MEMBRANE TRAFFICKING IN PROTOZOA: SNARE PROTEINS, H⁺-ATPASE, ACTIN, AND OTHER KEY PLAYERS IN CILIATES

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Abstract

Due to their well-defined pathways of vesicle trafficking and manyfold mutants ciliates have served as good model systems. Further studies required the development of databases, now available for *Paramecium* and *Tetrahymena*. A variety of key players have been identified and characterized based on BLAST search, domain analysis, localization, and gene-silencing studies. They include NSF (*N*-ethylmaleimide sensitive factor), SNAREs (soluble NSF attachment protein [SNAP] receptors), the H⁺-ATPase (V-ATPase) and actin, while Arf (ADP-ribosylation factor) and Rab-type small GTPases, COPs (*coatamer* proteins) and many others remain to be elucidated. The number of SNAREs, H⁺-ATPase subunits, and actins ever found within one cell type are unexpectedly high and most of the manifold vesicle types seem to be endowed with specific molecular components pertinent to trafficking. As in higher eukaryotes, multifactorial targeting likely occurs. It appears that, in parallel to higher organisms, ciliates have evolved a similar structural and molecular complexity of vesicle trafficking.

Key Words: Actin, Ciliate, H⁺-ATPase, Membrane, *Paramecium*, SNAREs *Tetrahymena*, Trafficking.

1. INTRODUCTION

In 1997, Hutton (1997) stated “it will be intriguing to learn whether homologues exist in these organisms [the ciliates] of the syntaxin, SNAPs, synaptobrevin, synaptotagmin, or other molecules, which have been

implicated in synaptic vesicle docking and exocytosis...” Now, detailed answers to many of these questions, and to some additional ones, can be presented.

1.1. State of discussion with higher eukaryotes

Intracellular vesicle trafficking is governed by multiple molecular components and presumably the latest common eukaryotic ancestor was already endowed with a multitude of them (Dacks and Field, 2007). Key players are SNAREs, that is, soluble *N*-ethylmaleimide attachment protein (SNAP) receptors, small monomeric GTP-binding proteins (GTPases, G-proteins), the vacuolar type H^+ -ATPase (V-ATPase), COP (*coatamer* or *coat protein*), and clathrin-type cytosolic membrane coats as well as elements of the cytoskeleton, including microtubules and filamentous actin (F-actin, microfilaments). In addition, many more proteins, some with modulatory or auxiliary function contribute to vesicle trafficking. Specific small GTPases of types Rab and Arf (ADP-ribosylation factor) can be assigned to specific sites which also contain specific phosphoinositides (Behnia and Munro, 2005). Components are exchanged on the way through the cell. This 4D-puzzle has been repeatedly reviewed (Behnia and Munro, 2005; Jackson and Chapman, 2006; Jahn et al., 2003; Malsam et al., 2008; Pfeffer, 2007). Figure 3.1 outlines the interactions of some of the principal molecules engaged in vesicle trafficking, as to be discussed in subsequent sections.

SNAREs are crucial for membrane-to-membrane interactions, that is, for docking of a vesicle to a target membrane (v- and t-SNAREs) and for final fusion (Jackson and Chapman, 2006; Jahn and Scheller, 2006; Jahn et al., 2003; Martens and McMahon, 2008). This became increasingly evident since the pioneer work of J. Rothman’s group from the early 1990s on (Nickel et al., 1999; Rothman, 1994; Rothman and Warren, 1994; Söllner et al., 1993a,b).

Until the early 1990s, other hypotheses, specifically for membrane fusion, have been preferably envisaged and it has been largely questioned whether membrane proteins may play any role at all in membrane interactions leading to fusion. For instance, fusion was explained by Ca^{2+} -mediated local lipid-phase transitions. In contrast, work with the ciliated protozoan cell, *Paramecium tetraurelia* had suggested at that early time already a decisive role for membrane-integrated and -associated proteins (Plattner, 1981, 1987, 1989; Vilmart and Plattner, 1983). This concept had been endorsed by numerous mutations in the sequence of the secretory pathway specifically in the ciliate, *P. tetraurelia* (Beisson et al., 1976, 1980; Bonnemain et al., 1992; Lefort-Tran et al., 1981; Pouphe et al., 1986; Vayssié et al., 2000, 2001). It should also be appreciated that such work from the Beisson group, with Jean Cohen and Linda Sperling (CNRS,

Figure 3.1 Principal mechanisms cooperating during vesicle trafficking, as exemplified by compartments endowed with SNAREs and H^+ -ATPase as well as with interacting F-actin. These components, analyzed mainly in *P. tetraurelia* and to a smaller extent in *T. thermophila*, are in the focus of the present review on trafficking in ciliates. The right side of the scheme refers specifically to exocytosis. Sequence from left to right. **Acidification:** Vesicles possess a set of v- (R-) SNAREs and a Ca^{2+} -sensor (not yet identified in ciliates) as well as an H^+ -ATPase (bright blue) undergoing conformational change as a consequence of luminal acidification (as shown in other cell types). **Activation:** The conformational change of the H^+ -ATPase allows for binding of an Arf-type small GTPase (dark blue) and its activator (red ball)—as shown in other cells, thus allowing for targeting. **Tethering:** Targeting to an appropriate compartment includes tethering. So far there is only evidence of some tethering effect of F-actin in ciliates, while exocyst (for constitutive exocytosis) and any other potential tethering components have not been clearly identified as yet. **Docking:** After tethering, docking ensues, involving pairing of the v- (R-) SNARE with the t- (Q-) SNAREs of which for simplicity only one type has been drawn. In *Paramecium*, we identified R-SNAREs of the type synaptobrevin (yet mainly as longin forms) and Q-SNAREs of the type syntaxin and SNAP-25-LP. As in other systems, in *Paramecium* only the (majority of the) first two possess a transmembrane domain which is a prerequisite to subsequent membrane fusion. **Ca^{2+} release and influx:** This occurs during stimulated exocytosis in response to a stimulus. As found with *Paramecium*, activation of cortical stores (alveolar sacs, green) causes Ca^{2+} release which precedes and entails a superimposed Ca^{2+} -influx (“SOC mechanism”). **Membrane fusion:** Increase of the local cortical cytosolic $[Ca^{2+}]$ activates the system for membrane fusion, provided SNARE zippering has preceded. It produces a membrane continuum, with mixture of the contents (inside the cell) or their release (exocytosis). Regrettably little is known on other key players, such as small G-proteins/GTPases and their regulators as well as of a Ca^{2+} sensor in ciliates.

Gif-sur-Yvette, France), served as a nucleation center for the development of the *Paramecium* genome project.

While cytoskeletal elements had been acknowledged early on as important components of intracellular trafficking in many systems, the significance of

SNAREs, of different vesicle coats, of small GTPases and, most recently, that of the H^+ -ATPase have been recognized only with some delay. Many such details are meanwhile known also from ciliates, mainly *Paramecium*.

1.2. State of research with ciliates

While basic concepts have been detected in other cells, from yeast to mammals, work with ciliates still does not yet cover all these fields. To mention just a few of the regrettable gaps in ciliate cell biology: Apart from their presence in *Paramecium* (Suchard et al., 1989) and *Tetrahymena* (Kersting et al., 2003; Leonaritis et al., 2005; Ryals and Kersting, 1999), almost nothing is known from ciliates, for example, on the distribution and turnover of phosphoinositides—another regulation principle known from higher eukaryotes (Behnia and Munro, 2005). Moreover, since the early recognition of a complex family of small GTPases in *Paramecium* (Fraga and Hinrichsen, 1994; Peterson, 1991) little detailed insight has been achieved. Molecular analysis of COPs is another gap to be filled.

In the years since the last review on vesicle trafficking in ciliates (Plattner and Kissmehl, 2003a) many new tools have become available and, thus, enabled the identification of important molecular aspects. This includes the cloning of the macronuclear genome of *Paramecium* and *Tetrahymena*, paralleled by key publications (*P. tetraurelia*: Arnaiz et al., 2007; Aury et al., 2006; Dessen et al., 2001; Zagulski et al., 2004; *Tetrahymena thermophila*: Coyne et al., 2008; Eisen et al., 2006; Orias, 1998). Databases are accessible as follows: <http://www.genoscope.cns.fr/paramecium> and <http://paramecium.cgm.cnrs-gif.fr> for *P. tetraurelia* and <http://www.ciliate.org/> for *T. thermophila*, respectively. For *Tetrahymena*, see also protein database, <http://www.tigr.org/tdb/e2k1/ttg/>. A database for the fish-pathogenic ciliate species *Ichthyophthirius multifiliis* is being elaborated (see internet). See also <http://www.genenames.org> for aspects of gene/protein designation, databases for protein types, and for specific protein domains.

In 1987, the first transformation of a *Paramecium* cell has been performed by microinjection of a cloned gene (Godiska et al., 1987). This was followed by complementation cloning (Haynes et al., 1996; Skouri and Cohen, 1997) and establishment of indexed genomic libraries (*P. tetraurelia*: Keller and Cohen, 2000; *T. thermophila*: Hamilton et al., 2006). Posttranscriptional homology-dependent gene silencing (siRNA technology) is possible (*P. tetraurelia*: Bastin et al., 2001; Galvani and Sperling, 2002; Ruiz et al., 1998; *T. thermophila*: Chilcoat et al., 2001; Howard-Till and Yao, 2006; Shang et al., 2002). In *Paramecium*, the mechanism behind may reflect the same principle that mediates faithful elimination of the IES (internal eliminated sequences) when, in an epigenetically controlled process, micronuclear genes are edited for storage in the macronucleus by comparison with the old macronuclear genome (Garnier et al., 2004; Meyer and Cohen, 1999).

With *Tetrahymena*, the efficient mass transformation achieved by electroporation (Gaertig et al., 1994) or by DNA-loaded particle bombardment (Cassidy-Hanley et al., 1997), eventually allowing also the production of germline transformants, is of big advantage. With *Paramecium* transformation of postautogamous cells by macronuclear injection is the rule, but from electroporation and particle bombardment also good results have been reported (Boileau et al., 1999). After adaptation to the specific code, proteins can be expressed as green fluorescent protein (GFP)-fusion proteins (Hauser et al., 2000a).

Wherever available, the exploitation of special databases, for example, for SNARE proteins, with the inclusion of all organisms analyzed, proved helpful in the molecular analysis of vesicle trafficking. Every time when we try to assign proteins, identified by molecular biology, to certain subcellular components we realize the importance of previous ultrastructural and functional analyses some of which have been conducted in admirable detail. Examples that were particularly helpful to us along these lines are the painstaking analyses by Richard Allen and his associates on the phagolysosomal and the osmoregulatory system in *Paramecium* (Allen, RD). <http://www5.pbrc.hawaii.edu/allen/>

Ciliates deserve special interest also for practical reasons as they are closely related to important protist groups which in part are animal and plant pathogens. An evolutionary relationship between ciliates and the apicomplexan parasites (*Plasmodium*, *Toxoplasma*) becomes increasingly robust in the literature, while this is somewhat less pronounced for heteroconts, such as plant pathogenic oomycetes and the large, nonpathogenic group of brown algae (phaeophyceae, kelp) (Baldauf et al., 2000).

Figure 3.1 can serve as a section summary as it presents the most important interaction partners during vesicle trafficking in eukaryotic cells, including SNAREs, H^+ -ATPase, actin, and GTPases, of which the first three have been elucidated to some extent in *Paramecium*, while information on G-proteins in ciliates is restricted.

1.3. *Paramecium* and *Tetrahymena* as model systems for membrane trafficking

Ciliates are highly organized cells, particularly with regard to vesicle trafficking, *Paramecium* being the best analyzed example for the time being (Allen, 1988; Allen and Fok, 2000; Fok and Allen, 1988), followed by *Tetrahymena* (Frankel, 2000). An outline of the main trafficking pathways is presented in Fig. 3.2. Widely different approaches have been applied particularly to *Paramecium*. It possesses not only numerous regularly arranged sites for the exocytosis of dense core-secretory vesicles (trichocysts) (Beisson et al., 1976; Plattner and Kissmehl, 2003a, b; Plattner et al., 1973), where exocytosis-coupled endocytosis also takes place (Allen and Fok, 1984a;

Figure 3.2 Main trafficking pathways in ciliates. (A) Three main vesicle trafficking pathways in ciliates, as analyzed mainly with *Paramecium* (to which the scheme refers), but to a considerable extent also with *Tetrahymena*. Green: exo-endocytotic pathways, mainly based on cited work with *Paramecium* (by J. Beisson and her then associates and by the present author and his coworkers) as well as with *Tetrahymena* (by A. Turkewitz). The general trafficking scheme is based on a figure by Kissmehl et al. (2007); therein the part concerning phago-lysosomal components (red) is based mainly on cited work with *Paramecium* (by R. Allen and A.K. Fok and their collaborators). Yellow: Unexpectedly, in the cited work on SNAREs, we found evidence of vivid trafficking in the contractile vacuole/osmoregulatory system of *Paramecium*. Abbreviations: a, ampulla; as, acidosomes; ci, cilia; cp, cytoproct; cv, contractile vacuole; ds, decorated spongiome; dv, discoidal vesicles; ee, early endosomes; er, endoplasmic reticulum; fv, food vacuole; ga, Golgi apparatus; gh, “ghosts” (from trichocyst release); oc, oral cavity; pm, plasmamembrane; ps, parasomal sacs; rv, recycling vesicles; sm, smooth spongiome; tr, trichocyst; trpc, trichocyst precursors. (B) The three main trafficking pathways depicted in Fig. 3.2A are shown here in more detail, each pathway with a remarkable number of membrane interactions by fusion and fission. Based on the scheme by Plattner and Kissmehl (2003a).

During cyclosis of a phagosome—the “food vacuole” serving digestion of food bacteria—several defined fusion/fission processes occur (Allen and Fok, 2000; Fok and Allen, 1988, 1990). In *Paramecium*, it has been shown that, after a nascent phagosome has pinched off, the first step is acidification by fusion with acidosomes (Allen and Fok, 1983a), followed by fusion with lysosomes and endocytotic vesicles (Allen and Fok, 2000; Fok and Allen, 1988, 1990). In addition, lysosomal membranes and enzymes are recycled (Allen and Fok, 1984b). Furthermore, two other sets of vesicles are recycled back to the nascent phagosome. First, pieces of membrane are detached as “discoidal vesicles” from the phagosome once it has achieved some degree of maturation (Allen and Fok, 1983b; Allen et al., 1995). Second, membranes from old phagolysosome are recycled from the cytoproct, the site of release of spent materials, also as discoidal vesicles (Schroeder et al., 1990). Additional small round vesicles occur along the oral cavity, particularly in zones with regular arrangement of cilia (“quadrulus” and “peniculus”) and some vesicles slide along the “oral fibers,” probably to the nascent food vacuole (Ishida et al., 2001).

Constitutive endocytosis by bristle coated pits/vesicles takes place by “parasomal sacs” that are stereotypically arranged on one side of the basis of cilia on the cell surface outside the oral cavity (Allen, 1988). Until now, one has generally assumed that constitutive endocytosis vesicles can assemble only at the sites of parasomal sacs. In this restricted area, the cell surface is not occupied by ciliary basal bodies or by alveolar sacs—the cortical Ca^{2+} -stores (Hardt and Plattner, 2000; Stelly et al., 1991). We now have evidence that there are potential sites for docking and detachment of small vesicles also outside this narrow region (Schilde et al., 2010), as outlined in Section 3.2. After pinching off vesicles travel to the “terminal cisternae” (Patterson, 1978), now considered as early endosomes that fuse with Golgi-derived vesicles to form late endosomes (Allen, 1988).

The contractile vacuole complex of *Paramecium* (Allen, 2000; Allen and Naitoh, 2002) mainly serves osmoregulation. It has been shown to perform cyclic membrane fusions, not only at the outlet of the contractile vacuole (the “porus”), that is, at the level of the cell membrane, but also at the sites where radial/connecting canals emanate from the vacuole (Tominaga et al., 1998a,b). Recently, we have found the unexpected occurrence of SNAREs in the contractile vacuole complex also outside these sites of periodic membrane fusions (Kissmehl et al., 2007; Schilde et al., 2006, 2008). Therefore, the contractile vacuole complex may contain many more fusion sites than previously assumed for this organelle.

Remarkably, some trafficking steps can take place in *Paramecium* in a highly synchronous manner (Plattner et al., 1993). In particular, exocytosis (Plattner et al., 1984, 1985b) and exocytosis-coupled endocytosis (Plattner et al., 1985a, 1992) can be massively triggered and, thus, studied under highly synchronous conditions. Many parameters, ultrastructural, biochemical, and biophysical, can then be analyzed correlatively within a subsecond

time range (Plattner and Hentschel, 2006). An example is the documentation of point fusion at a time when patch-clamp analysis could not yet approach that problem (Plattner et al., 1992).

As a summary of this subsection, Fig. 3.2 highlights the main vesicle trafficking routes in *Paramecium*—specifically one along the endoplasmic reticulum (ER), the Golgi apparatus, and secretory vesicles; another one going along the endo-/phago-/lysosomal system; and a third one including the contractile vacuole system. Vesicle trafficking includes a multiplicity of fusion/fission steps in a *P. tetraurelia* cell and will be similar in other ciliates. From the multitude of membrane interaction sites, one had to expect an abundance of specific molecular key players on the different membranes involved in the respective trafficking steps, as it has actually been found.

2. FACTORS INVOLVED IN THE REGULATION OF VESICLE TRAFFICKING

2.1. Identifying SNAREs—Criteria and methodology

More easily than most other proteins envisaged in this review, SNAREs can be generally identified by domain structure analysis. Criteria now to be outlined are illustrated in Fig. 3.3 and expanded to *Paramecium* SNAREs in Section 3.1.

2.1.1. General properties of SNAREs in other systems

Whenever a BLAST search of the *P. tetraurelia* database has revealed high similarity, sequences were completed and subject to detailed domain analysis including the following criteria (as used, for instance, to identify plant SNAREs; Lipka et al., 2007). (i) Most SNAREs are single-span transmembrane proteins with a C-terminal transmembrane domain. (ii) This is followed by a SNARE domain, ~60–70 aminoacids long, with “heptad repeats” centered around a “zero-layer.” The latter contains either an R- or a Q-residue—though with a few exceptions (Fasshauer et al., 1998; Sutton et al., 1998). The α -helical SNARE domain is able to coassemble with partner SNAREs to a quarternary transcomplex (SNAREs from opposite membranes). This is a prerequisite for membrane fusion (Jahn and Scheller, 2006). (iii) More distally, in the case of the Qa-SNARE syntaxin, a Habc domain of ~47–71 aminoacids follows; this domain allows consecutive binding of α -SNAP (unrelated to SNAP-25 and similar proteins) and consecutively of the SNARE-specific chaperone, NSF (*N*-ethylmaleimide sensitive factor) (Bock and Scheller, 1996; Rizo and Südhof, 2002; Xu et al., 1999). (iv) In R-SNAREs a longin domain of ~100–140 aminoacids may follow (“longins”; Filippini et al., 2001), for example, in most plant (Lipka et al., 2007) and ciliate (Schilde et al., 2006, 2010) R-SNAREs.

The longin domain is absent from “brevins” (e.g., synaptobrevin = VAMP [vesicle-associated membrane protein]), that is, in most animal R-SNAREs (Jahn and Scheller, 2006). (v) Cysteine residues in a specific C-terminal context may allow for fatty acylation (Magee and Seabra, 2005); this is the case with the SNAP-25-like proteins (SNAP-25-LPs) and with the Qbc SNARE proper, SNAP-25 (Gonzalo and Linder, 1998; Veit et al., 1996) as well as with the Qb SNAREs, Sec9 and Spo20, in yeast (Burri and Lithgow, 2004). The molecular size of SNAP-25-LPs, however, may deviate more or less from 25 kDa, as has been found in many species, from ciliates to mammals. Its Qb- and Qc-part each contain a SNARE domain and, by

Figure 3.3 (continued)

	-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6	+7	+8																																															
205	EKYKD	I	Q	L	S	R	S	V	Q	L	V	Q	L	F	V	D	L	A	I	L	V	H	A	Q	G	Q	I	D	N	I	E	I	N	L	S	A	K	T	Y	V	G	K	A	E	K	S	L	V	D	A	K	E	D	H	Q		PtSyx1-1						
207	EKYKD	I	Q	L	E	K	S	V	Q	L	V	F	Q	L	Q	D	L	A	I	L	V	G	T	G	Q	I	T	N	I	E	G	S	L	T	S	A	K	S	Y	N	N	K	A	K	D	D	L	I	D	A	E	G	D	H	K		PtSyx1-2						
202	D	K	Y	Q	D	I	Q	L	E	K	L	S	I	Q	Y	C	F	Q	M	L	S	E	I	A	F	L	T	Q	Q	E	G	E	I	S	I	E	Q	N	L	N	K	A	K	N	I	E	R	G	E	K	R	L	A	K	Q	E	I	H	K		PtSyx2-1		
202	D	K	Y	Q	D	I	Q	L	E	K	L	S	I	Q	Y	C	F	Q	M	L	S	E	I	A	F	L	T	Q	Q	E	G	E	I	S	I	E	Q	N	L	N	K	A	K	N	I	E	R	G	E	K	A	L	A	K	Q	E	I	H	K		PtSyx2-2		
213	EKYKD	I	V	K	L	E	K	S	V	Q	Q	M	L	F	A	D	M	A	V	L	V	K	N	O	G	E	L	I	D	N	I	E	Q	N	M	V	K	A	R	D	Y	I	K	G	E	D	E	R	K	A	K	N	H	K		PtSyx3-1							
213	EKYQ	D	I	V	K	L	E	R	S	V	Q	Q	V	Y	Q	L	L	V	D	M	A	V	L	V	K	N	O	G	E	L	I	D	N	I	E	Q	N	M	V	K	A	R	D	Y	I	K	G	E	A	Q	L	V	K	A	K	D	H	Q		PtSyx3-2			
223	E	R	D	Q	E	I	N	K	L	V	T	M	I	N	E	L	A	E	V	F	K	S	L	N	Q	L	V	I	D	Q	G	T	I	L	D	R	I	D	I	N	I	D	Q	A	V	F	N	V	K	A	N	E	E	L	K	A	E	D	Y	Q	N		PtSyx4-1
225	E	N	D	Q	E	I	N	K	L	V	M	I	N	D	L	A	E	V	F	Q	S	L	N	Q	L	V	L	E	Q	G	H	L	D	R	I	D	I	N	I	D	S	Y	K	N	I	K	A	N	H	L	Q	E	S	E	R	N	Q	N		PtSyx4-2			
183	E	K	L	V	M	Q	K	I	E	S	M	L	N	D	I	A	G	V	F	Q	R	V	G	T	M	V	R	L	Q	E	T	M	I	E	R	I	D	K	Y	I	D	E	A	Q	L	N	V	S	K	R	K	E	L	Q	E	S	H	K	R	I	S		PtSyx5-1
183	E	K	L	V	M	Q	K	I	E	S	M	L	N	D	I	A	G	V	F	Q	R	V	G	T	M	V	R	L	Q	E	T	M	I	E	R	I	D	K	Y	I	D	E	A	Q	V	N	V	S	K	R	K	E	L	Q	E	S	H	K	R	I	S		PtSyx5-2
169	D	R	N	D	R	I	K	S	L	G	E	K	L	K	M	N	E	L	F	I	E	M	N	R	L	V	I	E	Q	G	T	L	D	R	I	D	F	N	I	D	Q	I	F	T	R	I	Q	K	G	K	Q	Q	L	V	Q	A	N	T	K	Q	E		PtSyx6-1
199	E	R	Q	E	L	D	I	I	E	R	A	V	L	I	N	Q	M	G	E	L	I	D	Q	Q	G	E	L	D	F	Q	G	G	E	L	D	F	Q	G	G	L	N	E	A	K	A	N	I	G	V	S	N	L	V	G	A	E	N	Q	K		PtSyx7-1		
198	E	R	Q</

Figure 3.3 (continued)

Figure 3.3 Some molecular characteristics of *P. tetraurelia* syntaxins (A–C), their evolutionary connection (D) and intracellular localization (E) by different methods, based on the work by Kissmehl et al. (2007). (A) The 15 types of syntaxins found in *Paramecium* by sequence analysis and domain structure analysis show some diversification with regard to the presence of a syntaxin domain (green), but all forms contain a SNARE domain (red), and a transmembrane region (blue). Their molecular size also varies. From Kissmehl et al. (2007). (B) Molecular modeling of *PtSyx3*-1 and *PtSyx3*-2 in comparison to 1DN1 (syntaxin 1), from *R. norvegicus* (C. Danzer, Diploma work, University of Konstanz) reveals striking similarities with regard to the arrangement of α -helical structure in the SNARE domain (green), with the Q-residue in the zero-layer indicated, and the structure of the Habc domain (yellow); red—linker. Unpublished images from the series by Kissmehl et al. (2007). (C) Core structure of the SNARE domain of *PtSyx* paralogs. Note the zero-layer with the Q residue typical of syntaxins and an exceptional A in *PtSyx11*-1. Also note the heptad repeats (repetitive aminoacids, yellow, in positions 3/4/7 upstream and downstream from the zero-layer), with some exceptional aminoacids set in green. A series of such heptad repeats in each of the SNAREs would align to a quarternary complex (“SNARE complex”). From Kissmehl et al. (2007). (D) Relationships between the different *PtSyx*

backfolding, SNAP-25-type SNAREs can contribute the third and fourth α -helical SNARE domain to the quarternary SNARE complex (Fukuda et al., 2000; Jahn and Scheller, 2006; Malsam et al., 2008; Sutton et al., 1998). (vi) However, Qb- and Qc-domains may occur as independent proteins which, in that case, are membrane anchored by a C-terminal hydrophobic stretch (Lipka et al., 2007).

