, Pertile et al. 1999). Our data are consistent with this and suggest that Arf1 is recruited to the TGN via PI(4,5)P_2.

Godi *et al.* found that Arf1 could recruit PtdIns-4-OH-kinase isoform β (PI4Kβ) and a so-far unidentified PtdIns-4-phosphate-5-OH-kinase to the Golgi complex. As a result, high levels of PI(4,5)P_2 can be generated locally. The authors also showed also that these effects were independent of other known functions of Arf1 on COPI coat proteins and phopholipase D (Godi, Pertile et al. 1999). This sequential recruitment of two kinases by Arf1 to generate locally high concentrations of PI(4,5)P_2 fits the rationale that PI(4,5)P_2 which is mainly restricted to the plasmalemma can be produced already at the level of the TGN from where it is probably transported to the PM following the secretory pathway. This also points out that Arf1 can form a positive feedback loop to generate PI(4)P and subsequently also more PI(4,5)P_2 to enhance further recruitment of Arf1 to the TGN. Since PI(4)P is enriched at the TGN, the locally generated PI(4,5)P_2 may specifiy hotspots for budding of secretory cargo en route to the PM. By constrast PI(4)P may define budding sites for trafficking to endosomes

and lysosomes through recruitment of Arf1 GEFs. In this scenario, distinct phophoinositides may help to discriminate cargoes destined for transport to either the PM or to lysosomes in Arf1 dependent carriers.

In comparison, Arf6 binds to a much broader spectrum of negatively charged lipids including PS, PA, and PI(4)P. One distinct property of Arf6 in comparision to other Arfs is that Arf6 is a basic protein with a predicted pI in the range of 8.5-9.5. Other Arfs display pIs between 6.0 and 7.0. This positively charged surface may explain why Arf6 can bind to such a broad range of negatively charged lipid headgroups. Consistent with this it has been observed that Arf6-GDP, unlike Arf1-GDP, is still largely retained on the PM (Cavenagh, Whitney et al. 1996; Song, Khachikian et al. 1998).

Since Arfs get a myristoyl modification on the second amino acid glycine, it has long been believed that this modification contributes critically to membrane targeting (Haun, Tsai et al. 1993; Randazzo and Kahn 1995). However, our *in vitro* experiments show that membrane binding also involves basic and hydrophobic side chains within the NH_2-terminal amphipathic helix.

The hydrophobic side chains of the amino-terminal helix residues can insert into one layer of the membrane bilayer which results in an asymmetric distribution of lipids for membrane bending. Our mutagenesis studies show that mutation of with two hydrophobic amino acids into hydrophilic residues at positions 4 and 5 in the first helical turn of Arf1 and Arf6 do not affect membrane association, yet inhibit tubulation activity *in vitro*. In comparison, similar mutants within turn 2 of the helices of both Arf1 and Arf6 abolish both lipid binding and tubulation abilities. Both mutants were also cytosolic when over-expressed in COS7 cells, while their wild-type counterparts were associated with the Golgi (Arf1) or the PM (Arf6). The hydrophilic side of the helix contains positively charged lysine residues. Our mutagenesis experiments indicate that these charged residues contribute to liposome binding most likely via electrostatic interactions with negatively charged phospholipids headgroups (McLaughlin and Murray 2005). We suggest that the initial contact with the membrane and thus the on-rate of the reaction is largely driven by electrostatic interactions whereas the N-myristoylation and the hydrophobic side chains that partition into the cytosolic leaflet may stabilize the bound state and thus determine the off-rate

Class I and II Arfs have overlapping localizations at both the Golgi complex and endosomes. Arf1-Arf5 thus may facilitate clathrin- and COPI- vesicle formation by bending membranes at the Golgi apparatus and endosomes. Instead, Arf6 may aid generation of membrane curvature during the formation of CCVs and other types of clathrin-independent vesicles at the PM (Donaldson and Honda 2005). Arf6 can bind to and stimulate PIPKIγ for local generation of local $PI(4,5)P_2$, which serves as a hotspot for endocytosis at the PM (Krauss, Kinuta et al. 2003).