Since the assignment to v- or t-type membranes may be ambiguous, SNAREs are now generally subdivided more stringently according to the aminoacid in the center (zero-layer) of the SNARE-domain which either contains an Arg/R- or a Gln/Q-residue, flanked by the periodic heptad repeats (Sutton et al., 1998). Thus, SNAREs are subdivided into R-SNAREs (synaptobrevin and related forms, including longins, v-SNAREs members), Qa- (syntaxin), Qb-, Qc-, and Qb/c-SNAREs. In sum, with the exception of SNAP-25 and SNAP-25-LPs, SNAREs are normally, though not always, membrane-anchored by a C-terminal single membrane-spanning α -helical domain (Jahn and Scheller, 2006; Jahn et al., 2003; Lipka et al., 2007; Malsam et al., 2008; Melia et al., 2002). Vesicle docking and subsequent membrane fusion requires pairing and zippering of SNAREs, whereby at least one SNARE on each side has to have a transmembrane domain (Section 3.1.2). Zippering means the formation of a quarternary coiled-coil transcomplex proceeding from the peripheral (N-terminal) to the proximal (C-terminal) part of the SNARE molecules (Lin and Scheller, 1997; Melia et al., 2002; Pobbati et al., 2006; Sorensen et al., 2006). Arg/Gln in the zero-layer support stabilization by hydrogen bonding. This has also been found by *in vitro* studies with reconstituted recombinant SNAREs. Again detailed analyses are required to establish the consequences of the deviating SNAREs found in *P. tetraurelia*.

As we shall see, specificity of membrane interactions is not, or not solely determined by the organelle-specific SNAREs (see below). Rather, specific small GTPases are of crucial importance (Section 2.2) and possibly also subunits (SUs) of the H^+ -ATPase (Sections 2.4 and 3.3). Some “auxiliary” proteins are known to contribute, for example, α -SNAP for transient

paralogs (neighbor joining tree), with probability values indicated, can be interpreted rather clearly as representing waves of whole genome duplication (pink, green, blue) discussed in the text. Composed from material contained in Kissmehl et al. (2007). (E) Intracellular distribution of *PtSyx* species, as determined by expression as GFP-fusion proteins and by antibody labeling at the light and electron microscope level. For trafficking scheme, see Fig. 3.2A. Note association of *PtSyx* 9 and *PtSyx*10 with different vesicles probably interacting with food vacuoles; similarly uncertain is the assignment of *PtSyx*14 and *PtSyx*15 to the contractile vacuole system. Also note the presence of *PtSyx*1 all over the cell surface, including the oral cavity. Many other syntaxin isoforms can be clearly assigned to specific structures; they may be exchanged during trafficking, as is the case, for example, with syntaxins associated with early and later stages of the food vacuole. cph, cytopharynx; cs, cytostome; trp, trichocyst precursors; for additional abbreviations, see Fig. 3.2A. From Kissmehl et al. (2007).

binding of NSF to the SNARE complex, as well as Munc18 (Bethani et al., 2007), Munc13, complexin, α Rim, CAPS, etc., for some fine-tuning effects. In neuronal cells, they may also contribute to priming for subsequent fusion/exocytosis of neurotransmitter vesicles (Déak et al., 2009; Wojcik and Brose, 2007). In *Paramecium*, α -SNAP and Munc 18 occur, whereas some of these proteins, such as complexin, do not occur in the database.

2.1.2. SNAREs in *Paramecium*

On the basis of criteria just outlined, SNARE genes have been identified in *P. tetraurelia* (the only ciliate analyzed so far with this regard), followed by control of expression and intron verification (Kissmehl et al., 2007; Schilde et al., 2006, 2008, 2010). However, as in other systems, there occur also the following exceptions to the rules of SNARE characteristics. (i) A C-terminal hydrophobic aminoacid stretch may be absent (Kloepper et al., 2008); examples in *P. tetraurelia* are *PtSyb7* and *PtSyb12* as well as *PtSNAP-25-LP* (Tables 3.1 and 3.2). (ii) The zero-layer of the SNARE domain may contain an aminoacid other than R or Q (Fasshauer et al., 1998); for examples in *Paramecium*, consult Tables 3.1 and 3.2. (iii) A motif for potential fatty acylation (CAAX or other motifs) may be present, but in thorough analyses we could not verify fatty acylation where it would be expected, for example, in *PtSNAP-25-LP* (Schild et al., 2008).

These serious deviations made it even more important to include some additional, independent—though equally ambiguous—criteria for the identification of SNAREs in *P. tetraurelia*: (i) Control by Northern and/or Western blots, to verify transcription and translation, allowing for the recognition of potential pseudogenes. (ii) Expression as GFP-fusion proteins which in turn (iii) should be controlled by immunolocalization using antibodies against the endogenous protein. This may also require immunogold electron microscope (EM) analysis. Here, increased sensitivity can be achieved when GFP-labeling is combined with anti-GFP antibody labeling. (iv) Gene silencing will frequently disclose specific transport pathways although one has to bear in mind that some SNAREs can travel on rather different routes within one cell (Burri and Lithgow, 2004; Kloepper et al., 2008) and that they may have to use such routes to reach their final destination.

In our work with *Paramecium*, these approaches require mutual control for the following reasons. (i) GFP-fusion proteins may become mistargeted. (ii) Antigenicity or copy numbers of endogenous proteins may be too low for detection and (iii) discrimination between closely related paralogs by polyclonal antibodies may not be possible—in contrast to GFP expressions. (iv) Gene silencing may not discriminate between closely related genes, particularly with the most recently generated subfamily paralogs (also called “ohnologs” according to an author’s name). The difference in nucleotide sequence has to be >15% as a rule to achieve selective silencing (Ruiz et al., 1998).

Table 3.1 R-SNAREs and related forms found in *P. tetraurelia*

Type of SNARE ^{a)}	Transmembrane domain	SNARE-domain	Longin domain	amino acid 0-layer	Localization ^{b)}
<i>PtSyb1</i>	+	+	+	R	Endoplasmic reticulum
<i>PtSyb2</i>	+	+	+	R	Contractile vacuole complex
<i>PtSyb3-1</i>	+	+	+	R	Endoplasmic reticulum
<i>PtSyb4</i>	+	—	(+)	—	Small vesicles in cyclosis ^{c)}
<i>PtSyb5</i>	+	—	(+)	—	Trichocyst precursors?
<i>PtSyb6-1</i>	— ^{d)}	+	+	R	Cytopharynx, nascent food vacuole (acidosomes), cytoproct, parasomal sacs, endoplasmic reticulum, early endosome
<i>PtSyb7</i>	— ^{d)}	+	+	R	No localization achieved
<i>PtSyb8</i>	+	+	+	N	Acidosomes?, cytopharynx, nascent and early stage of food vacuoles
<i>PtSyb9</i>	+	+	+	H, N ^{e)}	Acidosomes?, cytopharynx (domain of food vacuole formation)
<i>PtSyb10</i>	+	+	—	N	Ciliary basis, cell membrane/alveolar sacs complex
<i>PtSyb11</i>	+	+	—	N	One side of cytostome, occasionally on food vacuoles, terminal cisternae
<i>PtSyb12</i> ^{f)}	—	—	—	—	Cytosolic ^{f)}
<i>Sec22</i>	+	+	+	R	Endoplasmic reticulum/Golgi apparatus

Notes: Data from Schilde et al. (2006, 2010); *Sec22*: Kissmehl et al. (2007). For more details on ohnologs, see Schilde et al. (2010).

^{a)} *PtSyb1*, 2, 4, 7, and 9 are represented each by two paralogs and *PtSyb* 3, 5, and 8 each by one, while *PtSyb6-2* is a fragment.

^{b)} Parasomal sacs = clathrin-coated pits, terminal cisternae = early endosomes, acidosomes = late endosomes studded with H⁺-ATPase for delivery to food vacuoles. For terminology, see also Section 1.3.

^{c)} "Small vesicles" are ~1 μ m in size and travel with the cyclosis stream.

^{d)} With a C-terminal CCXXF/Y motif.

^{e)} H in *PtSyb9-1*, N in *PtSyb9-2*.

^{f)} Prognosticated by sequence analysis, but questionable as a SNARE.

Table 3.2 Q-SNAREs and related forms found in *P. tetraurelia*

Type of SNARE ^a	Transmembrane domain	SNARE-domain	Syntaxin domain	Amino acid 0-layer	Localization ^b
Qa group					
<i>PtSyx1</i>	+	+	+	Q	Cell membrane, cytoproct, discoidal vesicles and additional recycling vesicles, nascent and early food vacuole
<i>PtSyx2</i>	+	+	+	Q	Contractile vacuole complex
<i>PtSyx3</i>	+	+	+	Q	Terminal cisternae, one side of cytostome
<i>PtSyx4</i>	+	+	+	Q	Discoidal vesicles, oral cavity (small vesicles), for nascent food vacuole formation?
<i>PtSyx5</i>	+	+	—	Q	Golgi apparatus
<i>PtSyx6</i>	+	+	+	Q	No result achieved
<i>PtSyx7</i>	+	+	—	Q	Food vacuoles
<i>PtSyx8</i>	+	+	—	Q	Endoplasmic Reticulum
<i>PtSyx9</i>	+	+	—	Q	Food vacuoles and interacting vesicles
<i>PtSyx10</i>	+	+	—	Q	Cyclosis vesicles
<i>PtSyx11</i>	+	+	+	A	Food vacuoles
<i>PtSyx12-1</i>	+ ^c	+	—	Q	Food vacuoles
<i>PtSyx13</i> ^d		Putative pseudogene			

Qc group					
<i>PtSyx14</i>	+	+	—	Q	Contractile vacuole complex ^e
<i>PtSyx15</i>	+	+	—	Q	Contractile vacuole complex ^e
Qb/c group					
<i>PtSNAP-25-LP</i>	—	+	Duplicate heptad repeat	Q/Q ^f	Contractile vacuole complex, Endoplasmic Reticulum, food vacuoles (except early stages), oral cavity, parasomal sacs, cell membrane ^g

Notes: From Kissmehl et al. (2007); *PtSNAP-25-LP* (Schilde et al., 2008).

^a *PtSyx1*, 2, 3, 4, 5, 7, 8, 9, 10, and 14 are represented by two paralogs each, in contrast to *PtSyx6*, 11, 12, and 15 as well as *PtSNAP-25-LP* (each with one paralog only).

^b For terminology, see Table 1 and Section 1.3.

^c With an extra-long C-terminal part beyond the transmembrane domain.

^d *PtSyx13* is a pseudogene.

^e Seen only after overexpression.

^f With two SNARE domains, each one with Q in 0-layer.

^g With many diffusely labeled sites beyond those indicated.

With ciliates, additional complications may be expected from the following experience with higher eukaryotes. Specifically, the promiscuous Qa-SNARE, syntaxin 6, can interact with other Qa-, Qb-, or Qbc-SNAREs or with R-SNAREs (Wendler and Tooze, 2001). While SNAREs frequently occur in specific membranes, they may also pair “illegitimately” with noncognate counterparts when reconstituted in liposomes (Brandhorst et al., 2006; McNew et al., 2000). From this one can conclude that SNAREs possess only limited intrinsic specificity. Proof-reading by some of the “auxiliary” proteins on the way through the cell may contribute to enhance organelle-specificity (Bethani et al., 2007). No SNARE specificity has been found for homotypic early endosome fusion (Brandhorst et al., 2006), in contrast to late endosomes where the SNARE domain is thought to be responsible for specificity (Paumet et al., 2004). In contrast, in the Golgi apparatus of yeast a combination of appropriate SNAREs mediates a high degree of specific interaction (“combinatorial specificity”) (Parlati et al., 2002). In sum, a mutual balance between the respective chances and pitfalls is mandatory to achieve reliable data on SNAREs. A cross-check of the data with those contained in a global SNARE database reachable under <http://www.mpibpc.mpg.de/english/service/bioinformatics/index.html> and design of corresponding evolutionary trees is advisable. This has been included in our work with *P. tetraurelia* SNAREs (Kissmehl et al., 2007; Schilde et al., 2006) in an attempt to round up the identification of *Pt*SNAREs. Thus, such data can be put in line with >3000 SNARE sequences (complete or fragmentary) that are globally available at this time (Kloepper et al., 2008).

The currently available *Paramecium* database contains many data on SNAREs based on two sources, that is, manual annotations mainly by our group (Kissmehl et al. 2007; Schilde et al., 2006, 2008, 2010) and comparative computer search in numerous genome databases (Kloepper et al., 2007, 2008). Note on the nomenclature used in *P. tetraurelia*: To give an example, the v-SNARE synaptobrevin is designated as *Ptsyb* for the coding gene and *PtSyb* for the protein, respectively. This is followed by the subfamily and the ohnolog number, for example, *PtSyb1-2*.

Results from plant molecular biology suggest that increasing diversification of secretory activity during evolution is accompanied by increased numbers of SNAREs (Rojo and Denecke, 2008; Sanderfoot, 2007). This may be expanded to the degree of complexity of the entire morphologically seizable trafficking system which in *Paramecium* is very high. The total number of *Pt*SNARE genes currently estimated on the basis of results from our group is well comparable to multicellular organisms up to man. However, such comparison requires clear definition which molecules are considered a SNARE and to what extent ohnologs are considered separately. This is discussed in more detail in Section 3.1.1. When compared with Fig. 3.2B, in *Paramecium* the number of SNAREs may even surpass the

number of specific membrane interaction sites currently known from structural studies. This suggests further functional (and unnoticeable structural) diversification, for example, of small vesicles associated with the digestive cycle (Schilde et al., 2010). One explanation for this wide diversification may be repeated whole genome duplications with subsequent differentiation. Only the last duplication appears to have created closely related subfamily members (ohnologs) which may serve gene amplification rather than neofunctionalization (Aury et al., 2006; Duret et al., 2008).

In practice, we have identified *Pt*SNAREs applying the following methodical arsenal. First, we performed BLAST searches in the *Paramecium* database. Then the putative genes were cloned and the corresponding cDNA was prepared to identify introns. The deduced aminoacid sequence served to specify in detail domains characteristic of the different SNAREs (Section 3.1) and, by molecular modeling, to check similarities with established SNAREs from other systems. Prognostication of immunogenic stretches of the protein served production of antibodies for immunolocalization at the light and EM level as well as for Western blot analyses from subcellular fractions, as far as available. This was complemented by over-expression as GFP-fusion proteins which also augmented chances for EM localization. Finally, posttranscriptional homology-dependent gene silencing was performed by feeding transformed bacteria (Section 1.2) or by microinjection of appropriate constructs into the macronucleus.

A most elegant method is the “antisense ribosome technology.” It was developed by Chilcoat et al. (2001) for posttranscriptional gene silencing in *Tetrahymena*. This method involves the generation of cells transfected with the genes to be analyzed by insertion into the 26S rRNA (“ribosome library”). Among ciliates, however, its use has largely remained restricted to *Tetrahymena*.

We finish this section by recommending for a short overall background information on the identification of SNAREs the review by Sorensen (2005). Though SNAREs are well defined by their insertion in membranes by a single C-terminal hydrophobic stretch and by specific domains, including a SNARE-domain with a defined zero-layer, etc., there are exceptions to most identification rules. Therefore, a combination of several molecular properties has to be considered, paralleled by *in situ* analysis (localization, gene silencing), to identify functional SNAREs also in ciliates.

2.2. Small GTP-binding proteins/GTPases and their modulators

As found with higher eukaryotic systems, from yeast to mammals, small GTPases of the Arf- and Rho-type may exert, independently from SNAREs, a dominant function in determining the specificity of membrane interactions (Behnia and Munro, 2005; Cai et al., 2007; Grosshans et al.,

2006; Novick and Zerial, 1997; Schwartz et al., 2007a,b; Zerial and McBride, 2001). In fact, specificity cannot be explained in full merely by SNAREs, as outlined in Sections 2.1 and 9. The Arf-type GTPases (in complex with their activators; see below) may be exceptional, as they may interact with SNARE complexes and also with COP-type coat proteins (Poon and Spang, 2008).

One of the biggest gaps in the analysis of membrane trafficking in ciliates concerns small GTPases and their modulators, including GAPs (guanine nucleotide activation proteins) and GEFs (guanine nucleotide exchange factors), etc. Arfs are a group of larger monomeric G-proteins that are involved in budding of COP-coated vesicles in the ER and the Golgi apparatus (Anders and Jürgens, 2008; Bonifacino and Glick, 2004; Pfeffer, 2007; Section 3.3). Monomeric G-proteins can also contribute to the recruitment of motor proteins (Jordens et al., 2005) and, thereby, to the motility from the early endosome on (Nielsen et al., 1999). Specifically, Arfs also interfere with the kinetics of the F-actin system (Doherty and McMahon, 2008; D'Souza-Schorey and Chavrier, 2006), including remodeling of cortical F-actin in dense core-secretory vesicle systems (Vitale et al., 2002). Therefore, monomeric G-proteins/GTPases may exert several functions along the secretory pathway, from vesicle budding till targeting and docking.

Since small G-proteins particularly of the Rab and Arf type are considered most essential determinants of vesicle targeting (Grosshans et al., 2006; Novick and Zerial, 1997), many of them serve as markers, for example, for specific stages of the endo-/lysosomal system: Rab5 is associated with early endosomes, Rab7 with late endosomes/lysosomes, and Rab11 with recycling endosomes (Behnia and Munro, 2005; Haas, 2007; Novick and Zerial, 1997).

Considering the particular importance of Arf molecules in vesicle targeting our ignorance with regard to ciliates is a highly regrettable gap. Apart from some GTP-overlay studies (Section 3.3.3) only a few genes have been partially cloned and their translation products tentatively characterized and localized (Surmacz et al., 2006). An exception is putatively Arf-specific GEFs in *P. tetraurelia*, with homologs in other ciliates. One form related to the mammalian type, ARNO, has been cloned (Nair et al., 1999), before a list of them has been derived from homology search (Mouratou et al., 2005). This, together with the number of small GTPases and GAPs to be expected, suggests considerable diversification in ciliates. All this may contribute substantially to the impressive differentiation of vesicle trafficking, also in ciliates. Since minute diversifications may have taken place during evolution, any premature assignment to specific localization and function should be avoided as long as any detailed analyses are missing.

In the *T. thermophila* genome (Elde et al., 2005) 69 different Rab protein genes, in addition to 8 dynamin-related genes, are found (Zweifel et al., 2009).

Also for *T. thermophila*, at a FASEB meeting (2009) Aaron Turkewitz and his team (L. Bright) presented evidence of a similar number of Rabs, and intracellular localization may hopefully soon be documented. Some of them are very conserved and some other ciliate specific, each group encompassing about one-fourth of the total number. This is a remarkable number, considering that somewhat over 60 Rabs have been identified in mammalian cells and 11 in yeast (Grosshans et al., 2006). No such precise estimates are available for *Paramecium* as yet (or for any other ciliate species), but the number may be even higher than in other ciliate and nonciliate species due to the most recent whole genome duplication.

Unfortunately, G-proteins associated with food vacuoles are not known as yet. Only at later stages food vacuoles in *Paramecium* are reported to acquire Rab7 (Surmacz et al., 2006) and, in analogy to mammalian cells, a Rab-interacting protein, together with the lysosomal marker, LAMP-2 (Wyroba et al., 2007). The Cda12p and Cda13p proteins that were found relevant for cytokinesis and conjugation in *T. thermophila* (Zweifel et al., 2009) are without any identified homolog in higher eukaryotes. From their function and localization (Section 9.3) they are considered functionally related to a Rab11-interacting protein—Rab11 being a determinant and marker for recycling endosomes (Ullrich et al., 1996).

To sum up this section one may state that information about small G-proteins/GTPases in ciliates, though of paramount importance for vesicle trafficking, is only rather fragmentary. The number of Rabs in *Tetrahymena* is known to exceed that in man. In *Paramecium*, examples of our fragmentary knowledge are Rab7, some Arf-related modulators, GEF- and GAP-proteins. It now appears mandatory to fully clone and to characterize these components functionally and to map them topologically. Partial sequences can be retrieved from the databases and used as a starting point, before any definitive identification and appreciation of G-protein subfamilies and their modulators can be achieved.

2.3. Actin

2.3.1. General considerations on actin participation in vesicle trafficking

The role of cortical F-actin in secretory vesicle docking has long been debated, from merely inhibitory (Aunis, 1998) to facilitation. Only quite recently the involvement of actin in the secretory cycle, from the Golgi apparatus (Cao et al., 2005) to vesicle docking (Vitale et al., 2002), release (Mitchell et al., 2008), pore closure (Larina et al., 2007) and “ghost” retrieval (Galletta and Cooper, 2009; Giner et al., 2007; Kaksonen et al., 2006; May and Machesky, 2001; Soldati and Schliwa, 2006) has become increasingly evident. To achieve such dynamics, actin filaments can associate with myosin (Bhat and Thorn, 2009). F-actin is essential in detachment

of endocytotic vesicles, not only for exocytosis-coupled endocytosis but also for other types, such as clathrin-coated and noncoated vesicle endocytosis (Galletta and Cooper, 2009; Miaczynska and Stenmark, 2008). For a more detailed discussion of what is known about the contribution of actin to phagocytosis in higher eukaryotes (May and Machesky, 2001; Soldati and Schliwa, 2006), see Section 6. What has to be expected along these lines for ciliates?

2.3.2. Actin in ciliates

The multitude of actin isoforms in *P. tetraurelia* is surprising (Table 3.3). Within ciliates, the highest number, up to 31, occurs in species with extensive macronuclear genome fragmentation during development (Zufall et al., 2006). We found nine subfamilies, subfamily *PtAct1* with nine paralogs, *PtAct5* with three, subfamilies *PtAct2*, 3, 4, 6, and 7 each with two isoforms, and subfamilies *PtAct8* and 9 with one form each (Sehring et al., 2007a,b, 2010). Even though a few of the numerous actin forms may also be classified as actin-related and actin-like proteins, they clearly outnumber the four actin genes reported from *T. thermophila* (Kuribara et al., 2006; Williams et al., 2006) and six from man (Pollard, 2001). From the abundant actin isoforms, members of seven subfamilies were investigated by immunofluorescence, by immuno-EM analysis, and as GFP-fusion proteins and nine subfamilies by gene silencing (Sehring et al., 2007a,b, 2010). These studies also yielded clues to the drug (in)sensitivity and to polymerization properties (Table 3.3) (Sehring et al., 2007b). This may be the reason why we have noticed in phalloidin-affinity labeling studies (Kersken et al., 1986) the questionable absence of phalloidin fluorescence label from some “classical” sites where actin would definitely have been expected. Concomitantly, using antibodies against common sequences mainly from *PtAct1* paralogs we have recognized many more actin-containing sites by immuno-EM localization studies. This included the occurrence of actin at some established crossroads of vesicle trafficking (Kissmehl et al., 2004).

However, in *Paramecium* the distribution of more widely different actin isoforms varies considerably (Table 3.3). This is in line with the involvement of actin in many phenomena. In higher eukaryotes this includes the arrangement of Golgi elements (Lin et al., 2005) and the formation of Golgi vesicles (Cao et al., 2005) as well as the endo-/phago-/lysosomal system (Kjeken et al., 2004) and thereby particularly the formation of (Yam and Thériot, 2004), and recycling vesicle formation from phagosomes (Damiani and Colombo, 2003) as well as delivery of the H⁺-ATPase via lysosomal extensions (Sun-Wada et al., 2009). Again in higher eukaryotes, actin also contributes to targeting of some SNAREs and of some SUs of the H⁺-ATPase (Section 3.3). In fact, in *Paramecium* many of these sites are endowed with actin with more or less pronounced selectivity.

Table 3.3 Characteristics of actin isoforms in *P. tetraurelia*

Actin type	Amino acid identity ^{a,b} %	ATP-binding site identity ^{a,b} %	Myosin binding site identity ^{a,b} %	Localization ^c
<i>PtAct1</i> ^d				Cytoproct
<i>PtAct1-1</i>	100	100	100	Cortex, cilia, cytoproct, cytostome, oral cavity, food vacuoles
<i>PtAct1-2</i>	100	100	100	Food vacuoles
<i>PtAct1-3</i>	100	100	100	
<i>PtAct1-4</i>	90	100	100	Cytosolic compartment
<i>PtAct1-5</i>	90	100	100	
<i>PtAct1-6</i>	60	80	65	Cytosolic compartment
<i>PtAct1-7</i>	75	95	70	
<i>PtAct1-8</i>	70	80	45	
<i>PtAct1-9</i>	65	45	65	Food vacuoles
<i>PtAct2-1</i>	60	85	85	Cilia, cytosolic compartment
<i>PtAct2-2</i>	60	i.c.p. ^e	i.c.p.	
<i>PtAct3-1</i>	45	55	50	Cilia, cortex, food vacuoles, cytosolic compartment
<i>PtAct3-2</i>	45	i.c.p.	50	

(continued)

Table 3.3 (continued)

Actin type	Amino acid identity ^{a,b} %	ATP-binding site identity ^{a,b} %	Myosin binding site identity ^{a,b} %	Localization ^c
<i>PtAct4-1</i>	30	25	70	Cortex, cilia, cytostome, oral cavity, nascent food vacuole
<i>PtAct4-2</i>	30	i.c.p.	i.c.p.	
<i>PtAct5-1^f</i>	40	75	60	Cortex, cytostome, oral cavity, food vacuoles, postoral fibers, cilia
<i>PtAct5-2</i>	40	i.c.p.	i.c.p.	
<i>PtAct5-3</i>	40	65	55	
<i>PtAct6-1</i>	30	60	55	Cytosolic compartment
<i>PtAct6-2</i>	30	i.c.p.	i.c.p.	
<i>PtAct7-1^g</i>	25	20	35	
<i>PtAct7-2</i>	25	i.c.p.	35	
<i>PtAct8-1</i>	35	55	45	Cortex, cytostome, cytopharyngeal fibers, ER/Golgi, food vacuoles, parasomal sacs
<i>PtAct9-1^h</i>	20	35	20	

Notes: Results from Sehring et al. (2007a,b, 2010).

^a Amino acid sequence derived from macronuclear DNA; numbers refer to amino acid sequence of *PtAct1-1*.

^b Rounded values ($\pm 5\%$).

^c For terminology, see Section 1.3.

^d Antibody labeling, without discrimination between *PtAct1* subtypes.

^e i.c.p., identical conservation pattern within subfamily.

^f Also designated ARP-1.

^g Also designated ARP2/4.

^h Also designated ARP10.

In *Paramecium*, surprisingly numerous actin isoforms are associated with the cell cortex (Table 3.3). Specifically *PtAct8-1* is the only actin associated with parasomal sacs (Sehring et al., 2007b). Silencing of the genes of another cortical form, *PtAct4*, distorts the endocytotic organelles derived from them (Sehring et al., 2010). This indicates mutual dependency of these isoforms, rather than complementation. Five of the *PtAct* subfamilies (*PtAct1-1*, 1-2, 1-9, 3-1, 5-1, and 8-1) are associated with food vacuoles (Table 3.3) and, thus, may interfere with vesicle budding and/or fusion. Isoforms are exchanged during cyclosis; for instance, *PtAct4-1* is restricted to nascent food vacuoles. Silencing only of some of these *PtAct* forms affects phagocytosis, while some of them (e.g., *PtAct1-1* and *PtAct1-9*) may be compensated for by other forms (Sehring et al., 2007b). Propulsion of food vacuoles in the cyclosis stream by an unilateral comet-tail seen with GFP-*PtAct1-2* and *PtAct1-9* (Sehring et al., 2007b) is another aspect pertinent to trafficking—a hypothesis suggested by unilateral arrangement (Section 6).

The presence of actin isoform 1 at the cytoproct of *Paramecium*, as determined by antibody staining (Sehring et al., 2007b), is in agreement with the following physiological findings. In *Paramecium*, cytochalasin B impedes closure of the cytoproct after defecation (Allen and Fok, 1985). In *Tetrahymena* another actin disruptive drug, latrunculin B, inhibits egestion of spent food vacuole contents (Sugita et al., 2009). The question which *PtAct1* isoform decorates the cytoproct requires elucidation. Table 3.3 also lists the conservation of ATP- and putative myosin-binding residues in *P. tetraurelia* actins (Sehring et al., 2007a) which both are relevant for dynamic actin functions. In different forms of *PtAct* both these properties fluctuate considerably and independently from each other.

We may summarize the situation in ciliates, notably in *Paramecium* as follows. Widely deviating actin isoforms can be associated with one specific type of vesicular organelle (e.g., the food vacuole), but the opposite also occurs, for example, *PtAct8-1*, associates with different organelles. Some of the actin layers, made of different types of actin, appear more dynamic than others; for instance, there occurs a coordinated exchange of actin isoforms during the phago(lyso)somal cycle in *Paramecium*. More aspects concerning the digestive cycle are discussed in Section 6.2. Clearly silencing of some of the actin genes affects specific vesicle trafficking steps.

2.4. H⁺-ATPase

To some extent, this H⁺-transport-ATPase is comparable to the mitochondrial ATP synthase (Dimroth et al., 2006) although—in contrast to the mitochondrial molecule—the V-ATPase hydrolyses ATP to induce rotation of the V₀ part inserted in the membrane and, thus, to translocate protons.

2.4.1. General aspects

The H^+ -ATPase (proton pump) is a hetero-oligomeric protein assembly consisting of a proteolipid (V0) and a catalytic part (V1) with exchangeable SUs. The V0 and V1 part are connected by a stalk containing an a-SU, but the V1 part can dissociate, thus leaving behind the V0 channel part (Boesen and Nissen, 2009) to which a role in membrane fusion has been assigned (Section 5.2). This complex molecule is classified as a V (vesicle)-type ATPase whose structure and modus operandi have been repeatedly reviewed (Beyenbach and Wieczorek, 2006; Forgac, 2007; Marshansky and Futai, 2008). These molecular assemblies are distributed over a variety of vesicular organelles undergoing trafficking, such as early and late endosomes as well as phago(lyso)somes (Hinton et al., 2009). H^+ -ATPases do not form a phospho-intermediate, in contrast, for example, to the monomeric Ca^{2+} -ATPases (calcium pumps, P-type ATPases) (Carafoli, 2005).

2.4.2. Aspects pertinent to *Paramecium*

In *P. tetraurelia* (the only protozoan species analyzed), the genes for the several SUs of the H^+ -ATPase have been cloned and the SUs localized by combined GFP- and antibody-techniques (Wassmer et al., 2005, 2006), as summarized by Wassmer et al. (2009) as well as in Table 3.4. Previously only the B-SU had been identified in *Paramecium multimicronucleatum* (Fok et al., 2002). A salient feature of our own work is the unsurpassed number of a-SUs, 17 versus 2 in yeast and 4 in the mouse (Wassmer et al., 2009). This multiplicity may be crucial for composing different holo-enzymes with different pumping kinetics (and related functions) in the multitude of organelles endowed with the H^+ -ATPase in *Paramecium* (Wassmer et al., 2009). This aspect is particularly intriguing as the luminal pH achieved can transduce a signal to the cytosolic side and, thus, determine trafficking specificity, as outlined in Section 3.3.

Biogenesis and targeting of the H^+ -ATPase and of its V0-SUs is a particular problem, as no signal peptide could be detected, also in *Paramecium* (Wassmer et al., 2005, 2006). Current views on proteins helping insertion into the ER membrane and escorting the V0 part and possibly parts of the “stalk” from the ER to the Golgi apparatus and beyond are discussed in Section 3.3. The many a-SU isoforms contain an ill-defined (conformational?) targeting motif in the C-terminal half of the molecule (Wassmer et al., 2006), rather than in the N-terminal half as reported for yeast (Kawasaki-Nishi et al., 2001). The a-SU isoforms are delivered selectively to different organelles in the *Paramecium* cell (Wassmer et al., 2005, 2006). If they were, in fact, to determine organelle-specific targeting, the problem arises, how the respective isoforms are instructed to go which way. Considering the interactions with other molecules, notably SNAREs, G-proteins and actin (Section 3.3), this may illustrate the complexity of membrane traffic regulation even in single-cell organisms, such as ciliates.

Table 3.4 Survey of compartments of *P. tetraurelia* cells endowed with an H⁺-ATPase and the SUs experimentally localized to the respective organelle

Organelle	Acidity ^a	SUs found in compartment ^b
ER region	No	a7-1, c1, c4, c5
Golgi apparatus	No	a8-1
Terminal cisternae ^c	Yes	a1-1
Cytostomal area ^d	?	a1-1, a4-1
Nascent food vacuole	No	—
Acidosomes	Yes	a4-1
Pinched off food vacuole (after fusion with acidosomes)	Yes	a4-1
Vesicles (lysosomes?) possibly contributing to food vacuole formation	Yes	a5-1, a6-1, a9-1
Matured food vacuole (intermediary stage)	Yes	a5-1, 6-1, 9-1, c1, c4, c5
Late food vacuole	No	—
Discoidal vesicles	No	—
Trichocyst precursors ^e	No	a3-1
Mature trichocysts ^{e,f}	No	a3-1, c1, c4, c5
Contractile vacuole system (decorated spongione)	No	a2-1, c1, c4, c5, F2

Notes: From Wassmer et al. (2005, 2006, 2009).

^a Determined by acridine orange. “No acidity” despite of the presence of H⁺-ATPase may be due to H⁺-binding by acidic contents (trichocysts and their precursors), presence of an H⁺-exchanger, or to expulsion of contents within short time periods (contractile vacuole system); see text.

^b Incomplete list enumerating only SUs actually localized experimentally by GFP fusion and/or antibody staining at the light and/or EM level.

^c Equivalent to early endosomes.

^d Containing ill-defined vesicles; see text.

^e Lack of acidity determined by electron microscopy, using antibodies against the hapten, dinitrophenol, after trapping a structural analog of this compound *in vivo* (Garreau De Loubresse et al., 1994).

^f Lack of acidity also registered by acridine orange (Lumpert et al., 1992).

In sum, the overall involvement of the H⁺-ATPase in vesicle trafficking is as follows. As known from higher eukaryotes this complex molecule and its SUs can—directly or indirectly—specifically contribute to vesicle trafficking. As discussed in detail in Section 3.3, this implies direct binding of SNAREs or of actin as well as binding of some monomeric G-proteins (Arf) and regulators that secondarily mediate specific organelle docking. In *Paramecium*, we have cloned and localized H⁺-ATPase SUs to specific vesicles (as discussed in the respective sections) and gene silencing experiments have been successful. Nevertheless the contribution to any specific vesicle interactions is still poorly understood in ciliates.

3. FEATURES OF SNAREs

3.1. Characteristics of *Paramecium* SNAREs

As we shall see, we are sometimes at the limits of identifying a molecule as a SNARE. This is so not only with ciliates where *Paramecium* is the only species analyzed with this regard up to now.

3.1.1. Overview of SNAREs in *Paramecium* as compared to other organisms

Tables 3.1 and 3.2 summarize the SNAREs identified and, to a large extent, localized in *P. tetraurelia* (the only ciliate species analyzed so far). Figure 3.3 presents some characteristics of *PtSyb* species and their localization. In *Paramecium*, the number of SNARE genes amounts close to the highest numbers known, as we shall see. Such numbers, however, require some comments. Most syntaxins and synaptobrevin(-like) *Pt*SNAREs are represented by subfamilies, mostly with two members each (Fig. 3.3, Tables 3.1 and 3.2).

On the aminoacid level, all of the SNAREs belonging to one subfamily differ from each other in the extreme by in between $>90\%$ (*PtSyx*; Kissmehl et al., 2007) and $\sim 85\%$ (*PtSyb*; Schilde et al., 2006). One may now assume that subfamily members would have to differ to a sufficient extent to be differentially localized and to exert differential functions—an experience which we made with various examples. If their nucleotide sequence differs by only $<15\%$, for instance, the aminoacid sequence will generally differ even less due to the degenerated genetic code. From a practical point of view, a difference of ≤ 15 between the open reading frames of two genes allows one to achieve simultaneous gene silencing (Ruiz et al., 1998). From our experience, this generally concerns ohnolog pairs. One may, thus, assume that rather similar isoforms may merely serve gene amplification for the same function (Aury et al., 2006). On this basis, one can estimate the number of “functionally distinct” SNAREs in *P. tetraurelia* as outlined below. Some uncertainty comes from missing information on the precise difference between some isoforms and whether lack of a transmembrane domain or of a SNARE domain, etc., would restrict function as a SNARE.

In detail, among Q-SNAREs we found only one gene each encoding *Pt*SNAP-25-LP (Schildt et al., 2008), *PtSyx*6, 11, 12, and 15 (Kissmehl et al., 2007). For the other Q-SNAREs, we found two ohnologs in type *PtSyx*1–5, *PtSyx*7–10, as well as in *PtSyx*14 (Kissmehl et al., 2007). *PtSyx*13 may be disregarded as a putative pseudogene. The 10 twin ohnologs together with the 5 singular forms (“singletons”) may result in 15 “functionally distinct” Q-SNAREs in *P. tetraurelia* if one assumes diversification

in function and localization, as discussed below. Similarly, we found 19 R-SNAREs (disregarding *PtSyb6-2* and *PtSyb12* because of fragmentation and/or of lacking clear domain structure), five of them being singletons (*PtSec22* [Kissmehl et al., 2007], *PtSyb3*, 5, 6, 8) and seven twin ohnologs, that is, *PtSyb1*, 2, 4, 7, 9, 10, 11 (Schilde et al., 2006, 2010). The number of “functionally distinct” (= sufficiently diversified in function and localization) R-SNAREs may, thus, be 12. In sum, out of a total of 44 *Pt*SNARE genes—the ones we have identified and characterized in any sufficient detail—only 27 may encode diversified “functionally distinct” SNARE proteins, or more likely close to 40, considering less similar ohnologs. The number of genes encoding *Pt*SNAREs and *Pt*SNARE-like sequences may be still higher, as concluded from database search (Kloepper et al., 2007), but detailed specification is still missing.

In total, the estimated number of SNAREs in *Paramecium* is comparable to the number determined for *Homo sapiens* that is, 41 (Kloepper et al., 2007), and plants (angiosperms) where similar estimates amount to 42 in *Arabidopsis thaliana* or to 47 in a poplar tree species (Lipka et al., 2007). These numbers exceed those in *Saccharomyces cerevisiae* where 26 SNAREs (7 Qa-, 6 Qb-, 8 Qc-, and 5 R-SNAREs) are established (Burri and Lithgow, 2004). An absolutely reliable comparison of diversification is difficult to achieve because not in all systems are functional and structural criteria sufficiently known. For specific SNARE types fully identified so far in *Paramecium*, refer Tables 3.1 and 3.2.

In other protists, the number of SNAREs is said to be lower than in higher organisms, but this conclusion has been reached from a restricted number of genomes from parasitic species (Yoshizawa et al., 2006) whose size is very likely reduced due to life style. In fact, only 17 SNAREs have been found in *Giardia lamblia* (Elias et al., 2008) and 18 SNARE-like proteins in *Plasmodium falciparum* (Ayong et al., 2007).

In a phylogenetic analysis, it was possible to extrapolate a set of ~20 primordial SNARE types in eukaryotes and ~30 in urmetazoans (Kloepper et al., 2008). For the latter, it has been assumed that evolution of a diversified endo-/phagosomal system has enforced the acquirement of additional SNAREs. When these data are compared with the “functionally distinct” SNARE types in *P. tetraurelia*, one can conclude that such an increase in the number of SNAREs may have been anticipated by ciliates, thus supporting the particular role of the evolution of an intricate exo-/endo-/phagosomal system as a driving force in evolution (Cavalier-Smith, 2002). For example, among Q-SNAREs we have several ones relevant for phago-lysosomal trafficking (*PtSyx7*, 9, 10, and 11) even without direct counterpart in metazoans; some other ones (*PtSyx4-1* and 4-2 and perhaps *PtSyx6-1*) are involved in processes resembling transcytosis (Kissmehl et al., 2007). Also among synaptobrevin-like *Pt*SNAREs several ones are dedicated to vesicle flow directly or indirectly bound to phagocytosis and recycling (Schilde et al., 2010).

The type of R-SNAREs also requires a comment. Most R-SNAREs are of the “brevin”-type in animal cells (Jahn and Scheller, 2006) and of the “longin”-type in higher plants (Filippini et al., 2001; Rossi et al., 2004); Section 2.1. Only some animal R-SNAREs are longins, for example, the TeNT-insensitive VAMP7 (TI-VAMP, Syb-LP1) occurring in some neuronal and nonneuronal cells (Galli et al., 2006). In *A. thaliana* targeting of VAMP7 is regulated by the longin domain (Uemura et al., 2005). In addition to TI-VAMP/VAMP7, the longins Ykt6 and Sec22 are widely distributed, from fungi to plants and man (Rossi et al., 2004).

In mammalian cells it has been found that the longin domain of VAMP7 folds back and, thus, blocks its SNARE domain when not engaged in a SNARE complex. Only in the open form—as occurring in a cis-SNARE complex after membrane fusion—can its longin domain bind the Arf-GAP, Hrb, which mediates clathrin binding and endocytosis (Pryor et al., 2008). In higher eukaryotes, the folding state of the longin domain, for example, of Sec22 (with a homolog in *P. tetraurelia* [Kissmehl et al., 2007]), is known to be important also for vesicle release from the ER and further targeting (Mancias and Goldberg, 2007). This may explain why N-terminal GFP labeling of another *Paramecium* longin, PtSyb10, inhibits exit from the ER and delivery to the cell membrane (Schilde et al., 2010). This is clearly an example calling for mutual control of trafficking pathway analysis by different methodologies.

Membrane fusion depends on the occurrence of one transmembrane domain in an R- (v-) and one in a Q- (t-) SNAREs: One type of SNARE each has to be anchored in one of the two membranes (Grote et al., 2000; McNew et al., 2000). Nevertheless, SNAP-25, in spite of a lacking transmembrane segment, also contributes to the fusion process as a whole. This has been demonstrated by application of antibodies and by truncation by the SNAP-25-specific *Clostridium* toxin, BoNT/E (Schuette et al., 2004), as outlined in Section 8.3.

3.1.2. Specific aspects of SNAREs in *Paramecium*

The aspects just discussed are particularly interesting if one considers the longin character of R-SNAREs in *P. tetraurelia*, including members of the PtSyb1, 2, 3, and 6–9 subfamilies (Schilde et al., 2006, 2010) as well as PtSec22 (Kissmehl et al., 2007). The question arises as to similar effects of the longin domain (Section 3.1.1), including targeting, in plants and in *Paramecium*—a question to be analyzed in future work. In Fig. 3.3, we give an example what *Paramecium* SNAREs look like and where they are localized. As Tables 3.1 and 3.2 show, some of the *Paramecium* SNAREs display various aberrant features. This may include absence of a transmembrane domain (PtSyb6 and 7), substitution of the R-residue in some PtSyb forms (PtSyb8–11) or of the Q-residue in one of the PtSyx molecules (PtSyx12). (Note that such aberrations also occur in established higher eukaryotic

systems [Fasshauer et al., 1998]). Even a SNARE domain may not be identifiable in some of the *Paramecium* R-SNAREs, such as *PtSyb4* and 5 which are prognosticated as SNAREs by overall homology (Table 3.1). Although they display distinct subcellular localization there may be functional implications yet to be analyzed. The expected lipidic anchor seems to be absent from *PtSNAP-25-LP* (Schilde et al., 2008). On the one hand, its abundance in the cytosol (apart from association with many membranes of trafficking compartments) and on the other hand the presence of other characteristic features may justify the inclusion of these molecules in the list of *PtSNAREs*. In fact, there are comparable examples in other cells.

However, what may the absence of important features imply in functional terms—may such SNAREs be functional? For the following reasons, it appears premature at this time to appreciate any role for the truncated SNAREs we found in *Paramecium*. (i) In yeast, the non-NSF type cochaperone, Sec 17 (different from the NSF homolog Sec 18) can complete fusion with normally nonfusogenic trans-SNARE complexes (Schwartz and Merz, 2009). (ii) Fragments of synaptobrevin can reduce the formation of dead-end syntaxin/SNAP-25 complexes (Pobbati et al., 2006). (iii) Soluble SNAREs can associate, in yeast, with a SNARE complex and thus drive vacuole interaction and fusion (Thorngren et al., 2004). Right away one would rather envisage some inhibitory effect in the latter two cases. (iv) In contrast to these situations, inhibitory SNAREs have been identified in mammalian cells as a set of t-SNAREs endowed with a transmembrane domain and occurring in addition to “normal” t-SNAREs; therefore, they may serve fine-tuning (Varlamov et al., 2004). Although no such analyses have been executed with ciliates, the examples clearly indicate that absence of a transmembrane domain would not necessarily entail an inhibitory/competitive role for SNAREs lacking a transmembrane segment.

Substitution of Q for R in the 0-layer of SNAREs in yeast reduces cell growth and protein secretion, but can be restored by an inverse substitution in a partner SNARE (Graf et al., 2005; Ossig et al., 2000). Similarly, a Q → R substitution in Syb of synaptic vesicles has no dramatic effect in hippocampal neurons (Déak et al., 2006). In fact, deviations from the orthodox 0-layer have been detected even in normal cells (Fasshauer et al., 1998; Sutton et al., 1998). However, the effect of deviating 0-layer aminoacids other than R and Q in *P. tetraurelia* is difficult to anticipate without detailed analysis. Deviations from the orthodox heptad repeat structure in quite a few *PtSNAREs* (Kissmehl et al., 2007; Schilde et al., 2006, 2010; Tables 3.1 and 3.2) may reduce SNARE specificity (Fasshauer et al., 1998; Graf et al., 2005; Paumet et al., 2004) and zippering, and, thus, fusogenicity.