A similar mechanism for the generation of membrane curvature is used by epsin and Sar1 in clathrin- and COPII-coated vesicle formation. Epsin was first identified as a binding partner for Eps15 (Chen, Fre et al. 1998), which has been later shown to fulfill different functions including cargo sorting, lipid binding, AP-2 and clathrin recruitment during CCV formation at the PM (Horvath, Vanden Broeck et al. 2007). Ford *et al.* recently demonstrated that the NH_2-terminal stretch of unstructured part can form an amphipathic helix upon $PI(4,5)P_2$ binding and subsequently initiates membrane curvature by inserting its hydrophobic side chains into one leaflet of the bilayer (Ford, Mills et al. 2002). Sar1 can not only initiate curvature formation at the early stage of budding but may also facilitate the final stage of fission by invading the neck region of COPII buds following recruitment and stabilization of Sec23/Sec24 and Sec13/31 coat complexes (Antonny 2006). Most evidence for budding via amphipathic helical insertion comes from *in vitro* studies. It has thus been suggested that additional factors, i.e. scaffolds are needed *in vivo* to stabilize membrane curvature. Hinrichsen *et al.* tried to answer this question by knocking down clathrin using RNAi in Hela cells (Hinrichsen, Meyerholz et al. 2006). Without clathrin, epsin and AP-2 still localized to punctuate spots at the PM. Further EM studies revealed that AP-2-domains were decorated with epsin but showed a flat surface and lacked membrane curvature, suggesting that the clathrin coat is essential for membrane invagination. Thus insertion of amphipathic helices by proteins such as epsin may be insufficient to form stable coated buds *in vivo*.

When we observed the tubule formation from lipid membrane sheets under a video-microscope, we found that Arf1-induced tubular structures were somewhat unstable and that tubule extension was always followed by fast retraction. *In vivo* Arf1 association with the Golgi is subsequently followed by recruitment of coat proteins, i.e. AP-1, GGA, COPI and clathrin. These factors may contribute to a more stabilized budding process (further discussed below). Whether Arfs work in exactly the same way as epsin and Sar1 or in a different manner is still an open question. Since Arf1 is utilized in both COPI- and clathrin-coated

vesicle formations at different sites within the Golgi complex, its precise role for membrane curvature in these two pathways may be much more complicated than previously anticipated.

One interesting question concerning membrane curvature by amphipathic helix insertion is how deep the hydrophobic side chains can insert into the lipid bilayer. To answer this question EPR (electron paramagnetic resonance) measurement could be used. We have mutated the fifth position residue phenylalanine to tryptophan for EPR experiments. PE labeled with a doxyl group at different positions of the fatty acid chain can be used to detect the quenching efficiency with regard to the shifted tryptophan spectroscopy signal upon liposome binding and insertion.

5.2.2 Assembly of GGA1- and clathrin-coated carriers

Arf1 recruits adaptor proteins like the heterotetrameric AP-1, COPI and monomeric GGA proteins. In the case of COPI-coated vesicles, βCOP can bind Arf1 for further assembly of coatomer vesicles. To understand its function in membrane deformation and adaptor-mediated coated-vesicle generation, we selected GGA1 and clathrin to investigate membrane curvature during coated-vesicle formation using a minimal *in vitro* system.

The truncated human GGA1(1-426) which contains the VHS, GAT and clathrin box in the unstructured second linker region can bind to PI(4)P- and PI(4,5)P_2-liposomes *in vitro*. The potential binding site for lipids may be localized within VHS domain since the VHS domain shares similarity with the ANTH/ENTH domains which are modules known to bind to PI(4,5)P_2. According to the known structure of PI(4,5)P_2 incomplex with ANTH/ENTH and AP-2, two putatively positively charged surfaces of yeast Gga2 VHS domain responsible for PI(4)P binding have been predicted on helices α1 and α8 (Demmel *et al,* submitted). Both yeast GGAs and mammalian GGAs can bind to the active form of Arf1 in its GTP-bound state via the amino-terminal GAT domain.