In conclusion, we have identified numerous SNAREs, type R, Qa, Qc, Qb/c, in *P. tetraurelia*, cloned the respective genes, localized the proteins, and largely probed their function by gene silencing (as discussed in

subsequent sections). These are the only data available on SNAREs in ciliates. Their number is about twice that assumed for the ur-eukaryote particularly when one also considers the twin isoforms originating from a recent whole genome duplication (“ohnologs”). These may now mainly serve gene amplification, in order to match the requirements for intense vesicle trafficking. A substantial number of *P*-SNAREs contributes to the extensive endo-/phago-/lysosomal system (Sections 4.2 and 6). Clearly, ciliates have increased their SNARE repertoire independently of, but in parallel to the evolution of multicellular organization.

3.2. Role of the SNARE-specific chaperone, NSF

3.2.1. General role of NSF

NSF is a hexameric AAA-type ATPase with characteristic domain structure (Hanson and Whiteheart, 2005; Whiteheart et al., 2001). NSF is generally believed to be engaged in disentangling SNARE complexes after fusion, so SNAREs can become amenable to reuse (Littleton et al., 2001). Another possibility, though less considered in the literature, is the establishment of SNARE complexes during membrane-to-membrane attachment (Ungermann and Langosch, 2005; own data in Section 3.2.2).

To appreciate the significance of NSF one has to bear in mind the following details. Fusion capacity *in vivo* depends on the assembly of a quarternary complex of α -helices from the SNARE domains. This includes a v (R)-SNARE and two or three t (Q)-SNAREs (Sections 2.1, 3.1, and 5), among them Qa-, Qb-, Qc-, and Qb/c-SNAREs (Fasshauer et al., 1998). Normally, a Qb/c with two pin-shaped (antiparallel) α -helical SNARE domains or, more rarely, separate Qb and Qc SNAREs are superimposed to a minimal SNARE pin of two membrane-anchored SNAREs (Fukuda et al., 2000). Thus, the minimum required for fusion is an R- and a Qa-SNARE (Grote et al., 2000; McNew et al., 2000). Specifically, *in vitro* studies have demonstrated that one type of v-/R-SNARE and one type of t-/Q-SNARE suffice to mediate fusion as long as they are inserted (by their carboxy terminus) in opposing membranes, so they can form “SNARE pins” (Fasshauer et al., 1998; Graf et al., 2005; Malsam et al., 2008; Melia et al., 2002; Sorensen et al., 2006). Beyond the fact that *in vivo* one SNARE complex is made of one v-/R- and three SNARE domains from two or three t-/Q-SNAREs (Section 2.1.1) (Fukuda et al., 2000; Jahn and Scheller, 2006; Malsam et al., 2008) it then has been found, moreover, that several such complexes are radially arranged around a potential fusion site (Section 5). All this arrangement depends on the SNARE-specific chaperone, NSF. NSF starts binding after preceding binding of α -SNAP to the Habc domain of syntaxin (Bock and Scheller, 1996; Rizo and Südhof, 2002; Xu et al., 1999) (Section 2.1.1).

3.2.2. NSF in *Paramecium*

We could for the first time identify NSF in *Paramecium* on the basis of its distinct domain structure (Kissmehl et al., 2002). This includes a conserved AAA domain, Walker A and B as well as SRH domains and some other features (Patel and Latterich, 1998; Whiteheart et al., 2001). Furtheron, we have demonstrated disturbance of vesicle trafficking by *PtNSF* gene silencing (Kissmehl et al., 2002). Then we showed that specifically the assembly of ultrastructurally defined, functional trichocyst exocytosis sites requires the activity of NSF (Froissard et al., 2002). This was demonstrated with the temperature-sensitive mutant, *nd9*, that does not differentiate such sites, even though trichocysts are docked at the cell periphery, when cultivated at a nonpermissive temperature of 28 °C (Beisson et al., 1980). During a 28 °C → 18 °C shift, functional exocytosis sites are normally assembled within ~2 h, but not when cells were silenced in the *NSF* genes (Froissard et al., 2002). Thus, in our system, NSF serves primarily establishing SNARE complexes for subsequent membrane fusion upon stimulation. (In addition, it may also serve dismantling SNAREs after fusion). Recently, in contrast to the mainstream hypothesis, a function of NSF as a chaperone before (!) fusion was also ascertained for neurotransmitter release (Kuner et al., 2008). This analysis relied on photolytic activation of a caged NSF peptide with adequate time resolution.

Normally NSF would hydrolyze ATP, paralleled by rapid release from its sites of action (Whiteheart et al., 2001). Therefore, to localize NSF at its potential sites of action, that is, fusion sites, in *Paramecium* we have elaborated a suitable protocol. We applied careful cell permeabilization and infiltration with the inhibitor, *N*-ethylmaleimide, and nonhydrolyzable ATP- γ -S (Kissmehl et al., 2002), followed by staining with antibodies against NSF. This resulted in hot spots indicating sites of repetitive fusion activity, including the cytoproct, the porus of the contractile vacuoles, and their connections with the radial canals—established sites of repetitive membrane fusion (Sections 6 and 9.1).

NSF gene silencing also allowed us to virtually see, in the EM, sites of vesicle interactions. Vesicles looked as if “frozen” at many sites where this process normally cannot be realized because of the high speed/low frequency. Figures 3.4–3.7 present some examples of this approach (H. Plattner, B. Schönemann, and C. Schilde, unpublished observation). In detail, we observed unusually extensive vesicle aggregates and stacks of rough ER, intermingled with aggregates of ribosomes (as described in fungi [Garrison and Boyd, 1974], chicken embryos [Birks and Weldon, 1971], or in crystals after isolation from bacteria [Avila-Sakar et al., 1994]), together with a substantial number of autophagosomes (Figs. 3.4 and 3.6). These features, including increased autophagy (Kuma et al., 2004), are clearly indications of inhibited metabolic activity, as one might expect from vesicle

Figure 3.4 Examples of effects of NSF silencing in *P. tetraurelia* cells, as analyzed by standard ultrathin section EM analysis. Ultrastructural changes in the region containing rough ER (rER), aggregates of polysaccharides particles (app) and of ribosomes (ar). (A) Unusually extensive vesicle aggregates (va) in between rough ER stacks and aggregates of ribosomes and of polysaccharide particles. Magnification 13,000 $\times$. (B) Considerable dilation of ER cisternae. “n” labels the nucleus. Magnification 8,500 $\times$. (C) Transition between rough and smooth ER (rER, sER) and an aggregate of ribosomes (ar). Magnification 13,000 $\times$. Unpublished data (see text).

trafficking impairment. The rough ER lumen may be dilated in some cells (Fig. 3.4B) or in continuity with smooth membrane aggregates, also in a form otherwise not seen (Figs. 3.5 and 3.6), as if ER $\rightarrow$ Golgi or reverse

Figure 3.5 Examples of effects of NSF silencing in *P. tetraurelia* cells, as analyzed by standard ultrathin section EM analysis. rER stacks end in an aggregate of smooth vesicles (asv), as in the framed area which is enlarged in the insert. Magnification 11,500 $\times$ (insert 22,000 $\times$). Unpublished data (see text).

trafficking was affected. Such situations in Fig. 3.5 display continuity of a mass of smooth vesicular/tubular membranes with emanating cisternae of rough ER. This is interesting, considering the occurrence of specific physical contact sites between the two ER forms and the Golgi apparatus in higher eukaryotes (Sparkes et al., 2009) and the assumption that a Golgi dictyosome forms at specific sites along the rough ER (Foresti and Denecke, 2008; Vivero-Salmerón et al., 2008). In the ER, experimental disturbance of the interaction between SNARE, COP (coatamer), and tethering molecules causes accumulation of vesicles at the ER boundaries (Zink et al., 2009). Similarly, in our case, NSF silencing may have inhibited in one way or another vesicle trafficking from the ER on, or it may have frozen vesicles recycling to the ER. Figure 3.6 presents varying aspects of the Golgi apparatus, from normal to barely identifiable, as well as increased autophagy (Fig. 3.6E). In fact, in higher eukaryotes, SNAREs are responsible not only for vesicle transport from the ER to subsequent organelles (mainly the Golgi apparatus) and back. In compatibility

Figure 3.6 Examples of effects of NSF silencing in *P. tetraurelia* cells, as analyzed by standard ultrathin section EM analysis. (A–D) show variations of the aspects of the Golgi apparatus and accumulations of small electron clear vesicles, and (E) shows large electron dense vesicle, in part representing autophagosomes (aps). app, aggregates of polysaccharide particles. Magnifications: $24,000\times$ (A, D), $29,000\times$ (B), $18,000\times$ (C). Unpublished data (see text).

Figure 3.7 Change of the surface membrane complex (cortex). Note numerous vesicles of variable size, including very small vesicles (arrows) between the cell membrane and the outer membrane of the alveolar sacs (as) and even at a basis of a cilium (ci) at the top right. app, aggregates of polysaccharide particles. Magnification 8,000 $\times$. Examples of effects of NSF silencing in *P. tetraurelia* cells, as analyzed by standard ultrathin section EM analysis. Unpublished data (see text).

with our observations, in HeLa cells a SNARE protein, syntaxin 18, has been shown to mediate the organization of ER subdomains including smooth/rough ER and ER exit sites (Iinuma et al., 2009).

In Fig. 3.7, numerous minute to medium-sized vesicles are attached to the cell membrane, to alveolar sacs and to some other membranes. At the cell membrane, this frequently occurs outside parasomal sacs so that additional sites now also have to be considered for constitutive exo- and endocytosis (Section 4.2). Vesicles arrested in contact with alveolar sacs may trace their biogenesis (Kissmehl et al., 2002). This is substantiated by the transfer of the SERCA-type Ca^{2+} -ATPase (pump) from the ER to alveolar sacs when expressed as a GFP-fusion protein in normal *Paramecium* cells (Hauser et al., 2000b).

The chaperone function of NSF, together with any other chaperones which may emerge in future studies, involves ATP requirement. Whether ATP is required for membrane fusion has been debated over a long time. Experiments with *Paramecium* cells and cortex fragments isolated from them have suggested that ATP may be required for vesicle priming, but not for fusion (Vilmart-Seuwen et al., 1986). Later on this has been shown more clearly in much more detailed work, including electrophysiological studies with chromaffin cells (Xu et al., 1999). However, it is not known whether such function has to be attributed solely to chaperones. In the case of NSF, ATP-dependent priming would presuppose that it becomes active before, rather than after fusion.

In sum, NSF can be characterized as a SNARE-specific chaperone that contributes to vesicle trafficking also in ciliates. However, NSF may exert its function not only after membrane fusion, to disentangle SNAREs from a cis-arrangement resulting from fusion (as most widely assumed), but in ciliates NSF operates also before fusion. Concomitantly, NSF is required to mediate exocytosis-competence to trichocyst docking sites. According to EM analysis, *NSF* silencing causes abolition of vesicle trafficking and thus results in the accumulation of vesicles at unexpected sites. Only “freezing” such interaction sites allows their visualization because they are normally too short-lived to be recognized.

3.3. “SNAREs and Co”—targeting of vesicle traffic from the ER to the Golgi apparatus and beyond

3.3.1. Basic background from higher eukaryotes

A set of potential key players participates in vesicle trafficking from the ER on: SNAREs, Arf-, and Rab-type small GTPases (G-proteins), COPs, the H^+ -ATPase as well as actin. Among them, in ciliates, COPs as well as small GTPases and their regulators have not yet been analyzed in any detail comparable to the other molecules.

In mammalian cells, right at the beginning of their travel, some longin-type SNAREs can bind to the signal recognition particle receptor, that is, a heterodimeric GTPase related to Arf which exerts a GAP activity (Schlenker et al., 2006) and mediates this first step of targeting. An Arf-GAP also has to bind to the longin domain of a VAMP7-type R-SNARE in normal rat kidney cells in order to mediate vesicle cycling between the Golgi apparatus and the ER (Pryor et al., 2008). Vesicle budding within the ER requires COPs (Cai et al., 2007; Rothman, 1994). This generally occurs at specific sites (Foresti and Denecke, 2008; Vivero-Salmerón et al., 2008) frequently seen in juxtaposition to the Golgi apparatus. We have followed up this aspect by *PtNSF* silencing (Section 3.2.2).

There are hardly any specific motifs relevant for specific vesicle targeting known from any of the systems analyzed so far (Pfeffer, 2007). Although orthologs of syntaxin display rather similar intracellular localizations in widely different cell types, even in that case no specific targeting motifs are known. However, substituting lipid anchors for transmembrane segments in the yeast Qa-SNAREs, Sso1 and 2, and the R-SNAREs, Snc1 and 2 (or their mammalian equivalents), does not affect targeting, though it blocks membrane fusion (Grote et al., 2000; McNew et al., 2000). In mammalian cells the transmembrane domain (plus flanking positively charged aminoacids) is essential for trafficking from the ER on, as is binding of SNAP-25 (Yang et al., 2006). The length of the transmembrane domain has also been reported as crucial for targeting of some syntaxins, for example, types 3, 4, and 5 in some mammalian systems (Watson and Pessin, 2001).

More specific membrane interaction may be mediated by complex molecular interactions, including not only SNAREs, SUs of the H^+ -ATPase (see below), and monomeric G-proteins (Grosshans et al., 2006), but probably also components that are currently only partially known. For instance, “accessory/auxiliary proteins” may interact with SNAREs (Bethani et al., 2007; Medine et al., 2007; Rizo et al., 2006; Weninger et al., 2008). It is also unknown whether posttranslational modifications, such as phosphorylation of SNAREs, might contribute to targeting. Specifically for syntaxin 6, in mammalian cells, a motif within the middle part of the cytoplasmic domain has been found relevant for sorting (Watson and Pessin, 2000).

Overall, the mode of targeting by which specific membrane interactions are achieved is assumed to be complex. Very likely it requires the interaction of several different components, and is, therefore, poorly understood for the time being. Clearly microtubule rails with associated motor proteins may contribute to efficient delivery (Soldati and Schliwa, 2006), but hardly to specific targeting proper (Hirschberg et al., 1998). The H^+ -ATPase has recently been attributed a key role in targeting vesicle flow (Maranda et al., 2001; Marshansky and Futai, 2008; Recchi and Chavrier, 2006). In mammalian (AtT-20, anterior pituitary) cells, it was shown by inhibitor studies that the H^+ -ATPase directs separate pathways for lysosomal and secretory proteins at the level of the trans-Golgi network (Sobota et al., 2009).

How is specific protein targeting and subsequently specific membrane interaction possible, considering the absence of a signal peptide sequence, for instance, in SNAREs and in the SUs of V0 (proteolipid) part of the H^+ -ATPase? In fact, this is also the case in *Paramecium* (Kissmehl et al., 2007; Schilde et al., 2006, 2008, 2010; Wassmer et al., 2005, 2006). Some information along these lines is available from other eukaryotes, mainly yeast, where V0-SUs are inserted into the ER membrane (Forgac, 2007) by interaction with several assembly factors including the “vacuolar ATPase assembly integral membrane protein,” VMA21 (<http://www.uniprot.org/uniprot/Q3ZAQ7>), and also quite recently identified factor, Voa1p. This is a double membrane-spanning glycoprotein with a signal peptide of 24 aminoacids and a dileucyl motif for ER retention (Ryan et al., 2008). H^+ -ATPase components can escape from this strong interaction only after full assembly. This is enabled by another assembly factor, Vma21p, which binds to COPII-type coats and thus mediates budding for delivery to the Golgi apparatus (Malkus et al., 2004). ER/Golgi SNAREs of the types Sec22 and syntaxin may be included in such complexes (Mossessova et al., 2003; Springer and Schekman, 1998). This is facilitated by the longin domain of Sec22 (Liu et al., 2004) by means of a conformational motif (Mancias and Goldberg, 2007).

From the Golgi apparatus on, the V1 part of the H^+ -ATPase associates with syntaxin 1 which in kidney cells is, thus, delivered to the cell surface (Schwartz et al., 2007b). Association of the H^+ -ATPase B- (Zuo et al., 2008) and C-SU with F-actin is another regulatory aspect (Beyenbach and

Wieczorek, 2006). Note that syntaxins, though conventionally designated as t-SNAREs, also have to travel in vesicles from the ER on for final delivery to the respective target membranes. An additional interaction partner leading to site-specific membrane delivery is clathrin, for example, for vesicle targeting to specific domains of the cell membrane (Deborde et al., 2008). It remains to be established, also with ciliates, whether there is any crosstalk to other components determining vesicle targeting.

Another aspect of increasing complexity is the budding of different secretory vesicle types from different sites of the Golgi apparatus (Muniz et al., 2001; Spang, 2009). This also concerns glycosylphosphatidyl inositol (GPI)-anchored proteins, such as variant surface antigens (Section 4.2). In yeast, GPI-anchored proteins are suggested to be transported, from the ER on, in separate vesicles to the Golgi apparatus and from there to the cell membrane; this may be mediated by specific COP interactions (Mayor and Riezman, 2004; Muniz et al., 2001). Whether this would hold true for protozoan variant surface antigens and which subset of SNAREs, GTPases, etc., would be involved is not known.

3.3.2. Aspects pertinent to ER/Golgi trafficking in ciliates

Only parts of this puzzle are known from the currently best analyzed ciliate, *P. tetraurelia*. The H^+ -ATPase can be recognized from the ER on (Wassmer et al., 2005, 2006). A longin-type Sec22 ortholog occurs (Kissmehl et al., 2007), probably also somehow intermingled between the ER and the numerous Golgi units. Unfortunately, *Paramecium*'s Golgi apparatus is scattered in so minute dictyosomes (Estève, 1972), as it is in *Tetrahymena* (Kurz and Tiedtke, 1993), that localization of specific components to such elements is difficult to achieve. By EM work, smooth coats comparable to COP-type vesicles also occur at the ER/Golgi interface and on the Golgi apparatus of *Paramecium* (Allen and Fok, 1993; Garreau De Loubresse, 1993). Similar situations are visible also in micrographs from *Tetrahymena* (Kurz and Tiedtke, 1993). However, molecular identification of COP SUs as well as of specific components of the clathrin-type coats, both at the trans-side of the Golgi apparatus, is still missing. Such coats could mediate formation of secretory organelles (trichocysts) and of lysosomal vesicles, respectively, as known from higher eukaryotes.

During *NSF* gene silencing, in *Paramecium*, we observed that the rough ER, at some sites, is continuous with a tightly entangled mass of smooth, tubulo-vesicular membranes without any visible association with a Golgi apparatus (Fig. 3.5; Section 3.2.2). This could represent sites where normally vesicle budding and fusion would occur (Section 3.3.1). At other sites, the rough ER is considerably swollen, probably because transport of luminal proteins fails after *NSF* silencing.

There are several trivial, though basic open questions concerning passage through the Golgi apparatus in ciliates. Which are the molecular components of the two types of membrane coats? Do lysosomal enzymes go through the

Golgi apparatus? To what extent is the Golgi apparatus involved in trichocyst biogenesis? Is *PtAct8*, a resident of the Golgi apparatus (Sehring et al., 2007a), involved in Golgi trafficking, in analogy to higher eukaryotes (Cao et al., 2005; Carreno et al., 2004)? Again we are confronted with fundamental questions that have been settled for higher eukaryotes already quite some time ago (Section 3.3.1), yet only in part for ciliates.

Several reports have dealt with the release of acid hydrolases, probably of lysosomal origin, from *Tetrahymena* cells (Banno et al., 1993; Taniguchi et al., 1985). More detailed analysis of a cysteine protease revealed expression as a preproprotein, that is, with the potential of a passage through the Golgi apparatus (Herrmann et al., 2006).

As known from higher eukaryotes, on the way through the cell, SUs of the V1 part of the H^+ -ATPase molecule can be exchanged (Marshansky and Futai, 2008), depending on the local “cellular environment” (Qi and Forgac, 2007). Thus, different luminal pH values can be produced in the different compartments. In the *Paramecium* cell, SUs of the catalytic V1 part are also exchanged on the way through ER, Golgi, and beyond (Wassmer et al., 2005, 2006, 2009). The unsurpassed number of 17 genes encoding in *Paramecium* the α -SU (Wassmer et al., 2006) that forms part of the V0/V1 connecting stalk may, by numerous combinations with other SUs, endow the holo-enzyme with locally different H^+ -transport kinetics. (This aspect has not yet been analyzed in any detail.) Based on results from higher eukaryotic cells one now assumes that this can entail a transmembrane signal by conformational change of the holo-enzyme depending on the luminal pH. In detail, a conformational change can allow for the docking of specific Arf proteins and their activators which in turn can mediate specificity of membrane interaction—or at least part of the specificity (Maranda et al., 2001; Recchi and Chavrier, 2006). Although this has been shown primarily for delivery of cargo from early to late endocytotic vesicles in mammalian cells it may be a mechanism common to many trafficking steps (Brown et al., 2009; Hurtado-Lorenzo et al., 2006) including phagosome processing (Steinberg et al., 2007).

In conclusion, complex interactions may represent a basic scenario to explain in part the specificity of transport steps from the ER, via Golgi apparatus, to the secretory pathway as well as to phago- and lysosomes. In fact, in *Paramecium*, H^+ -ATPase components occur in different combinations from the ER on as well as along the secretory pathway and in the many compartments of the endo-/lysosomal apparatus (Wassmer et al., 2005, 2006, 2009), as summarized in Table 3.4.

3.3.3. Dense core-secretory vesicle biogenesis and trafficking in *Paramecium* and *Tetrahymena*

As usual with dense core-secretory vesicles, the biogenesis of ciliate “extrusomes” starts in the ER. As to the passage of trichocyst precursor elements through the Golgi apparatus, there is different evidence available. First,

immuno-EM localization of the main contents proteins suggests transit through the trans-Golgi in *Paramecium* (Garreau De Loubresse, 1993; Vayssié et al., 2001) and in *Tetrahymena* (Turkewitz et al., 1991). Second, an antigen connecting the trichocyst matrix with the organelle membrane can also be localized to the Golgi apparatus (Momayezi et al., 1993). Third, the glycosylation pattern derived from lectin binding studies (Glas-Albrecht et al., 1990), particularly the inclusion of fucosylation sites (Allen et al., 1988), suggests such a pathway.

In *Paramecium*, the trichocyst membrane is probably endowed with a v(R)-SNAREs, type *PtSyb5* (Schilde et al., 2010), as discussed below. Small G-proteins have also been addressed by GTP-overlays and differences between secreting and nonsecreting cells have been reported (Peterson, 1991). Any molecular details, however, await exploration. Along these lines one has to consider not only docking and fusion capacity required for exocytosis, but also the multiple fusions of trichocyst precursor vesicles (Garreau De Loubresse, 1993; Gautier et al., 1994).

Strikingly, trichocysts contain an H^+ -ATPase (Wassmer et al., 2005, 2006) although they are not remarkably acidic compartments (Garreau De Loubresse et al., 1994; Lumpert et al., 1992). Also precursor vesicles are not acidic (Garreau De Loubresse et al., 1994). Here, protons may contribute to the assembly of trichocyst matrix proteins (tmx, trichynins) in crystalline form, and this may be linked to posttranslational processing which in turn enables docking at the cell membrane (Gautier et al., 1994). In fact, the Me^+/H^+ exchanger, monensin, blocks maturation and transport of trichocysts (Garreau De Loubresse et al., 1994). Whenever in mutants, or by experimental manipulation proteolytic protrichynin cleavage fails, EM morphology of trichocysts is aberrant, and no delivery to the cell membrane takes place (Gautier et al., 1994; Pollack, 1974; Pouphile et al., 1986). The mostly highly acidic trichynins (Tindall, 1986) may trap protons and, thus, obscure their presence in color assays. Exchange of protons for other ions may also be relevant for trichocyst matrix assembly.

With dense core-secretory vesicles of *Tetrahymena* (“mucocysts”) one would expect a similar situation, as they also contain acidic proteins (Bowman et al., 2005a; Chilcoat et al., 1996) and maturation by proteolytic cleavage also occurs (Turkewitz et al., 1991; Verbsky and Turkewitz, 1998). In fact, several aspects relevant for *Paramecium* are also relevant for *Tetrahymena* (Bowman et al., 2005a; Bradshaw et al., 2003; Turkewitz et al., 1991). Again different mutations are available (Bowman and Turkewitz, 2001; Orias et al., 1983). On this background, it is surprising that the assembly of the mucocyst contents core (Grl = granule lattice) proteins can undergo crystallization still in the ER (Cowan et al., 2005). It would be interesting to see whether mucocysts dispose of a similar endowment with an H^+ -ATPase as trichocysts and how this contributes to processing and condensation of contents.

In their tip region, *Tetrahymena* mucocysts accumulate an independent, soluble group of proteins (Grt = granule tip) defined by a C-terminal

β/γ -crystallin domain (Bowman et al., 2005b; Rahaman et al., 2009). Homolog proteins also occur in *Paramecium* (unpublished observation). Whereas crystalline matrix proteins serve rapid contents decondensation, thus expelling trichocysts upon stimulation in a Ca^{2+} -dependent manner (Section 7), the function of crystallin-like soluble proteins remains to be determined. It is worth noting that Grt proteins are described as sticky (Rahaman et al., 2009) and that trichocyst tips contain soluble, inherently sticky secretory lectins (Haacke-Bell and Plattner, 1987) whose trafficking pathway has not been analyzed as yet. In *Tetrahymena*, Grl and Grt proteins, respectively, are assumed to be sorted along independent pathways (Rahaman et al., 2009).

Acidification or pH-dependent maturation of secretory matrix components—which one determines trafficking? To decide this question one would have to manipulate components of the H^+ -ATPase and the endopeptidase (Collins and Wilhelm, 1981) responsible for secretory protein processing. In fact, inhibition of the subtilisin or cathepsin family proteases in *T. thermophila* causes a delay in the processing of the mucocyst matrix proteins (Bradshaw et al., 2003). (Note, however, that Elde et al., 2007, by contrast, assume the absence of a “convertase” in *T. thermophila* and *P. tetraurelia*, based on database search.) Subtilisin-type endoproteases are established enzymes for secretory proprotein processing in mammalian cells (Rouillé et al., 1995). Remarkably, in *Tetrahymena*, cathepsin follows the secretory pathway (Section 4.2). Also the peptide cleaved off from the Grl proteins in *Tetrahymena* go the constitutive exocytotic pathway (Bowman et al., 2005a), just as in mammalian (pancreatic β -) cells (Arvan et al., 1991). Also in this cell-type acidification by the secretory organelle H^+ -ATPase is mandatory for secretory proprotein $\rightarrow$ protein conversion by acid endoprotease activity (Sun-Wada et al., 2006). Thus, the answer to the question on top says that, also in ciliates, both factors are relevant as they condition each other.

Biogenesis of mature chromaffin granules by homotypic fusion of precursor organelles requires syntaxin 6 (Urbé et al., 1998). For *Paramecium* we have suggestive evidence for the relevance of *PtSyb5*, based on the fact that overexpression as a GFP-fusion protein alters trichocyst processing (Schilde et al., 2010) in a way reminiscent of mutants described in *Paramecium* (Vayssié et al., 2001) as well as in *Tetrahymena* mucocysts (Bowman and Turkewitz, 2001).

We have evidence for a crosstalk between the luminal and the cytoplasmic side of trichocysts, some details being presented in Section 3.3.3. What may the signals be? In mammalian gland cells interaction of the luminal with the cytoplasmic side may be mediated by syncollin and a luminal GPI-anchored protein (Kalus et al., 2002) or by the transmembrane protein, phogrin, as well as by different luminal scaffolding proteins anchored by an α -helix (Dikeakos and Reudelhuber, 2007). In *Paramecium*, some monoclonal

antibodies recognize, after fast freezing and freeze-substitution, fragile periodic connections situated between the trichocyst matrix and the membrane (Momayezi et al., 1993) so that a kind of transmembrane crosstalk appears possible. When trichynin precursors are not cleaved, as in the *tl* (trichless) mutant (Pollack, 1974), these are secreted by numerous small trichocyst precursor vesicles along the constitutive pathway (Madeddu et al., 1994).

Looking back at the transport from the ER on, via the Golgi apparatus and beyond we emphasize the following essential findings. At least some of the SNAREs, together with some of the H^+ -ATPase SUs are inserted into the ER membrane and escorted to the Golgi apparatus by very intriguing molecular interactions (not yet known in detail from ciliates). From there, molecules can be passed over by vesicle flow to other parts of the trafficking pathway, including secretory components. Although there are reasons to assume a similar scenario for ciliates, these aspects have not yet been analyzed in detail. Specifically from ciliates we know that proper secretory contents processing and assembly is required for delivery further on along the secretory pathway. In *Paramecium*, this depends on the activity of the organellar H^+ -ATPase. Up to now, there is only circumstantial evidence for the occurrence of an R-SNARE in the trichocyst membrane.

4. EXOCYTOSIS AND ENDOCYTOSIS

4.1. Exo- and endocytosis in general

From yeast to man, membrane interactions leading to exocytotic membrane fusion require a teamwork of a multitude of proteins including SNAREs and a variety of auxiliary proteins. These include SM proteins (Sec1/Munc18 complex) as well as Rab-type GTPases and their modulators and effectors, etc. (Cai et al., 2007; Spiliotis and Nelson, 2003; Südhof, 2007). However, in the details requirements differ between stimulated and constitutive exocytosis inasmuch as the latter uses an octameric protein complex for tethering vesicles to the cell membrane whose biogenesis it controls. This “exocyst” complex, detected in yeast (Terbush et al., 1996), has homologs up to mammals (Grindstaff et al., 1998; Yeaman et al., 2001), whereas no information is available so far for ciliates.

In the course of stimulated exocytosis, “clear” and “dense core” secretory vesicles liberate their secretory contents, that is, neurotransmitters and proteins, respectively. The time period required is much shorter for clear vesicles. Ca^{2+} requirements and sensors are also different for the different types of stimulated exocytosis (Section 7), as are other parts of the molecular machinery engaged in membrane interaction. This is also the case with the subsequent retrieval of vesicle membranes by exocytosis-coupled endocytosis. The steady-state delivery of membrane components by “unstimulated” (constitutive) exocytosis

needs no Ca^{2+} signal (Burgoyne and Clague, 2003; Jaiswal et al., 2009; Schwartz and Merz, 2009).

For constitutive endocytosis, in the past few years a variety of mechanisms—with different cytoplasmic coats or without a coat visible in the EM—have been established, although many details are still unsettled (Kirkham and Parton, 2005; Mayor and Pagano, 2007). In human cells, some of these processes are modulated by dynamin, clathrin, and actin, but reportedly no Ca^{2+} is required (Jaiswal et al., 2009). A precisely timed study with chromaffin (PC12) cells, molecules emerge at sites of constitutive endocytosis in the following sequence: clathrin, dynein, and actin (Felmy, 2009). This is complemented by an elegant recent study with higher eukaryotic systems where molecules proposed to be required for endocytosis have been subject to a detailed analysis (Ohya et al., 2009). Thereby SNAREs, Rab GTPases, and regulators of their nucleotide exchange/activation cycle, in addition to Rab effector molecules, have been probed individually and collectively in an *in vitro* assay. The data support the concept that, Rab proteins, more than SNAREs, determine organelle-specific membrane interactions.

In ciliates, only a selection of these mechanisms is ascertained; for details, see the respective sections. Exo-/endocytosis encompasses (i) stimulated dense core-vesicle exocytosis (“extrusomes”; trichocysts in *Paramecium*, mucocysts in *Tetrahymena*) and (ii) rapidly ensuing detachment of empty membranes (“ghosts”) from the cell membrane and their internalization (exocytosis-coupled endocytosis). This is independent of endocytosis by bona fide clathrin-coated vesicles (parasomal sacs) near ciliary bases.

This section can be summed up as follows. The clear distinction between stimulated and constitutive exo-/endocytosis becomes evident also in ciliates. The respective sites are endowed with very different molecular components relevant for vesicle trafficking, as will be specified in the subsequent Sections 4.2 and 4.3.

4.2. Constitutive endocytosis and exocytosis in ciliates

4.2.1. Parasomal sacs as sites of constitutive exo-endocytosis

Parasomal sacs are bristle-coated omega-shaped profiles stereotypically associated unilaterally with ciliary bases in *Paramecium* (Allen, 1988) and in *Tetrahymena* (Elde et al., 2005). They serve constitutive endocytosis. In *Paramecium*, parasomal sacs contain SUs of the H^+ -ATPase (Wassmer et al., 2006). The coat is most likely made of clathrin (though molecular proof is scant), which would be in line with its presence in the *Paramecium* and in the *Tetrahymena* databases. Involvement of clathrin-associated adaptor protein, type AP2, in *Tetrahymena* (Elde et al., 2005) strongly supports a clathrin-mediated mechanism. In *Paramecium* the situation may be similar, probably with the involvement of dynamin (Wiejak et al., 2004). (However, dynamin can equally support some other internalization modes, as

known from higher metazoans [Mayor and Pagano, 2007; Miaczynska and Stenmark, 2008]). Accordingly, the dynamin activating protein phosphatase, type 2B (calcineurin), as specified in *P. tetraurelia* by Fraga et al. (2010), is enriched at parasomal sacs of *P. tetraurelia* (Momayezi et al., 2000). In an unrooted phylogenetic tree dynamin of the ciliates, *P. tetraurelia* and *T. thermophila*, cluster only to a small extent with each other; however, they do not at all cluster with any other group, from mammals down to the apicomplexan relatives (Breinich et al., 2009). This is just one out of many examples of independent diversification in protozoa in general and in ciliates in particular.

Occurrence of SNAREs in parasomal sacs of *P. tetraurelia* had to be expected from proteomic analysis of clathrin-coated vesicles isolated from human cells. This has revealed NSF, different syntaxins (types 6, 7, and 8), and Rab-type monomeric G-proteins (Borner et al., 2006). In polar epithelia, specific SNAREs (TI-VAMP) are dedicated to the delivery of GPI-anchored proteins to the apical surface (Pocard et al., 2007). Manipulation of SNAREs occurring in parasomal sacs membranes of *P. tetraurelia*, type SNAP-25-LP (Schilde et al., 2008) and *PtSyb6* (Schilde et al., 2006), may contribute in the future to unraveling the poorly understood pathway of variant surface antigens and of other GPI-anchored proteins in ciliates. The association of actin, type *PtAct8* with parasomal sacs (Sehring et al., 2007a) fits well the situation in coated pits/vesicles of higher eukaryotes (Giner et al., 2007; Kaksonen et al., 2006).

Constitutive exocytosis delivers glycoproteins to the cell surface, among them the “variant surface antigens” which, in ciliates, are also called “immobilization antigens,” as antibodies generated against them can immobilize the respective “serotype” (Beale and Preer, 2008). As analyzed in more detail in *Paramecium*, they undergo permanent turnover (Klöppel et al., 2009) since they are internalized via parasomal sacs (Flötenmeyer et al., 1999), probably for degradation. On the biogenetic pathway, in *Paramecium*, native variant surface antigen molecules may be transported to the same sites for insertion into the cell membrane by constitutive exocytosis (Capdeville, 2000; Flötenmeyer et al., 1999). Only recently, we have found evidence of additional potential sites for constitutive exocytosis and endocytosis, some also near the ciliary bases, but separate from parasomal sacs (Schilde et al., 2010). This evidence comes mainly from NSF silencing experiments (Section 3.2) that can disclose cryptic sites of membrane interactions by “freezing” their otherwise very fast dynamics.

Variant surface antigens of the *Paramecium* surface are fixed by a GPI-anchor (Azzouz et al., 1995, 2001) which, in contrast to other systems, is attached to the phospholipid bilayer by a ceramide residue (Benwakrim et al., 1998). Clathrin-mediated endocytosis would be one of several currently heavily discussed internalization pathways of GPI-anchored proteins (Campana et al., 2005; Frick et al., 2007; Linden et al., 2008). This pathway

also holds for (mammalian) prion protein (Langhorst et al., 2008)—a molecule smaller than variant surface antigens. Since chances for transport via clathrin-coated vesicles increases with the bulkiness of the GPI-attached protein (Bhagatji et al., 2009), this may well explain the pathway we found for *Paramecium* variant surface antigens (larger than prions) by immunogold EM analysis (Flötenmeyer et al., 1999). Circumstantial evidence also suggests that newly synthesized variant surface antigens are delivered to the same sites by constitutive exocytosis (Flötenmeyer et al., 1999). This would not easily be recognizable on EM micrographs when both, exo- and endocytosis, alternate in a cycle because assembly of a clathrin coat requires a much longer time period, up to 1 min (Ehrlich et al., 2004), than exocytosis. Interestingly, in *Trypanosoma*, unequivocal evidence for the internalization of variant surface antigens, also GPI-anchored, via clathrin-mediated endocytosis has been obtained (Overath and Engstler, 2004). In protozoa, this internalization pathway may be used because—to our current knowledge—some other pathways known from mammalian cells (Kirkham and Parton, 2005; Mayor and Pagano, 2007) appear to be absent (or they are not yet identified).

4.2.2. Early and late endosomes

In *P. primaurelia* soluble fluorescent wheat germ agglutinin, a lectin, is first accumulated below the plasmamembrane, followed by delivery to food vacuoles (Ramoino et al., 2001). Applying exogenous peroxidase and EM analysis in *P. multimicronucleatum* reveals the transfer to disk-shaped vesicles below the basis of cilia (terminal cisternae = early endosomes) and transfer to late endosomes (Allen et al., 1992). The “early” vesicles possess an antigen of the cell membrane. Molecular biology clearly provides an even more distinct picture as we find proteins specific for the different stages of vesicle internalization (Tables 3.1–3.4 and below).

How is the molecular equipment of the terminal cisternae, that is, the early endosomes, in *Paramecium* (Allen, 1988)? We could localize *PtSyb11* (Schilde et al., 2010), *PtSyx3* (Kissmehl et al., 2007), and H^+ -ATPase SU-a1 (Wassmer et al., 2005, 2006) to this organelle. No specific SNAREs for homotypic fusion between early endosomes have been found in higher eukaryotes (Brandhorst et al., 2006). This may be even more valid for *Paramecium* where the stereotype arrangement of these well-defined structures would not suggest occurrence of homotypic fusion. If so, the dual endowment with R- and Q-type SNAREs, *PtSyx3* and *PtSyb11*, may indicate additional vesicle input, for example, from the Golgi apparatus (Allen, 1988).

From NSF gene silencing experiments, we have recently obtained evidence of trafficking of smooth-surfaced vesicles to/from other sites of the cell membrane, even within the normally very narrow cleft between the cell membrane and alveolar sacs (Schilde et al., 2010; H. Plattner,

B. Schönemann, and C. Schilde, unpublished observation). Near the ciliary basis there may occur some particular sites predetermined for vesicle trafficking, as *PtSyb10* is enriched there in patches (Schilder et al., 2010). This aspect is discussed in Section 9.2.

The most crucial points of this section can be summarized as follows. Specific SNAREs, H^+ -ATPase, and actin isoforms are clearly engaged in constitutive endocytosis in ciliates, as we find specific paralogs associated with the established sites, the parasomal sacs. From NSF silencing experiments with *Paramecium* cells, we also obtained evidence for the relevance of SNAREs for constitutive exocytosis and/or endocytosis outside the established sites.

4.3. Stimulated exocytosis and exocytosis-coupled endocytosis in ciliates

4.3.1. Assembly of exocytosis sites

After delivery to the cell surface, an estimated period of several minutes may suffice to acquire exocytotic fusion capacity of trichocysts (Plattner et al., 1993). When *Paramecium* cells of the temperature-sensitive mutant, *nd9* (Beisson et al., 1980; Froissard et al., 2001), are transferred from a nonpermissive to a permissive temperature ($28^\circ\text{C} \rightarrow 18^\circ\text{C}$), almost all sites achieve exocytosis competence, paralleled by assembly of “rosettes” (aggregates of intramembraneous particles/integrated proteins seen in freeze-fractures; Section 5.2) within hours (Froissard et al., 2002). This does not contradict our previous estimation of much shorter times for the individual process (Plattner et al., 1993), as this seeming discrepancy is observed with all steps of the exo-endocytosis cycle due to a certain degree of asynchrony (Knoll et al., 1991a; Plattner and Hentschel, 2006).

Interestingly, NSF gene silencing during the $28^\circ\text{C} \rightarrow 18^\circ\text{C}$ transfer of *nd9* cells can suppress rosette assembly and acquirement of fusion competence (Froissard et al., 2002); Section 5. This implies that NSF, in this case, is required to establish SNARE complexes, rather than to disassemble them after exocytosis. The latter has been found with some other systems and then tacitly generalized (Littleton et al., 2001). In fact, the sequence we described has also been found with bovine chromaffin cells (Xu et al., 1999). Even more strikingly, recent electrophysiological studies with neuronal cells analyzed under conditions of sufficient time resolution concludes an ongoing interaction of NSF with SNAREs to maintain them in an assembled state ready for fusion (Kuner et al., 2008). Since trichocyst docking sites are newly formed over many cell divisions independently of any exocytosis, this also implies a primary role of NSF for SNARE assembly, rather than disassembly after fusion (although NSF may also support SNARE rearrangement after trichocyst exocytosis).

4.3.2. Dynamics of exocytosis in *Paramecium*

In *Paramecium*, an appropriate stimulus provokes the immediate release of trichocysts. Such a stimulus can be the contact with a predatory ciliate, such as *Dileptus*, whose attacks are survived selectively by exocytosis-competent cells, as detected by Harumoto and Miyake (1991). Based on this work, subsequent studies by Knoll et al. (1991b) have shown that local trichocyst release keeps the predator at a distance, thus allowing the *Paramecium* cell to escape. While the actual chemical stimulus is not known, a mechanical stimulus does not produce this phenomenon. In contrast, it can be perfectly mimicked by polyamines such as aminoethyl-dextran, AED (Plattner et al., 1984, 1985b). Meanwhile, AED has been accepted as a standard secretagogue for *Paramecium*.

The dynamics of synchronous trichocyst exocytosis and exocytosis-coupled endocytosis has been thoroughly analyzed by quantitative quenched flow/cryofixation/freeze-fracture EM analysis (Knoll et al., 1991a; Plattner and Hentschel, 2006; Plattner et al., 1997). Synchronous trichocyst exocytosis upon stimulation with AED occurs within 80 ms, followed by ~ 270 ms for endocytotic membrane resealing and still longer times for pinching off trichocyst “ghosts.” Again, the individual fusion/resealing pore has a much shorter life-time than registered for the overall phenomenon in the entire cell population analyzed. For the individual fusion event, we estimate a time requirement of ≤ 1 ms, that is, below the methodical time resolution which was available to us (Plattner et al., 1993). With liposomes, this has been substantiated *in vitro* by fast kinetics analysis (Kasson et al., 2006) and *in vivo*, by patch-clamp analysis, with mammalian cells (Breckenridge and Almers, 1987), as summarized by Sorensen, 2005). The open time of a fusion pore (opening to full width) is about in the range of ~ 1 s. It is similar when recorded by electrophysiology with mammalian dense core-vesicle systems (Fang et al., 2008) as it is for the stimulated exo-endocytosis coupling in *Paramecium*. In the latter case, coupling is accelerated with increasing $[\text{Ca}^{2+}]_0$ (Plattner et al., 1997), as it is in mammalian systems (Henkel and Almers, 1996; Rosenboom and Lindau, 1994), as discussed in Section 7.

What is the molecular background of these processes? No ATP is required for membrane fusion *per se* during trichocyst exocytosis (Vilmart-Seuwen et al., 1986). There is currently unanimous agreement on this aspect also in other systems (Sorensen, 2005) though initially this issue had been hotly debated.

In *Paramecium*, *PtSyx1* is considered the t-type SNARE required for trichocyst exocytosis, as silencing of this gene greatly reduces the stimulated exocytotic response (Kissmehl et al., 2007). Strikingly, *PtSyx1* is scattered over the entire “somatic” (nonciliary) cell membrane, whereas one would expect concentration at the sites with rosettes, directly above the trichocyst tips. (The aspect of potential microdomain arrangement is discussed in Section 5.) The rather ubiquitously distributed *PtSNAP-25-LP* is also recognized over the

entire cell boundary (Schilde et al., 2008). Both, *PtSyx1* and *PtSNAP-25-LP* may also mediate any other membrane fusion events occurring at the cell surface.

How about other components for stimulated exo-/endocytosis and other membrane fusion processes? Unfortunately, the v-type SNARE pertinent to trichocyst exocytosis could not be identified unequivocally so far, but *PtSyb5* is a realistic candidate (Section 3.3). Another open question pertinent to trichocyst exocytosis concerns the nature of the Ca^{2+} -sensor which has to be expected at such sites, as outlined in Section 7. Finally, the relevance of calmodulin occurring at trichocyst exocytosis sites (Momayezi et al., 1986; Plattner, 1987) expects elucidation. Calmodulin is known to be mandatory for the assembly of a functional trichocyst exocytosis site (Kerboeuf et al., 1993). In mammalian cells, calmodulin binds to synaptobrevin (Quetlas et al., 2002) and, thus, can affect the arrangement of SNAREs. By interaction with the auxiliary protein, Munc13, calmodulin also drives priming for neurotransmission (Dimova et al., 2009). However, from its Ca^{2+} -binding properties and kinetics, calmodulin is generally considered inappropriate to serve as a Ca^{2+} -sensor for a rapid exocytotic response. Beyond this, calmodulin is a Ca^{2+} -sensor for the different forms of endocytosis in nerve terminals (Wu et al., 2009) and, thus could exert the same function in ciliates. In sum, calmodulin may be a multifunctional component of dense core-vesicle docking/exocytosis sites also in ciliates. Actin is another modulator of exocytotic fusion pore dynamics, for example, in pancreatic acinar cells (Larina et al., 2007). Remarkably, actin flanks trichocyst docking sites in *Paramecium* (Kissmehl et al., 2004) and trichocyst docking is reportedly inhibited by cytochalasin B (Beisson and Rossignol, 1975).

Combining fast freezing technology during stimulated synchronous trichocyst release with EM analysis (Plattner and Hentschel, 2006) has shown that membrane fusion probably occurs within a submillisecond time scale and presents itself as a ~ 10 nm large spot (“fusion pore”). All this corresponds to the temporal and spatial resolution achievable by the method used (Knoll et al., 1991a; Section 5). Subsequently, the pore expands and thus allows access of extracellular Ca^{2+} to the trichocyst contents. This entails vigorous expansion of the trichocyst matrix (Bilinski et al., 1981) by Ca^{2+} binding to specific matrix proteins (Klauke et al., 1998), as commented in Section 7.2.

Not only trichocyst membranes (“ghosts”) are internalized by exo-endocytosis coupling, but also dischargeable intact trichocysts can be caused to dedock and eventually to redock. In nondischarge strains of *P. tetraurelia*, trichocysts can be detached from the cell surface and brought to redocking under conditions described (Pape and Plattner, 1990). This observation is supplemented by the following experiments. Secretory contents release is blocked in a *P. caudatum* mutant (Watanabe and Haga, 1996) because of

defective Ca^{2+} -binding to secretory matrix proteins (Klauke et al., 1998). In normal cells, contents release can also be inhibited by exocytosis stimulation under conditions unfavorable to matrix expansion, thus resulting in “frustrated exocytosis” (Klauke and Plattner, 2000). This has been visualized by the styrene dye FM1-43 that is spontaneously incorporated into the cell membrane and diffuses into the secretory organelle membrane upon fusion (Henkel et al., 1996). In this case, trichocyst membranes fuse with the cell membrane, just as during exocytosis, but without contents release. This “frustrated exocytosis” is followed by resealing, internalization, and redocking of the intact organelles which can be stimulated to perform normal exocytosis. The organelle must have a signal indicating its state (Section 3.3.3) because empty “ghosts” would go the degradation pathway. All this has specified for the first time membrane detachment as a distinctly regulated step.