To check the cooperative effect of membrane bending by Arf1-GTP and GGA1, we first had to exclude the possibility that GGA1 itself can tubulate liposomes. Actually, GGA1 did not generate tubular structures from lipid membrane sheets by itself. Similar results have been seen for Rab11 and other proteins that can bind to, but not tubulate membrane. Interestingly, when Arf1-GTP was injected together with GGA1(1-426) and clathrin into the lipid membrane sheet chamber, massive, very stable straight tubules were formed. These tubules were similar to those generated by dynamin (Roux, Uyhazi et al. 2006) and F-BAR (Itoh,

Erdmann et al. 2005) proteins, suggesting a possible protein oligomerization on the tubule. Further cryoEM structure analysis of the protein decoration pattern on the lipid membrane tubule could help to clarifiy this.

Recent systematic and combinatorial RNAi knock down experiments in Hela cells showed only a weak phenotype when any single Arf was knocked down. Combined knock-down of at least two Arfs in coordination was needed to induce a severe defect in secretory or endocytic traffic (Volpicelli-Daley, Li et al. 2005). If the bending capacity of the oligomerized Arfs is critical for vesicle trafficking, we speculate that homo- or hetero-oligomerization of Arfs is the driving force to initiate vesicle budding on the donor membrane. Curved membranes at this early stage may be further stabilized by adaptor proteins such as GGAs, and coat proteins like clathrin or COPI, until the final fission step takes place. Fission may either be mediated by GTP hydrolysis of Arf itself, as in the case of Sar1, or facilitated by a specialized fission protein like dynamin (Jones, Howell et al. 1998). We will further study the Arf1-GGA-clathrin-coated vesicle budding process *in vitro* and its physiological relevance *in vivo*.

To finally confirm the importance of the *in vitro* tubulation capacity of Arf1 in the secretory pathway from the TGN, tubulation-deficient mutants of Arf1 will be over-expressed in COS7 cells, and a temperature-sensitive, thermo-reversible folding mutant of vesicular stomatitis virus glycoprotein (ts VSVG) will be used to check whether the secretory pathway is affected. Using temperature block, the G protein could be localized to the ER (39.5°C), to the vesiculo-tubular clusters (15°C), to the TGN (20°C), and to the PM (37°C). To make sure that any possible defect in the secretory transport is a direct result of tubulation deficiency, myristoylation will be checked by using tritium-labeled myristic acid to feed COS7 cell, and immuoprecipitation is followed by fluorography detection.

5.2.3 Arf GAP

The selective membrane traffic is regulated by Arfs which themselves are regulated by their nucleotide-cycle. GEFs sit on cognate membranes and recruit Arfs and promote GTP-loading, whereas GAPs can induce GTP hydrolysis that leads to the disassembly and dissociation of vesicle coats (Zeghouf, Guibert et al. 2005; Nie and Randazzo 2006). All Arf GAP proteins can be divided into six groups according to their primary structure (Randazzo and Hirsch 2004). The last four subgroups are called AZAP, for Arf GAPs with ankyrin repeats and PH (pleckstrin homology) domains. The first subgroup Arf1 GAP-1 has an ALPS (for Arf GAP-1 lipid packing sensor) motif that forms an amphipathic helix. This amphipathic helix can sense

the membrane curvature of formed coated vesicles and determines the time-point of Arf1 GTP hydrolysis to induce coat disassembly (Bigay, Gounon et al. 2003; Bigay, Casella et al. 2005). ASAP-1 and ACAP-2 in the AZAPs subgroup have an NH_2-terminal a BAR domain followed by PH domain. BAR domains are protein modules that sense or induce membrane curvature (Gallop and McMahon 2005). PH domains are specific phosphoinositide binding modules responsible for membrane targeting (Lemmon 2004). The combination of BAR and PH domains may target these proteins to membranes with specific phosphoinositides and induce membrane curvature generation. Actually, ACAP-1 and ACAP-2 have already been shown to tubulate LUVs *in vitro* (Jackson, Brown et al. 2000; Nie, Hirsch et al. 2006). This membrane deformation capacity of Arf GAPs adds another layer of sophisticated complexity of membrane curvature regulation during the process of coated-vesicle formation.