We summarize this section on exo- and endocytosis as follows. The highly efficient machinery of stimulated exocytosis is only partly understood on a molecular level, also in ciliates. Actin is found around docking sites underneath the cell membrane (Section 2.3.2). *PtSyx1* is involved in stimulated exocytosis, though it is distributed all over the somatic cell surface. Quite uncertain is the type of v-SNARE, possibly *PtSyb5*, in the trichocyst membrane (Section 3.3.3). No evidence could be found in *Paramecium* for any contribution of the proteolipid part of the H^+ -ATPase to exocytotic membrane fusion, as elaborated in Section 5. Finally, we observe a specific difference between the detachment of intact trichocysts and their “ghosts” formed by exocytosis.

5. POSSIBLE SNARE ARRANGEMENT IN MICRODOMAINS AND MEMBRANE FUSION

5.1. General aspects

As mentioned, for fusion to occur, two of the SNAREs forming a SNARE pin have to be anchored by a transmembrane domain in the opposing membranes. Concomitantly, exchange of a transmembrane domain by a lipid anchor in R- or Qa-SNAREs inhibits fusion (Grote et al., 2000; McNew et al., 2000). Note that Qa/b-SNAREs, such as *PtSNAP-25-LP*, have no transmembrane domain.

Using widely different methods, the number of SNARE molecules of any type occurring on the donor and receptor side has been estimated as about six (Han et al., 2004; Montecucco et al., 2005). This would suffice to form a kind of crown of SNARE pins for tethering, zippering, and subsequent fusion of membranes. At neurotransmitter release sites, indirect methods have suggested the occurrence of at least 3 (Lu et al., 2008), more likely 5–8, or a maximum of 10–15 SNARE pins (Montecucco et al., 2005). According to the Jackson

group, the transmembrane domains of 5–8 syntaxins finally line a fusion pore (Han et al., 2004). SNARE complexes required for fusion can be accommodated in a $\sim 3\text{--}4$ nm large area (Martens and McMahon, 2008). Some other measurements revealed formation of larger SNARE microdomains that define sites for vesicle docking and fusion (Lang et al., 2001). A cluster of ~ 75 syntaxins, 50–60 nm in size can be assembled within ~ 10 min (Sieber et al., 2007) by homo-oligomerization via interaction of the α -helical SNARE domains (Sieber et al., 2006). Clustering syntaxin 1 (equivalent to *PtSyx1*) in the cell membrane can be mimicked in reconstitution studies by adding cholesterol (Murray and Tamm, 2009) under conditions that differ from those characteristic of classical “rafts” or microdomains induced by scaffolding proteins. The authors assume this a potential regulatory aspect of localized exocytosis site formation. In this case, it remains unclear how interaction with SNAREs of the opposed membranes would not be sterically hindered during fusion. For further comments, see below.

By contrast, synaptobrevins are reported not to be liable to oligomerization (Bowen et al., 2002). However, this is what we see with *PtSyb10* in the plasmamembrane (!) surrounding the basis of cilia (Schilde et al., 2010), as outlined in Section 5.2. Are such microdomains formed by SNAREs only in the cell membrane? For the time being, this remains an open question.

May SNAREs at the cell surface exert still other functions? In neuronal cells, syntaxin1, together with the scaffolding proteins reggie/flotillin clusters the (normal) Alzheimer amyloid precursor protein in the cell membrane, thus facilitating normal trafficking and processing (Sakurai et al., 2008). This would mean an alternative, indirect function of SNAREs, that is, microdomain assembly of a specific protein to prepare its piecemeal removal by endocytosis. Whether the reggie/flotillin-related scaffolding protein stomatin (occurring in the *Paramecium* database) can serve such a function or, alternatively, mediate formation of microdomains for intracellular signaling, as executed by reggie/flotillin-based microdomains (Langhorst et al., 2005), remains to be analyzed with ciliates. Remarkably, in mammalian cells, in such microdomains GPI-anchored proteins are enriched; among them is the prion protein, PrP^C. This in turn can modulate, in neurons, $[\text{Ca}^{2+}]_i$ dynamics upon stimulation (Powell et al., 2008). Therefore, SNAREs can induce microdomains and these may perform, in addition to membrane-to-membrane interactions, other functions such as signaling. Currently, such aspects remain hypothetical paradigms yet to be analyzed with ciliates.

5.2. Aspects concerning ciliates

In *Paramecium*, a microdomain-type assembly has been observed with Syb10 (Schilde et al., 2010). We have combined expression of *PtSyb10* as a GFP-fusion protein, monitoring by fluorescence imaging, and EM-analysis by applying anti-GFP antibodies and proteinA-gold-conjugates. Possible

functional implications are discussed in Section 9.2. Another question concerning microdomain assemblies is the molecular identity of “rosette” particles seen on freeze-fractured exocytosis sites of trichocysts and mucocysts of *Paramecium* and *Tetrahymena*, respectively (Orias et al., 1983; Plattner et al., 1973), as well as in some other ciliates (Bardele, 1983). If a rosette particle would represent densely packed aggregates of single membrane-spanning domains of any type of SNAREs, one particle is estimated to accommodate ~ 75 molecules (Plattner and Kissmehl, 2003b). This figure may just coincidentally be identical with that of plasmalemmal syntaxin 1 in the aggregates described by Sieber et al. (2007). It is noteworthy, however, that a rosette is an infallible indication of the presence of an exocytosis-competent trichocyst underneath (Beisson et al., 1976; Pouphe et al., 1986; Vayssié et al., 2001). Nevertheless, rosette particles still could represent integral membrane proteins unrelated to SNAREs.

Work with *Paramecium* has gradually developed to a general view of membrane fusion (“focal fusion concept”)—importantly with the inclusion of proteins in the formation of a point-like fusion pore (Knoll and Plattner, 1989; Plattner, 1981, 1987, 1989; Plattner and Knoll, 1993), rather than an extended diaphragm (the intermediate fusion structure then commonly assumed). While point fusion had been described already by others with *in vivo* (Heuser et al., 1979) and *in vitro* systems (Verkleij et al., 1979), any essential role of membrane proteins had been envisaged for the first time in the work with *Paramecium*. This has been endorsed by the analysis of secretory mutants obtained by the Beisson group (Beisson et al., 1976, 1980; Lefort-Tran et al., 1981; Pouphe et al., 1986) and by the evident dependency of fusion capacity on defined protein arrangements at fusion sites, the rosettes (Plattner et al., 1973; Vilmar and Plattner, 1983). While all this currently may appear trivial it was far from being so at that time. Focal fusion (point fusion) has then unequivocally been demonstrated with improved temporal and spatial resolution by patch-clamp analysis (Breckenridge and Almers, 1987; Neher and Marty, 1982). For the time being, the discussion still goes on whether lipids (Fang et al., 2008), proteins (Han et al., 2004; Jackson and Chapman, 2006) or, alternatively, both (Chapman, 2008) may line the fusion pore—probably another trivial aspect, once settled.

In *Paramecium*, c-SUs (proteolipid, V0 part) of the H^+ -ATPase are clearly not present at preformed exocytosis sites in *Paramecium* (Wassmer et al., 2005) (whereas the H^+ -ATPase is present in the trichocyst membrane [as discussed in Section 3.3]). Thus, it was not possible to expand a concept derived from homotypic (vacuolar) membrane fusion in yeast (Bayer et al., 2003; Mayer, 2002; Peters et al., 2001) to exocytosis. Interestingly, the perception of this hypothesis of membrane fusion is distinctly divided—believers cluster among H^+ -ATPase experts and doubters among most of those dedicated to the analysis of membrane fusion.

Expulsion of trichocysts is enabled by instantaneous rearrangement of crystalline matrix components (Sperling et al., 1987; Section 7.2) before trichocyst exocytosis sites are rapidly resealed within ~ 270 ms after exocytosis (~ 80 ms), that is, ~ 350 ms after stimulation (Knoll et al., 1991a). This membrane fusion event initiates “ghost” retrieval and is also of the focal type (Plattner et al., 1992). For technical reasons, it could be demonstrated only at a much later time, by patch-clamp analysis, that endocytotic membrane resealing in mammalian cells is also of the point-fusion type (“fission pore”) (Rosenboom and Lindau, 1994; Roux and Antonny, 2008).

The essential points of this section are the following. The molecular identity of “rosettes,” the prominent freeze-fracture particle aggregates seen at preformed exocytosis sites in the cell membrane of ciliates—an indicator of exocytosis competence—has not yet been elucidated. t-SNAREs contributing to exocytosis are *PtSyx1* and possibly *PtSNAP-25-LP*, while *PtSyb5* is a candidate for a trichocyst v-SNARE. No such role could be assigned to V0-type SUs of the H^+ -ATPase as they do not occur at trichocyst exocytosis sites.

6. PHAGOCYTOSIS

The general background, mainly derived from macrophages, is as follows. Though phagosome formation, for instance in macrophages, is perceived as an indentation of the cell membrane, formation and detachment of a phagocytotic vacuole (phagosome) requires delivery of additional membrane involving NSF (Coppolino et al., 2001) and specific SNAREs (Braun et al., 2004; Hackam et al., 1998). Substantial membrane material is contributed by fusion of Rab11-positive recycling vesicles (Braun and Niedergang, 2006; Cox et al., 2000; Huynh et al., 2007) with the participation of synaptotagminV (Vinet et al., 2008) as a Ca^{2+} -sensor and VAMP3 as a v-SNARE (Bajno et al., 2000). Whereas early endosomes contribute little, if any membrane material, fusion with late endosomes/lysosomes follows at a later stage (Bajno et al., 2000), thus forming a phagolysosome. This transition can be followed by the respective Rab-type G-proteins (Haas, 2007; Novick and Zerial, 1997). In macrophages, fusion of a phagosome with late endosome/lysosome also delivers the H^+ -ATPase into the phagolysosomal membrane thus formed (Sun-Wada et al., 2009).

Phagosome formation is paralleled by the assembly of an actomyosin coat (May and Machesky, 2001; Soldati and Schliwa, 2006), regulated by an Arp protein (Niedergang et al., 2003), and formation of a dynamin–amphiphysin complex for pinching off (May and Machesky, 2001), that is, for closing the vacuole and its detachment. Further on, there occurs multiple input and

exchange of components. Concomitantly, proteomics of phagosome membranes from macrophages yields a variegated picture (Garin et al., 2001).

6.1. Phagocytosis in ciliates

Phagocytosis has been analyzed in most detail by Richard Allen and Agnes Fok and their associates in *P. multimicronucleatum* using electron microscopy, including labeling with monoclonal antibodies (Allen, 2000; Allen and Fok, 2000; Fok and Allen, 1988, 1990). The phagocytosis cycle is sketched in Figs. 3.2 and 3.5. In their pivotal study, Allen et al. (1995) describe the expansion of the nascent phagosome membrane by membrane delivery in the form of “discoidal vesicles.” Pinching off is supported by F-actin (Cohen et al., 1984; Kersken et al., 1986), followed by fusion with “acidosomes” (Allen and Fok, 1983a) and retrieval of acidosomal constituents by discoidal vesicle membrane formation (Allen and Fok, 1983b). This membrane replacement was sensitive to cytochalasin B, but not to acidification (Fok et al., 1987). Then, lysosomal components are delivered and later on retrieved (Allen and Fok, 1984b). This is complemented by retrieval of an early population of discoidal vesicles (Allen et al., 1995), before the spent food vacuole discharges its contents at the cytoproct (Allen and Wolf, 1974), with an additional, late retrieval of a second population of discoidal vesicles (Schroeder et al., 1990). See Fig. 3.8 for a selection of molecular components involved in phago(lyso)some cycling in *P. tetraurelia*, as subsequently discussed.

Application of cytochalasins to *Tetrahymena* (Gronlien et al., 2002a) and *Paramecium* (Allen and Fok, 1985; Cohen et al., 1984; Kersken et al., 1986; Zackroff and Hufnagel, 1998) inhibits phagocytosis. The different steps, from pinching off, acidosome and lysosome fusion as well as membrane retrieval from the cytoproct, have different cytochalasin sensitivity (Allen and Fok, 1985; Fok et al., 1985, 1987). Generally F-actin is a well-established contributor to phagosome formation, trafficking, and processing. In ciliates, the different sensitivity to disrupting drugs must be caused by the participation of different actin isoforms with different drug sensitivity (Sehring et al., 2007a), as specified in Section 2.3.2 below, and in Table 3.3.

Phago(lyso)some biogenesis requires more detailed elucidation. In *Paramecium*, in addition to recycling “discoidal vesicles” one can see, in time-resolved microscopy, small vesicles associated with the oral cavity; they vigorously travel directionally along to local cytoskeletal elements (Ishida et al., 2001). In this region, we found vesicles with *PtSyb8*, *PtSyb9*, and *PtSyb10* (Schilde et al., 2010). Also among *PtSyx* proteins, such as *PtSyx7*, *PtSyx9*, *PtSyx10* we found some that are specifically dedicated to the phagocytotic system (Kissmehl et al., 2007). Since for these vesicles no information is available about specific markers, for example, small G-proteins, it is impossible at this time to classify them accordingly, but

Figure 3.8 The phago(lyso)somal cycle of *Paramecium*. (A) Formation and maturation of the phago(lyso)somal vacuole (“food vacuole”), with the change of luminal pH from acidic (after fusion with numerous small acidosomes, as) to near neutral and neutral, for final discharge at the cytoproct (cp). For other abbreviations, see Fig. 3.2A. Scheme adapted from Wassmer et al. (2009). (B) SNAREs, actin, and H^+ -ATPase SU isoforms contributing to the phago-/lysosomal cycle in *P. tetraurelia* cells. Scheme as in Fig. 3.2B. Superimposed are data combined from Kissmehl et al. (2007), Schilde et al. (2006, 2008, 2010), Sehring et al. (2007a), and Wassmer et al. (2005, 2006). Magnification of micrographs $\sim 600\times$. Source of the basic scheme as in Fig. 3.2A; micrographs from Sehring et al. (2007b).

they may contribute to *Paramecium*'s phagosome formation in one or the other way. The organelle, commonly called the "food vacuole," receives its H^+ -ATPase only after pinching off, by fusion with acidosomes. These are considered late endosomes (Allen et al., 1993). This distinction from other endosomes is compatible with several facts. (i) Acidosomes are endowed with H^+ -ATPase (Allen and Fok, 1983a, 1993), just like late endosomes. (ii) In *Paramecium*, they may be formed as follows: The early endosomes (terminal cisternae) although also containing an H^+ -ATPase (Wassmer et al., 2006), receive additional input from Golgi vesicles (Allen, 1988) (Sections 1.3 and 4). (iii) Recycling endosomes are generally devoid of any H^+ -ATPase in other systems (Gagescu et al., 2000; Hinton et al., 2009), but—as stated—they contribute to phagosome formation in mammalian cells, as they do in the form of discoidal vesicles in *Paramecium*. In sum, in *Paramecium* late endosomes and recycling vesicles contribute to food vacuole formation. The contribution of the small cytopharyngeal vesicles mentioned above remains to be established. For a more stringent classification of additional contributors one would have to know the orthologs of the organelle-specific Rab proteins, types 5, 7, and 11, as established in higher eukaryotic cells (Section 2.2).

Food vacuoles travel through the cell, mostly steadily but locally in a saltatory manner, by cytoplasmic streaming (cyclosis). Velocities registered vary from ~ 1 to $2 \mu\text{m}$ (Nishihara et al., 1999) or ~ 2 to $4 \mu\text{m s}^{-1}$ (Sikora, 1981) in *P. bursaria*, whereas in *P. multimicronucleatum* maximally $\sim 6 \mu\text{m s}^{-1}$ has been measured (Ishida et al., 2001). This is fast enough to postulate active propulsion (see below).

6.2. Involvement of actin in phagocytotic cycle of ciliates

Recent analyses have revealed a considerable number of actins and actin-related proteins in *T. thermophila* (Kuribara et al., 2006) and an even much higher number of actins (subdivided into nine subfamilies) in *P. tetraurelia*, with a particularly high number of subfamily 1 members (Sehring et al., 2007a,b). Analysis of potential binding sites for drugs that disrupt (such as latrunculin A) or stabilize F-actin (phalloidin, jasplakinolide) have prognosticated widely different sensitivities, from fully sensitive to insensitive (Sehring et al., 2007a). The numerous isoforms occurring in *Paramecium* are specified in Table 3.3, those contributing to phagocytosis in Fig. 3.8B.

In *Paramecium* and *Tetrahymena*, F-actin disrupting drugs, such as cytochalasins, do not inhibit the indentation of a phagocytotic cup, but they can slow down detachment of a phagosome (Cohen et al., 1984; Kersken et al., 1986) in a dose-dependent manner (Fok et al., 1985) and fusion with acidosomes (Allen and Fok, 1983a). It thus appears that closing of a phagocytotic vacuole requires a specific actin isoform still to be determined. Specifically, *PtAct4* is involved in phagosome/food vacuole formation

(Sehring et al., 2010)—a role it may share with Act1 in *T. thermophila* (Williams et al., 2006). *PtAct4-1* is seen in association with nascent food vacuoles and its silencing reduces food ingestion (Sehring et al., 2010). Since *PtAct4* has no prognosticated binding capacity for disrupting drugs (Sehring et al., 2007a), other actin paralogs, such as *PtAct1-1* and *PtAct1-2*, may come into play.

Altogether there seems to occur some finely tuned selectivity for actin isoforms along the phagocytotic pathway in *Paramecium*, whereas this is not so well known from higher eukaryotes. During cyclosis, actin isoforms are exchanged in *P. tetraurelia* (Sehring et al., 2007b): Actin types 1, 3, 4, 5, and 8 were seen on food vacuoles (Fig. 3.8B). *PtAct1-2* and *PtAct1-9* as well as *PtAct5-1* can either fully cover the phagosome as a smooth layer or alternatively form a smooth, but only partial cover, or alternatively produce speckles. *PtAct1-2* and *PtAct1-9* can also produce a comet tail-like structure on the lee-side relative to the organelle movement (Sehring et al., 2007b) to which it probably contributes by propulsion in analogy to *Listeria* actin tails (Tilney and Portnoy, 1989). Any sequence of these different aspects of actin arrangement during phago(lyso)some cyclosis in ciliates has not been established as yet. For comparison, in macrophages the lifetime of such a tailed aspect of (bacteria-free) phagosomes is short (Zhang et al., 2002). In a more extensive study, also with macrophages, it was found that varying actin assemblies on phagosomes can regulate docking of, and fusion with lysosomes, while comet-tails can propel the organelle (Liebl and Griffiths, 2009).

According to Nishihara et al. (1999) any contribution of actin to cyclosis of endosymbiotic algae in *P. bursaria* is unlikely, based on cytochalasin insensitivity. However, on the one hand, this may be explained by drug insensitivity of the relevant actin isoform in those cells. On the other hand, in analogy to plants (Shimmen and Yokota, 2004), actomyosin engagement would be expected. In fact, in *Tetrahymena*, requirement of the nonconventional myosin, myo 1p, for cyclosis has been established (Hosein et al., 2005). Scrutinized analysis of actin subtypes with regard to latrunculin A and myosin binding in *Paramecium* revealed that most of the actins associated with food vacuoles (*PtAct* types 1-1, 1-2, 1-9, 3, 4, 5, and 8; Table 3.3) possess full (*PtAct1-1* and 1-2), partial (*PtAct1-9*, 4, 5), or no (*PtAct3*, 8) latrunculin A-binding sites (whose similarity to cytochalasin binding, however, is not established in ciliates), whereas myosin binding generally decreases from *PtAct1-1* to *PtAct8* (Sehring et al., 2007a). (Note that the relevance of our prognostication of myosin binding capacity would need more detailed experimental confirmation.) In sum, there exists some inherent capacity to exploit actin/actomyosin for cyclosis in ciliates.

Defecation is reported to be insensitive to cytochalasin in *Paramecium* (Cohen et al., 1984). This contrasts with the occurrence at the cytoproct of *PtAct1-1* (Sehring et al., 2007b) which possesses drug binding sites

(Sehring et al., 2007a). The discrepancy may be resolved by assuming a role for *PtAct1* isoforms in the process of pinching off discoidal vesicles (known to be supported by F-actin [Cohen et al., 1984; Kersken et al., 1986]), rather than food vacuole contents discharge proper. If so, the cytoproct could be clogged for ongoing food vacuole docking and discharge.

In sum, F-actin isoforms can contribute to specific interactions of the food vacuole membrane with different components of the vesicle trafficking apparatus. To our knowledge, involvement of so widely different isoforms is not known from higher eukaryotic cells, although several aspects correspond to our observations with *P. tetraurelia*: (i) A regulatory function of actin during phagocytosis is generally acknowledged (Damiani and Colombo, 2003; Soldati and Schliwa, 2006). (ii) Actin has been found in phagosome membrane proteomics (Garin et al., 2001). (iii) Phagosomes undergo repeated cycles of actin assembly and disassembly (Yam and Thériot, 2004).

As already mentioned, phagosomes receive multiple input from the formation stage (nascent food vacuole) on (Allen and Fok, 2000; Fok and Allen, 1990). As determined with *P. multimicronucleatum*, the membrane of the nascent food vacuole is formed mainly from recycling vesicles of dual origin, that is, discoidal vesicles derived from the membrane of spent phagosomes discharging at the cytoproct (Schroeder et al., 1990) and those derived from an earlier stage of phagosome development (Allen and Fok, 2000). Acidosomes, $\sim 0.8 \mu\text{m}$ in diameter and endowed with H^+ -ATPase molecules, fuse right after detachment of a nascent phagosome (Allen and Fok, 1983a; Allen et al., 1993). Although the signal for fusion is not known, control of the accessibility of interacting vesicle membranes by superficial actin could be crucial. Additional small vesicles (Ishida et al., 2001), endowed with distinct v-SNARE populations (Table 3.1) interact with oral fibers (Schilde et al., 2010). Localization coincides with specific actin isoforms (Table 3.3) (Sehring et al., 2007b, 2010) and their fusion may finally also contribute to food vacuole formation. To sum up, different stages of food vacuole formation are associated with specific SNAREs (Kissmehl et al., 2007; Schilde et al., 2006, 2008, 2010), but not all stages are fully characterized with regard to actin binding.

In retrospect, specific interactions of vesicles with selective actin isoforms may contribute to vectorial vesicle trafficking by mechanisms yet to be determined. The actin assemblies of varying appearance occurring on food vacuole membranes in *Paramecium* (Sehring et al., 2007b; Fig. 3.8B) could interfere with vacuole-vesicle fusion, as shown in yeast (Eitzen et al., 2002). The association of *P. tetraurelia* phagolysosomes and associated vesicles containing different actin isoforms (Sehring et al., 2007b) is paralleled by the different cytochalasin B sensitivity in *P. multimicronucleatum*. Acidosome and lysosome fusion as well as membrane retrieval from the cytoproct all display different sensitivity to cytochalasin B (Allen and Fok, 1983a;

Fok et al., 1985, 1987), while they are insensitive to abolition of luminal acidity (Allen et al., 1995). It now would appear mandatory to discriminate between actin isoforms of different drug sensitivity (Sehring et al., 2007a). Beyond differences within one species, comparative analyses in the future will have to consider the wide divergence of sequences of actins in different ciliate groups (Kim et al., 2004; Zufall et al., 2006).

6.3. Role of H^+ -ATPase, SNAREs, and G-proteins in phagocytotic cycle of ciliates

The changing acidity in food vacuoles of *Paramecium* during a throughput time of ~ 20 –40 min (depending on species and strain) is illustrated in Fig. 3.8A. The precise origin of acidosomes is not established on a molecular basis, but the dominant proposal says they may be derived from subciliary early endosomes (the terminal cisternae in *Paramecium* [Patterson, 1978]). However, acidosomes are believed to represent late endosomes (Allen, 1988) as they receive additional input from Golgi-derived vesicles (Allen et al., 1993). The latter would be compatible with the likely biogenetic pathway of the H^+ -ATPase molecule (Sections 2.4 and 3.3). Comparison of SNAREs and of SUs of the H^+ -ATPase in acidosomes and food vacuoles in *P. tetraurelia* shows that the acidosomal a4-1 SU is rapidly removed from the phagosome, while other SUs are included (Wassmer et al., 2006; Fig. 3.8B). This corresponds to the morphological observation of a retrieval of acidosomal components in *P. multimicronucleatum* (Allen and Fok, 1983b). In *P. tetraurelia*, later food vacuole stages contain SUs a5, a6, and a9. Therefore, there must be several cycles of delivery and removal of H^+ -ATPase components (Wassmer et al., 2006) whose specific pathways and functional implications remain to be elucidated. In the end, old food vacuoles retain some of their H^+ -ATPase molecules, though their contents are no more remarkably acid (Wassmer et al., 2009).

Acidification, first to $pH \leq 5$, then to higher values (Fok and Allen, 1988), serves—in the presumable absence, or lack of evidence of an oxidative burst in protozoa—inactivation of ingested food bacteria and digestion by lysosomal enzymes if one takes into account their more or less acidic pH optima also in *Paramecium* (Fok and Paeste, 1982; Fok et al., 1982). Hydrolytic enzymes are delivered by fusion with lysosomes, whereas at a later stage of phagolysosome maturation lysosomal components are retrieved (Allen and Fok, 2000).

Also during cyclosis, input from constitutive endocytosis (Allen and Fok, 1980) and, after exocytosis stimulation, from trichocyst “ghosts” ensues in *Paramecium* (Lütke et al., 1986). As mentioned in Section 4.3.2, “frustrated exocytosis” involves transient membrane fusion with the cell membrane without contents release, but with membrane resealing and detachment of intact trichocysts. Interestingly, frustrated exocytosis allows trichocysts to

undergo recycling (Klauke and Plattner, 2000). This fact implies that a specific membrane signal, normally formed or exposed after contents release, is required for delivery into the phagocytotic pathway. Though this signal is not yet known it may depend on a connection between the trichocyst matrix and the trichocyst surface (Section 3.3.3).

By proteomics analysis of macrophage phagosome membranes, a plethora of constituents showed up, among them the SNARE-specific chaperone, NSF, together with its adaptor protein, α -SNAP, but surprisingly no SNAREs of the v-(R-) or t-(Q-)type (Garin et al., 2001). Actin, SUs of the H^+ -pump, Rab-type, and Arf-like G-proteins have also been detected. Apart from SNAREs this fits well with origin and activity of phagosomes. For instance, phagocytotic ingestion of bacteria does require NSF (Coppolino et al., 2001) although NSF should remain attached only transiently (Section 3.3). The phagocytic system of ciliates would be particularly suitable for time-sequence analyses of phagosome formation and maturation, as it is amenable, after magneto-bead ingestion, to magnetic separation with rather precise timing (Vosskühler and Tiedtke, 1993). In *Tetrahymena*, the exchange of small GTPases has thus been detected by $[\alpha\text{-P}^{32}]\text{-GTP}$ overlays (Meyer et al., 1998) or by partial sequencing (Maicher and Tiedtke, 1999). Unfortunately, this has not been pursued in full by genomics/proteomics analysis.

In mammalian systems, changing lumenal pH in phagosomes is paralleled by interaction with varying monomeric G-proteins and this can act as a timer controlling the transition between different maturation stages (Steinberg et al., 2007). G-proteins probably condition specific vesicle attachments and fusions, as outlined in Section 2.2. A more comprehensive proteome analysis of *T. thermophila* whole phagosomes, isolated after latex feeding, has detected—beyond lysosomal enzymes—several specific G-proteins (including Sar1 and Rab1, 7, and 13 members), a longin-type synaptobrevin, actin binding proteins, and various H^+ -ATPase SUs (Jacobs et al., 2006). This is clearly compatible with the selective localization of specific types of syntaxins (Table 3.2; Kissmehl et al., 2007), synaptobrevins—which actually are longins (Table 3.1; Schilde et al., 2006), actin (Table 3.3; Sehring et al., 2007a,b), and H^+ -ATPase SUs (Table 3.4; Wassmer et al., 2005, 2006), as analyzed *in situ* in *P. tetraurelia* cells by molecular biology.

For the association of calmodulin with phago(lyso)somes/food vacuoles in ciliates and other cells as well as the potential role it may exert, see Section 7.

6.4. Autophagy

In *P. tetraurelia*, autophagocytosis was abundant after NSF gene silencing (Kissmehl et al., 2002; H. Plattner, B. Schönemann, and C. Schilde unpublished observation). This may be due to the following effects. (i) Cells may

become energetically compromised due to disturbance of heterophagocytotic food processing; mammalian cells, for instance, react by increased autophagy when energetically compromised (Galluzzi et al., 2008; Kuma et al., 2004). (ii) Formation of autophagosomes *per se* does not require SNAREs (Ishihara et al., 2001; Klionsky and Emr, 2000); their double membrane envelope closes with the aid of a triple-A ATPase different from NSF (Babst et al., 1998). (iii) Autophagosomes may pile up because subsequent fusion with lysosomal elements does, in fact, require SNAREs for maturation to autophagolysosomes and intraorganellar digestion (Ishihara et al., 2001; Klionsky and Emr, 2000).

In retrospect the endo-/phago-/lysosomal system in ciliates is most complex. During evolution, this may have contributed to the “invention” and association of a great number of isoforms of SNAREs, H^+ -ATPase SUs, and actin in this subcellular compartment system. In fact, numbers of such molecules undergoing association/dissociation with food vacuoles are equivalent to, or even higher than known from most metazoans and higher plants, as discussed in Section 3.1.1. Our findings with *P. tetraurelia* are illustrated in Fig. 3.8.

7. CALCIUM-BINDING PROTEINS AND CALCIUM SENSORS

7.1. Comparison of Ca^{2+} -signaling in ciliates with other cells

Upon cell stimulation, Ca^{2+} transmits many different signals, in a direct or indirect way, in connection with widely different cell activities (Berridge et al., 2003; Clapham, 2007; Laude and Simpson, 2009), including exo- and endocytosis (Henkel and Almers, 1996). This also holds true for ciliates, but only with *Paramecium* has Ca^{2+} signaling been studied in some detail (Plattner and Klauke, 2001). Briefly, trichocyst exocytosis requires Ca^{2+} mobilization from alveolar sacs, the cortical Ca^{2+} stores, tightly followed and superimposed by Ca^{2+} -influx (Hardt and Plattner, 2000; Klauke et al., 2000; Mohamed et al., 2002). Locally, $[Ca^{2+}]_i$ increases to $\sim 5 \mu M$ (Klauke and Plattner, 1997). The more Ca^{2+} is present in the outside medium during stimulation, the more are all steps of an exo-endocytosis cycle accelerated, including endocytosis, that is, the final removal of trichocyst “ghosts” (Plattner et al., 1997). The very recently identified Ca^{2+} -release channels of alveolar sacs membranes are arranged such as to mediate a very rapid local Ca^{2+} signal upon stimulation and, thus, an efficient exocytotic response (Ladenburger et al., 2009).

All this clearly resembles dense core-secretory vesicle handling in mammalian cells in several regards. For instance, exocytosis and exo-endocytosis coupling in these systems is also accelerated by increased extracellular

[Ca²⁺] and the time periods required are about the same (Henkel and Almers, 1996; Rosenboom and Lindau, 1994). Concomitantly, a Ca²⁺-activated protein (Ca²⁺-sensor) must accelerate exocytosis. It will also promote exocytosis-coupled endocytosis by binding the adaptor for clathrin binding, AP-2, on its C2B domain (Schwartz, 2004). Does a Ca²⁺-sensor of any kind occur in *Paramecium* and in other ciliates?

7.2. Synaptotagmin as a Ca²⁺-sensor

Generally, the Ca²⁺ signal can be transmitted by binding to a variety of proteins, for example, low-capacity/high-affinity Ca²⁺-binding proteins with specific Ca²⁺-binding motifs. Among them are theoretically available several proteins with EF-hand motifs, as found in calmodulin, or proteins with C2-domains, as in the established Ca²⁺-sensor synaptotagmin. Particularly, the latter type is considered relevant for membrane fusion (Martens and McMahon, 2008). Such a function has been demonstrated not only for exocytosis (Chapman, 2008; Lynch et al., 2008; Paddock et al., 2008) but also *in vitro* by reconstitution studies (Lynch et al., 2007; Martens and McMahon, 2008; Tucker et al., 2004). Therefore, synaptotagmin is believed to mediate the final reaction of the exocytotic machinery to the Ca²⁺ signal which arises when mammalian cells, for example, different neuronal cell types, are stimulated (Chapman, 2008; Lynch et al., 2007, 2008; Martens and McMahon, 2008; Paddock et al., 2008).

As the local Ca²⁺ signal ranges from slightly below 10 μ M, for example, in chromaffin and some other glands (Voets, 2000), to ≥ 100 μ M in some nerve terminals (Neher, 1998), different synaptotagmin-type Ca²⁺-sensor isoforms may be in action (Sugita et al., 2002). The delay between Ca²⁺ signal formation and the actual exocytotic response reflects the Ca²⁺ binding kinetics (Heinemann et al., 1994). The signal we recorded in *P. tetraurelia* during trichocyst exocytosis (Klauke and Plattner, 1997) resembles that in chromaffin cells. Could synaptotagmin be involved? To address this question, we have to scrutinize this molecule.

Synaptotagmin is inserted by an N-terminal stretch in synaptic and other vesicle membranes (Perin et al., 1991). It represents the only established Ca²⁺-sensor pertinent to vesicle fusion that has been analyzed in any depth. Since its detection by Matthew et al. (1981) its mode of action is increasingly unraveled. Its two C2 domains, C2A and C2B, follow the N-terminal transmembrane stretch, and bind Ca²⁺ rapidly at concentrations emerging upon stimulation. Thereby Ca²⁺ ions bind to short loops protruding from eight-stranded β -barrels of the C2 domains (Chapman, 2008). Upon Ca²⁺-binding, particularly the conformational change of the C2A domain causes its partial penetration into the opposite phospholipid bilayer. This binding prefers phosphatidylserine and phosphatidyl inositol 4,5-bisphosphate (PIP₂), both enriched on the cytoplasmic side, of the vesicles to be fused

and a target membrane. Synaptotagmin also favors the interaction with SNAREs (Martens et al., 2007), notably with SNAP-25, and thus increases Ca^{2+} -sensitivity (Lynch et al., 2007; Nagy et al., 2008). This step actually comes into play only after SNARE-pin zippering (Section 3.1.2). Then, synaptotagmin can, *in vitro* and *in vivo*, significantly accelerate membrane fusion. Although many details have been ascertained in many systems (Chapman, 2008; Lin and Scheller, 1997; Lynch et al., 2007, 2008; Martens and McMahon, 2008; Paddock et al., 2008; Pobbati et al., 2006; Sorensen et al., 2006; Tucker et al., 2004) the actual process of membrane fusion remains elusive. The role of synaptotagmin may be to force lipids into some unstable rearrangement (“perturbation”) prone to fusion. The multifarious activity of synaptotagmin evidenced by all these studies and derived from the multiple sites of occurrence in cells (below) prompted us to look carefully for synaptotagmin in *Paramecium*.

Synaptotagmins or related proteins are usually found in different subcellular regions (Adolfsson et al., 2004) down to the Golgi region (Ibata et al., 2000). They occur in widely different cell types (Li et al., 1995) including plant cells where they contribute to cell membrane biogenesis (Schapire et al., 2008). In neurons and related cells, they participate not only in exocytosis but also in maturation of secretory vesicles (PC12 [chromaffin] cells: Ahras et al., 2006) and endocytosis, including recycling at nerve terminals (Jorgensen et al., 1995; Nicholson-Tomishima and Ryan, 2004; Poskanzer et al., 2003). Different paralogs—probably with different Ca^{2+} -binding characteristics—may be in use, for example, synaptotagmin V for delivering early endosome membrane to a forming phagosome (Vinet et al., 2008) or synaptotagmin VII for fusion of lysosomes with phagosomes (Czibener et al., 2006).

Proteins similar to synaptotagmin, but with only one or with more than two C2 domains have also been found, from moss to man (Craxton, 2007), but their function has remained enigmatic so far. Multiple C2-domain proteins (related to synaptotagmin, but distinct from other protein families with C2-domain) may be restricted to some intracellular sites of vesicle trafficking (Martens and McMahon, 2008) where their role remains to be analyzed, just as the precise relevance of local $[\text{Ca}^{2+}]_i$ for intracellular membrane fusions (see below).

The absence of synaptotagmin from yeast cells (Schwartz and Merz, 2009) suggests that constitutive fusion processes can take place in the absence of a Ca^{2+} -sensor, if not by supplementation by an unknown functional surrogate. Generally, constitutive exocytosis is considered a Ca^{2+} -independent process (Burgoyne and Clague, 2003; Jaiswal et al., 2009). Beyond that, a variety of intracellular fusion processes do not need Ca^{2+} (Hay, 2007). This may apply to some steps of the endo-/phagosomal pathway in some cell types. In contrast, for instance, in neutrophils phagosome-lysosome fusion requires a recordable increase in $[\text{Ca}^{2+}]_i$ (Jaconi et al., 1990). On these fundamentals we can now inspect the situation in ciliates.

7.3. Calcium and calcium sensors in ciliates

May calmodulin be of interest in the present context? We have detected in *Paramecium* 13 genes encoding putative calmodulin isoforms (R. Kissmehl, unpublished research). Besides several other sites, calmodulin has been localized, from the light to the EM level, to trichocyst docking sites (Momayezi et al., 1986; Plattner, 1987). Association of calmodulin with docking sites reflects its requirement for the assembly of functional trichocyst exocytosis sites (Kerboeuf et al., 1993). Its association with digestive vacuoles and with various other vesicles participating in trafficking (Fok et al., 2008; Momayezi et al., 1986) clearly assigns to calmodulin a role also for other vesicle trafficking pathways. This will also include the contractile vacuole complex which is labeled by microinjected fluorescent calmodulin *in vivo* (Momayezi et al., 1986), particularly since we now know this organelle to participate in vesicle trafficking (Section 9.1).

Any direct contribution of calmodulin to exocytosis, however, is not established (Burgoyne and Clague, 2003). Its role may be indirect, for example, by binding to specific SNAREs (Quetglas et al., 2002) (Section 4.3). Also any specific role of calmodulin in other vesicle trafficking steps remains to be established. An exception may be the requirement of Ca^{2+} /calmodulin for completion of the vacuole docking/fusion process, as found in yeast cells (Mayer, 2002; Peters and Mayer, 1998). This recalls findings with phagocytotic vacuoles in ciliates, as they bind calmodulin in *T. thermophila* (Gonda et al., 2000) and *P. tetraurelia* (Momayezi et al., 1986; Plattner, 1987), the latter having been confirmed recently by Fok et al. (2008) for *P. multimicronucleatum*. Furthermore, in neuronal cells, calmodulin in conjunction with the “auxiliary” protein, Munc13, can form a Ca^{2+} sensor/effector complex (Junge et al., 2004). Any such effects would be worthwhile exploring in ciliates.

In *Paramecium*, the Ca^{2+} -increase occurring at trichocyst exocytosis sites upon AED stimulation amounts to $[\text{Ca}^{2+}] \sim 5 \mu\text{M}$ (Klauke and Plattner, 1997). This is in the range of a synaptotagmin type with a Ca^{2+} -sensitivity as in an average gland cell. For instance, $10 \mu\text{M}$ is required to activate the readily releasable pool of chromaffin cells (Voets, 2000). At the cell membrane level, in some neuronal systems, sensitivity to Ca^{2+} may be enhanced by specific point mutations in the syntaxin 1A molecule (Lagow et al., 2007) or by close association of syntaxin 1A with SNAP-25 and Ca^{2+} -channels (Hagalili et al., 2008). This disturbingly complex situation in higher eukaryotic systems can set only a quite enigmatic frame for our expectations in ciliates.

Trichocyst exocytosis is considerably faster (Knoll et al., 1991a; Plattner and Kissmehl, 2003b) than any other dense core-secretory vesicle system (Kasai, 1999). Once docked, trichocysts all belong to a “readily releasable pool” (>95% of all trichocysts present). While we had to expect the occurrence of a Ca^{2+} -sensor comparable to synaptotagmin in *Paramecium*, the only

sequence with closest similarity found in the database contains eight, rather than two C2 domains (R. Kissmehl, unpublished results). In no higher eukaryotic system has the function of synaptotagmin-related proteins with more than two C2 domains been elucidated up to now. This also holds true for *Paramecium*. Whereas synaptotagmin is the established Ca^{2+} -sensor in metazoans and in plants (Schapire et al., 2008), only details of this hypothesis being currently a subject of scrutinized analysis, no equivalent molecule could be identified in ciliates as yet.

The fast response of *Paramecium* to exocytosis stimulation is supported by the vigorous expulsion of trichocyst contents. This is due to rapid decondensation of the crystalline trichocyst matrix proteins (Section 3.3) in response to extracellular Ca^{2+} when it gets access through the exocytotic opening (Bilinski et al., 1981). This decondensation/expulsion process is based on the Ca^{2+} -binding capacity of some of the contents proteins (Klauke et al., 1998) of which several ones are probably derived from one precursor. A similar mechanism, though less vigorous, may subside the release of *Tetrahymena* mucocysts which also contain acidic Ca^{2+} -binding proteins (Chilcoat et al., 1996; Turkewitz et al., 1991; Verbsky and Turkewitz, 1998).

Considering the additional involvement of synaptotagmin in exocytosis-coupled endocytosis in higher eukaryotes, by binding adaptor protein 2 (AP2), this can explain the acceleration of exo-endocytosis coupling in dependency of extracellular $[\text{Ca}^{2+}]$ in a variety of systems (Section 4). We do not know whether this would also include ciliates where such coupling is equally fast (Section 4.3).

In summary, there is some agreement that only some, but not all intracellular fusions may require Ca^{2+} (Burgoyne and Clague, 2003) and a Ca^{2+} -sensor (Hay, 2007). All these restrictions also concern *Paramecium* cells—almost the only ciliate for which we have some information available. As in other cells, calmodulin may contribute to intracellular membrane interactions. Moreover, in *Paramecium* calmodulin enables the assembly of functional trichocyst docking sites (Kerboeuf et al., 1993). Whereas synaptotagmin is a well-established Ca^{2+} -sensor for exocytosis particularly in neuronal cells, in ciliates as well as in other protozoa Ca^{2+} -signal transduction by a sensor protein with C2 domains remains poorly understood at this time.

8. ADDITIONAL ASPECTS OF VESICLE TRAFFICKING

8.1. Guidance and support by microtubules

In this section, we shall address the auxiliary role of microtubules as a kind of long-range targeting aid, the unsettled role of some additional molecules with potential relevance for vesicle trafficking in ciliates and finally the

considerable restrictions of using allegedly organelle- or molecule-specific drugs as a tool for analyzing vesicle trafficking in ciliates. This problem is largely exemplified by the effects on the microtubule system.

In metazoans, microtubules are considered relevant for fast and directional movement of vesicles of different kinds, rather than for their precise targeting (Hirschberg et al., 1998). In a variety of higher eukaryotic cells, docking of dense core-secretory vesicles takes place by saltatory movement along microtubule rails (Lacy, 1975) and, hence, with the involvement of motor proteins (Soldati and Schliwa, 2006). Only the recruitment, but not the release of dense core-secretory vesicles is accelerated by the presence of an intact microtubule system in pancreatic acinar cells (Schnekenburger et al., 2009). Endocytosis, at least from some distance from the cell membrane on (Nielsen et al., 1999), also involves microtubules (Bananis et al., 2000; Matteoni and Kreis, 1987). Microtubules contribute not only to trafficking from the early to the late endosome (Aniento et al., 1993), but also to phagosome formation (Harrison and Grinstein, 2002; Khandani et al., 2007) and intra-Golgi trafficking (Cai et al., 2007).

8.1.1. Microtubules in ciliates

Cytoplasmic microtubules are arranged in a complex pattern in *Paramecium* (Adoutte et al., 1991; Allen, 1988; Allen and Fok, 2000; Fok and Allen, 1988, 1990), in *Tetrahymena* (Gaertig, 2000), and in other ciliates. They may contain tubulin with different posttranslational modifications in ciliates (Libusova and Draber, 2006) such as *Paramecium* (Adoutte et al., 1991) and *Tetrahymena* (Gaertig, 2000; Penque et al., 1991). Ciliates may represent a useful model for analyzing the functional meaning of the numerous posttranslational modifications that are also found in metazoans (Westermann and Weber, 2003). Moreover, some of the numerous paralogs generated in *Paramecium* after gene duplication acquire specific new functions (Aury et al., 2006). This complex scenario still awaits more detailed analysis.

Microtubules flank the oral cavity from where a separate population emerges, called the (post)oral fibers. In this region, at least three types of vesicles associate with microtubules (Schroeder et al., 1990). This differentiation is supported by the differential endowment with SNAREs (Schilde et al., 2010). Additional microtubules run perpendicular to the oral cavity (Adoutte et al., 1991) and still others connect the oral cavity with the cytoproct (Allen and Wolf, 1974), as summarized by Allen and Fok (2000). This set of microtubules serves recycling of membrane materials as “discoidal vesicles” generated from the spent phagosome membrane after contents release, thus supporting the formation of a nascent food vacuole. This is true for *Paramecium* (Schroeder et al., 1990) and for *Tetrahymena* (Sugita et al., 2009).

Another chemically defined microtubule population runs around the contractile vacuole and elongates over the radial canals of the osmoregulatory system in *Paramecium* (Adoutte et al., 1991) and in *Tetrahymena* (Gaertig,

2000). In addition, in *Paramecium*, single distinct microtubules have been observed to emanate from the cell periphery, deep into the cytoplasm (Adoutte et al., 1991), and may serve trichocyst docking (Aufderheide, 1977; Plattner et al., 1982).

Among oral (and intracytoplasmic) microtubules in *Tetrahymena* there are glycinated and glutamylated forms (Gaertig, 2000). Different microtubule subpopulations made of differently posttranslationally modified tubulin participate in phagosome processing in *T. thermophila* (Wloga et al., 2008). Microtubule subpopulations differ in sensitivity to depolymerizing drugs, concomitantly different steps of the digestive cycle in *Paramecium* have widely different drug sensitivities (Fok et al., 1985). Also their cold sensitivity differs (Adoutte et al., 1991).

As mentioned, in *P. tetraurelia*, the different microtubule populations connected to the oral cavity are associated with vesicle types endowed with different proteins. The first population, possibly comprising two or more vesicle types (also with the additional uncertainty of discrimination from acidosomes), contains SNAREs type *PtSyb6* (Schilde et al., 2006), *PtSNAP-25-LP* (Schilde et al., 2008), *PtSyb8*, *PtSyb9*, and *PtSyb10* (Schilde et al., 2010) as well as *PtSyx3* and *PtSyx4* (Kissmehl et al., 2007). Furthermore, these vesicles differ in their H^+ -ATPase SUs; among others, they contain SUs type a1 and a4 (Wassmer et al., 2006) and they are associated with the oral filament system. This is made not only of microtubules, but it also contains *PtAct5* and *PtAct8* (Sehring et al., 2007b). Some of these vesicles are vigorously catapulted from their origin to the periphery of the respective microtubule population, just as previously described by structural video-analysis (Ishida et al., 2001). How they are associated with microtubules and by which motor proteins they are propelled remains open for the time being.

In *Paramecium*, discoidal recycling vesicles travel along microtubules to the cytopharynx. This is less evident for the population derived from partially matured phagosomes (Allen et al., 1995). Discoidal vesicles originating from the cytoproct are more clearly connected to a specific set of microtubules by dynein (Schroeder et al., 1990). Microtubule binding also occurs with the small cytopharyngeal vesicles, also probably contributing to phagosome biogenesis, since microtubules also emanate longitudinally and perpendicularly to the oral cavity (Adoutte et al., 1991) and these small vesicles are seen to travel forcefully and unidirectionally, as described above.