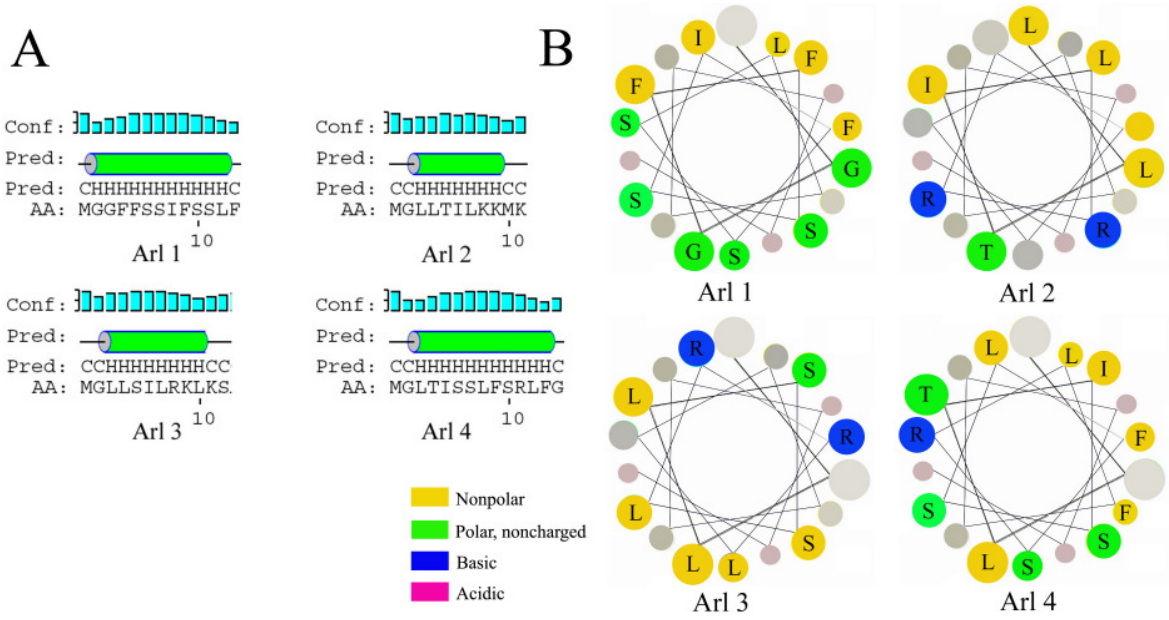

Figure 5-1 Arl 1-4 secondary structure prediction and helical wheel analysis

The same procedure like the one used in **Figure 4-11** and **4-12**. Sequences from rat were used.

5.2.4 Arf like proteins (ARLs)

Arf-like proteins (ARLs) were first identified using degenerate oligonucleotide primers (Clark, Moore et al. 1993; Schurmann, Massmann et al. 1995). They share 50-60% sequence identity with Arfs and are structurally related. So far, more than ten genes have been identified that encode Arls in the human genome. They are classified into different groups according to their primary sequence (Pasqualato, Renault et al. 2002). Although the function of most Arls is not known, initial data suggested that some Arls, for example Arl1, localize to the Golgi complex where they regulate membrane trafficking (Lu and Hong 2003; Lu, Tai et al. 2004). Although some Arls are suggested to lack myristoylation, most of them contain a conserved glycine at positions for myristate modification. One exception is Arl3p which

needs NH_2-terminal acetylation for its native localization at the Golgi apparatus (Setty, Strochlic et al. 2004). Their secondary structure prediction also shows an amino-terminal helix with amphipathic properties as illustrated in **Figure 5-1**. Since myristoylation is not needed for Arfs to tubulate liposomes, we can speculate that Arls can also bend membranes.