The motor proteins, kinesin and dynein, that drive the anterograde ($- \rightarrow +$) and retrograde ($+ \rightarrow -$) vesicle transport, respectively, along microtubules (Hirokawa and Takemura, 2005; Vallee et al., 2004) also occur in ciliates. There is much less information about kinesin and its potential contribution to vesicle transport than about dynein. *T. thermophila* expresses ~ 25 and *P. tetraurelia* ~ 26 dynein heavy chains (Wilkes et al., 2008). In *Tetrahymena*, DYH1 encodes a cytoplasmic form required for phagocytosis (independent of oral ciliary activity) (Lee et al., 1999). In *P. multimicronucleatum*, dynein has been

identified by biochemical analysis as a two-headed cytosolic form (Schroeder et al., 1990). In *P. bursaria*, its silencing inhibits the formation/detachment of a food vacuole, thus indicating a process operating in $+\rightarrow-$ direction. Nishihara et al. (1999) also report cyclosis inhibition by the bona fide dynein ATPase inhibitor, erythro-9-[3(2-hydroxynonyl)] adenine (“EHNA”). Accordingly, formation and/or transport of food vacuoles through the cell may be facilitated by microtubules. In agreement with this, nocodazole, an established microtubule depolymerizing agent in ciliates (Plattner et al., 2009), greatly reduces cyclosis in *Paramecium* (Nishihara et al., 1999).

In *Paramecium*, the basal bodies of oral ciliary assemblies called “quadriculus” and “peniculus” are associated with selective dynein isoforms, as these microtubules stain with specific antibodies (Asai et al., 1994). This is the site of vivid transport of small vesicles (Ishida et al., 2001)—a site also stained with GFP constructs of *PtAct*, *PtSyx*, *PtSyb*, and SNAP-25-LP (specified above). These vesicles are transported along with the formation of food vacuoles (Ishida et al., 2001). In *Paramecium*, the oral cavity microtubules and the postoral fibers stained differentially when a battery of antibodies was probed (Adoutte et al., 1991); postoral fibers also contain *PtSyb9*-positive structures (Schilde et al., 2010). The precise, posttranslationally modified microtubule subtype involved in the different steps mentioned is not always known as yet.

In higher eukaryotes, microtubules may equally contribute to ordered vesicle trafficking from deep inside the cell to the periphery as their disruption causes disintegration of the Golgi apparatus (Pfeffer, 2007) and abolition of saltatory transport of secretory vesicles (Lacy, 1975). In *Paramecium*, some microtubules emerge from ciliary basal bodies and hang into the cell interior vertically to the cell surface (Glas-Albrecht et al., 1991; Plattner et al., 1982). During saltatory docking (Aufderheide, 1977), these microtubules can guide trichocysts to the cell periphery. Interestingly, docking follows an inherent polarity of trichocysts, tip first, from the plus to the minus end of microtubules. (An exception is some exocytosis-incompetent mutants, such as *ptA2*, that do not dock their aberrant trichocysts [Pouphile et al., 1986].) This microtubule-guided polarity in *Paramecium* is opposite to that in most higher eukaryotic cells (Soldati and Schliwa, 2006). However, plus to minus docking has later on been observed also in MDKC (renal) cells (Bacallao et al., 1989; Bré et al., 1990). This is supported by the observation that chromaffin granules, isolated from bovine adrenal medulla and injected into *Paramecium* cells, travel to the plus end (Glas-Albrecht et al., 1991) which, in chromaffin cells, would carry them to the cell membrane. When similar experiments were conducted with chromaffin granules injected into sea urchin egg cells, granules were docked at the cell membrane (Scheuner et al., 1992), as to be expected from the minus-to-plus orientation of microtubules emanating from the cytocenter in that system.

In summary, in ciliates microtubules relevant for exo-endocytosis do not emerge from a cytocenter, but microtubule organizing centers are associated with ciliary basal bodies. The interaction of dense core-secretory vesicles with microtubules evidently follows, or imposes, an inherent directionality—another novel finding one can derive from the cited work with ciliates. Unfortunately, analysis of kinesins has been largely neglected with ciliates.

8.1.2. Additional aspects concerning microtubules in ciliates

In *P. multimicronucleatum*, the different steps of the phagosomal cycle, from acidosome fusion to fusion with lysosomes, probably involve rather different populations of microtubules and microfilaments. This may be inferred from the different sensitivity of the different steps to disrupting drugs (Fok et al., 1985, 1987). An intriguing interaction of cytoskeletal elements, microtubules and actin, is observed in *P. tetraurelia* cells by immunogold EM localization in different areas of intense vesicle trafficking (Kissmehl et al., 2004)—in agreement with the effects of some drugs aiming at microtubule and microfilament function (Beisson and Rossignol, 1975). Mechanisms and functions of these interactions remain to be elucidated in detail.

Most recently, Cda12p-containing vesicles relevant for cytokinesis (Section 9.3) have been documented in *Tetrahymena* to travel along cortical microtubules to their site of integration into the cleavage furrow (Zweifel et al., 2009). This microtubular arrangement is considered equivalent to the cytospinde described in more detail in *Paramecium* where it assembles just prior to cytokinesis (Delgado et al., 1990; Iftode et al., 1989).

In sum, microtubules form an unspecific long-range guidance system for vesicle trafficking. Ciliates contain several regularly arranged subpopulations of microtubules, with different posttranslational modifications and varying drugsensitivity. In *Paramecium*, trichocysts are docked, tip first according to their inherent polarity, along microtubules from the plus- to the minus-end. (In contrast, almost all cells of higher eukaryotes operate in the opposite direction.) In ciliates, subsets of differently modified microtubules are relevant for phagosome formation, that is, to handle the multiplicity of vesicles that are seen around the oral cavity and which all contain different membrane protein signatures.

8.2. Additional potential key players

Annexins are a family of proteins interacting with phospholipids/biomembranes subsequent to Ca^{2+} binding (Gerke et al., 2005). In *Paramecium*, antibodies generated against different common sequences from mammalian annexins revealed two binding sites (Knochel et al., 1996). One antibody bound to the cytoproct and the other one to trichocyst tips. Later on, another

family of proteins with similar binding characteristics, the copines, has been detected, first in *Paramecium* (Creutz et al., 1998) and then in higher eukaryotic, including mammalian cells. In none of these systems, the precise localization and function has been ascertained (Tomsig and Creutz, 2002). Only more recently it was found that binding of different target proteins, including cytoplasmic actin and SNAP-25, by copines may play a functional role (Tomsig et al., 2003). Similarly, annexin A2 favors actin polymerization during endosome biogenesis, also in mammalian cells (Morel et al., 2009). This can be appreciated if one considers the relevance of actin for endocytosis (Sections 2.3 and 4). To sum up, the function of Ca^{2+} -dependent phospholipid binding proteins, such as annexins and copines, awaits much more detailed exploration—not only in ciliates.

8.3. Pharmacology of vesicle trafficking

Many drugs are applied in cell biology of higher eukaryotes to unravel specific functions and to pinpoint their localization. A recent thorough evaluation of data published on the effects of different drugs in ciliates warns us of lacking, unspecific or even adverse effects of important drugs that otherwise are of standard use (Plattner et al., 2009). Therefore, application of such drugs to ciliates appears highly problematic, unless the effects are strictly established with the species under investigation, with the strict support by molecular biology.

In animal cells, neurotoxins derived from *Clostridium botulinum* and *Clostridium tetani* cleave certain SNAREs, by the metallo-endoprotease activity of their light chain, to inactive fragments (Montecucco and Schiavo, 1995; Niemann et al., 1994). In *Paramecium*, our experience is as follows. The SNAREs investigated are not sensitive to the *Clostridium* proteases tested, that is, Botulinum and Tetanus toxins (BoNT, TeNT) and their respective subtypes, respectively (Schilde et al., 2008). In unpublished work, we had previously injected the light chains of several *Clostridium* toxins and achieved inhibition of trichocyst docking by BoNT/E (D. Vetter, Diploma work, University of Konstanz). Considering its specificity (Brunger et al., 2008) we had expected cleavage of SNAP25-LP which, however, did not occur (Schilde et al., 2008). These results are in line with the insensitivity of related longin-type SNAREs in higher plants (Bassham and Blatt, 2008; Foresti and Denecke, 2008).

In *P. tetraurelia*, several actin forms are predicted insensitive to the F-actin stabilisator, phalloidin, and also to depolymerizing drugs (Sehring et al., 2007a). Thus, only a subfraction of microfilaments will be affected at specific sites of a ciliate cell. Similarly, among a variety of “antimicrotubule” drugs only some are active at low standard concentrations (Pape et al., 1991). However, the widely different subtypes of posttranslationally modified microtubules at specific sites have not yet been analyzed accordingly. For this aspect, see Section 8.1. Among H^+ -ATPase inhibitors, only concanamycin B (Gronlien et al., 2002b), but not

bafilomycin, is efficient in *Paramecium* (Fok et al., 1995). Application of brefeldin A, a blocker of binding between the Arf-GDP and the COP coat (Anders and Jürgens, 2008), would be a standard assay to identify in the light microscope the Golgi apparatus by reversible dispersal. However, unpleasantly high concentrations are required in *Paramecium* so that control by EM immunogold labeling was advised to assign different proteins to the Golgi apparatus (Kissmehl et al., 2007). This is not exceptional as several Arf types are brefeldin A-insensitive also in plants (Anders and Jürgens, 2008; Foresti and Denecke, 2008).

We now can summarize as follows. In ciliates, insensitivity to otherwise established drugs may frequently be caused by aberrant binding sites (Plattner et al., 2009). This greatly restricts the repertoire of tools available for ciliate cell biology, while it opens up a wide field for future research. Examples are the insensitivity of the ciliate Golgi apparatus (or rather its formation by vesicle trafficking from the ER) to brefeldin A and of some SNAREs to *Clostridium* neurotoxins, the SNARE-specific endopeptidases, etc.

9. EMERGING ASPECTS OF VESICLE TRAFFICKING IN CILIATES

In this section, we shall present mainly two aspects whose future development will provide important contributions to basic cell biology and to that of ciliates. (i) Unexpectedly, the contractile vacuole complex turned out to be an organelle with rather intense vesicle trafficking. (ii) Although in principle biogenesis of cilia is known to take place, in part, by vesicle trafficking, detailed information, particularly on SNAREs, is scarce. (iii) Cytokinesis is another activity of cells with considerable contribution by vesicle trafficking. Now important new aspects emerge on these aspects which will deserve enforced investigation.

9.1. Contractile vacuole complex

The contractile vacuole complex/osmoregulatory system of ciliates is made up of a contractile vacuole and emanating radial canals that are continuous with a tightly attached tubular “spongiome.” It disposes of two preformed sites of cyclic membrane fusion/fission. These are the “porus” for exocytotic fluid expulsion and the connection of the radial canals with the vacuole (Allen and Naitoh, 2002). Electrophysiology has ascertained by capacitance measurements their periodic dis-/reconnection from/to the vacuole (Tominaga et al., 1998a,b). Precisely these sites, together with the vacuole docking site at the cell membrane, are labeled by antibodies against NSF, provided dissociation of NSF is inhibited in carefully permeabilized cells by adding the NSF

inhibitor, *N*-ethylmaleimide, and nonhydrolyzable ATP- γ -S (Kissmehl et al., 2002). Note that normally NSF would be released from membrane docking/fusion sites with each cycle (Section 3.2).

Strikingly, under such conditions the other parts of the contractile vacuole complex were also labeled with antibodies against NSF, though much more faintly (Kissmehl et al., 2002). This is compatible with the localization, at the light and EM level, of some SNAREs (as GFP fusion proteins), particularly of *PtSyb2* and *PtSyx2* (and possibly of some other SNAREs) (Kissmehl et al., 2007; Schilde et al., 2006). These two are both restricted to the contractile vacuole complex where they occur on the membranes of the vacuole, of the radial canals (including ampullae), and on the widely branching tubular system of the smooth spongiome (Kissmehl et al., 2007; Schilde et al., 2006). A similar distribution is found with the Qb/c-type SNARE called “SNAP-25-like protein,” *PtSNAP-25-LP* (Schilder et al., 2008) as well as with an inositol 1,4,5-trisphosphate (InsP₃) receptor (Ladenburger et al., 2006). In contrast, the decorated spongiome, with its abundant H⁺-ATPase molecules (Fok et al., 1995, 2002; Wassmer et al., 2005, 2006) mediating a “decorated” appearance in the EM, does not display any labeling for *PtSyb2* or *PtSyx2*.

Although the biogenesis of the contractile vacuole system still remains to be elucidated, a tentative interpretation of the facts reported is as follows. Based on Section 3.3 one may assume that assembly of components of the contractile vacuole complex would begin in the ER, followed by delivery via Golgi-derived vesicles. Within the spongiome one may envisage lateral segregation, perhaps enabled by the tendency of H⁺-ATPase molecules to form dimers and higher order linear clusters (Strauss et al., 2008). This in turn could drive segregation into the decorated spongiome and this process may drive its tubularization (Allen et al., 1989).

In the contractile vacuole system, SNAREs may serve unexpectedly vivid membrane trafficking to allow for intense turnover of membrane components. Alternatively or additionally, SNAREs may be permanently required in diastole to revoke membrane vesiculation or tubularization occurring during systole, as described by Allen and Fok (1988)—a hypothesis to be tested in future work. H⁺-ATPase constituents may be delivered in vesicles as outlined in Section 3.3 to any level below the decorated spongiome, before they may be trapped in these terminal branches by lateral segregation, as outlined above.

In summary, the contractile vacuole system now appears as an unexpectedly dynamic system—far beyond its impressive systole/diastole cycle. *PtSyb2* and *PtSyx2* are SNAREs exclusively found in this complex organelle, in all of its parts except the decorated spongiome (where the H⁺-ATPase exclusively resides). There is no evidence of actin in this organelle.

9.2. SNAREs and ciliary function

Detailed knowledge on vesicle trafficking for cilia biogenesis is scarce. This will be an important field for future research. We have recently obtained some fragmentary data for *Paramecium*, as will be discussed below, after setting a baseline available from other systems.

9.2.1. Cilia in vertebrates

As known from higher eukaryotes, many constituents of cilia, or rather of their membranes, are delivered by vesicular transport close to the ciliary basis. Here, vesicles are integrated into the cell membrane by fusion for further transport in “rafts” (Rosenbaum and Witman, 2002). Fusion likely requires SNAREs, though information on SNAREs responsible specifically for ciliogenesis is scanty. Indirect evidence, or rather a postulate, refers mainly to the ongoing biogenesis/turnover of the “outer segments” of photoreceptors (Chuang et al., 2007) and to the formation of the primary cilium of nonsensory epithelia (Follit et al., 2006; Zhang et al., 2007).

Delivery of rhodopsin to the retina outer segment (sensory cilium) has been supposed for some time already to involve SNAREs although more stringent evidence came up only very recently. Biogenesis of rhodopsin transport carriers destined for the sensory cilium depends on a ciliary-targeting complex comprising Arf4 and Rab11, a Rab11/Arf effector protein and an Arf-GAP (Mazelova et al., 2009a). Targeting of these carriers to near the basis of the rod outer segment (periciliary plasmamembrane) is regulated by Rab8 and its effector Sec6/8 (exocyst; Section 3.3) as well as by syntaxin 3. Based on fluorescence images this was proposed to serve vesicle delivery to the periciliary membrane (Mazelova et al., 2009b). Rab8 (Moritz et al., 2001) and Syx3 (Chuang et al., 2007) were known already previously to participate in vesicle delivery for raft formation in rod outer segments. Syx3 stays excluded from these ciliary derivatives (Baker et al., 2008), whereas SNAP-25 is reported to be found within primary cilia (Low et al., 1998). By contrast, Mazelova et al. (2009b) localized SNAP-25 to the inner rod segment. Concomitantly, no SNAREs show up in the ciliary proteome (Blacque et al., 2005; Pazour et al., 2005). This is in agreement with the assumption that newly added membrane components are incorporated into the cell membrane below the onset of cilia, as distinct membrane domains (“rafts”), for subsequent intraciliary transport (Blacque et al., 2008; Rosenbaum and Witman, 2002).

Similar discrepancies emerge with the biogenesis of the primary cilium. Rab8, together with its GDP/GTP exchange factor—both promoting ciliogenesis—are on the one hand reported to mediate vesicle transport to the primary cilium and on the other hand to enter the cilia (Nachury et al., 2007). Similarly, exocyst components (Section 3.3) were scattered all over the primary ciliary membrane after overexpression (Zuo et al., 2009). In the

absence of any vesicle traffic inside cilia (Rosenbaum and Witman, 2002) this may be due to overexpression and/or other experimental corollaries.

9.2.2. Cilia in ciliates

Specifically in *Paramecium*, we found a candidate SNARE for ciliary function, *PtSyb10*, which is enriched in patches (“microdomains”) close to the basis of cilia, but still on the somatic cell surface (Schilde et al., 2010). A similar situation is seen within the oral cavity. Our finding of *PtSyb10*–GFP clusters in the periciliary cell membrane is surprising insofar as synaptobrevins are v-SNAREs and not liable to clustering (Bowen et al., 2002); Section 5.

Simultaneous silencing of the *Ptsyb10-1* and *Ptsyb10-2* genes significantly reduces rotations that normally accompany depolarization-induced backward swimming (“ciliary reversal”). However, ciliary activity is co-regulated by somatic channels. The actual component causing the failure observed after *Ptsyb10* silencing has not been identified as yet. Interestingly, in a *P. tetraurelia* pawn-B mutant (unable of ciliary reversal), the d4-662 transcript relevant for ciliary reversal—probably an activator of some voltage-dependent channels—was found to be delivered close to the cell membrane when expressed as a GFP-fusion membrane protein (Haynes et al., 2000). Among the exuberant K^+ -channel genes detected in *P. tetraurelia* (Haynes et al., 2003) there are some that are activated by depolarization (some with, some others without Ca^{2+} -dependency) (Kung and Saimi, 1982; Saimi et al., 1999). Such channels mediate a delayed rectification in the context of Ca^{2+} -current activation by depolarization that causes ciliary beat reversal, although they are—in contrast to the ciliary voltage-dependent Ca^{2+} -channels—localized exclusively to the somatic cell membrane (Kung and Saimi, 1982; Machemer, 1988).

In this context SNAREs could play a dual role according to some anecdotal, but paradigmatic situations in higher eukaryotes. (i) A modulator of comparable K^+ -channels in mammalian neuronal cells and cardiac myocytes is escorted by the R-SNARE VAMP7/TI-VAMP (Section 3.1) from the Golgi apparatus to the cell membrane (Flowerdew and Burgoyne, 2009). (ii) Gating of voltage-dependent cation channels is modulated by SNAREs in widely different organisms, from plants (Grefen and Blatt, 2008) to mammals (Bezprozvanny et al., 2000; Leung et al., 2007; Ramakrishnan et al., 2009). Clearly, more data are required to fully understand the situation in *Paramecium*. On a speculative basis, we consider it possible that Syb10 clusters found near ciliary bases in *P. tetraurelia* cells may be engaged in the regulation of ciliary activity, notably of the ciliary reversal response. This presupposes delivery of the respective membrane components by vesicles. The *PtSyb10* clusters we observe may also contribute to form and maintain microdomains with functionally important ion channels and signal transduction (Section 5).

In full agreement with a role for these *PtSyb10* clusters in vesicle delivery is the following observation by EM analysis (Fig. 3.7). After NSF silencing

we could observe small vesicles in close association with the cell membrane, including sites near the ciliary basis, where they normally are not visible (Schilde et al., 2010). The *PtSyb10* microdomain we observe reminds us of the recent description of “platforms,” in the nonflagellar cell membrane, that are enriched in specific proteins and destined for delivery into the flagellar membrane in *Trypanosoma brucei* (Emmer et al., 2009).

How can an R (v)-SNARE, such as *PtSyb10*, ever be enriched in the cell membrane? Such a situation could indicate a steady-state situation under conditions of intense membrane delivery with only partial retrieval of components. Such a situation has been found in some nerve terminals of the nematode, *C. elegans* (Dittman and Kaplan, 2006).

To sum up, in no cell system has the contribution of SNAREs to the biogenesis of cilia, specifically of their membrane, been explored in any satisfying detail. We have obtained some evidence of the participation of *PtSyb10* in the delivery and/or clustering of components relevant for ciliary reversal in *Paramecium*.

9.3. Cytokinesis

In an elegant study, Eric Cole and his collaborators (Zweifel et al., 2009) have identified in *T. thermophila* two proteins, Cda12p and Cda13p, that are relevant for vesicle trafficking during cytokinesis and conjugation. They produced cells with ribosomes endowed with antisense RNA-transfected 26S-rRNA according to Chilcoat et al. (2001) for downregulation, as well as N-terminally GFP-tagged fusion proteins for overexpression. They found the GFP-fusion proteins, complemented by anti-GFP antibody labeling for immunogold EM analysis, at distinct subcellular locations. Cda12p was localized to subcortical compartments fusing with endocytotic vesicles that were labeled by the exogenous sterene dye FM4-64 (Section 4.3.2). Cda13p was assigned to a structure considered the trans-Golgi network and to multivesicular bodies (late endosome/lysosome intermediates). Antisense technology with Cda12p yielded defects in processing of endocytotic vesicles and in cytokinesis, whereas Cda13p proved important for conjugant separation and subsequent cytokinesis. One can conclude from this that—out of a plethora of vesicles occurring in *T. thermophila* (Frankel, 2000)—particular vesicles, including some derived from early and late endosomes and possibly from the Golgi apparatus, contribute to cell membrane biogenesis during cytokinesis and conjugation in ciliates (Zweifel et al., 2009).

In summary, the recent findings with *Tetrahymena* complement our knowledge about vesicle trafficking in higher eukaryotes. There, the Q- and R-SNAREs, syntaxin 2, and endobrevin, have been identified in mammalian cells as being relevant for cytokinesis (Low et al., 2003).

10. CONCLUDING REMARKS

In ciliates, essential components of membrane trafficking have been identified, based on work mainly with *Paramecium* and *Tetrahymena*. Most data are compatible with those from other systems, while they also reveal important new aspects. Remarkably, the secretory mutants analyzed previously at the molecular level, particularly in *Paramecium* may all act more or less upstream of the direct membrane interactions; some of the mutations concern integral membrane proteins of unknown function. This indicates that secretory activity is regulated by a much broader spectrum of components than envisaged here. During eukaryote evolution, among the key players regulating membrane trafficking, not only Rab GTPases (Gurkan et al., 2007), but also SNAREs have widely diversified already at the level of ciliates. The same holds true for actin and some H^+ -ATPase SUs, particularly the a-SU relevant for exchangeable binding of other SUs. One also has to bear in mind the expectation of numerous “auxiliary proteins” that may interact with the SNARE machinery and that still await identification in ciliates.

What are unsettled questions of general relevance beyond ciliates? What determines specificity, targeting, and molecular interaction of different molecules on the way through the cell? Also inhibitory SNAREs need more careful investigation. Which SNAREs are relevant for biogenesis of cilia? What is the detailed interaction of auxiliary proteins in SNARE-mediated membrane fusion? Which function has Ca^{2+} -binding proteins with a number of C2-domains different from synaptotagmin? How do membranes ultimately fuse and which molecules, lipids, and/or proteins, line the fusion pore? What is the role of Ca^{2+} -dependent phospholipid-binding proteins, annexin-like proteins, and copines? As with other systems, this aspect remains in abeyance. This list could be expanded by many detailed questions for all of the different cell systems under investigation up to now.

With ciliates, some of the most significant gaps concern our uncertainty about the identity of COP-like coats. Another aspect requiring detailed analysis is the unambiguous identification of the R (v)-SNARE for dense core-vesicle exocytosis and the Ca^{2+} -sensor mediating exocytosis. What is the identity of “rosette” particles—a still enigmatic freeze-fracture/EM feature correlated with exocytosis capacity—and which function would they play? Are rosette particles microdomain assemblies of syntaxin1 (in analogy to reports from neuronal systems)? Is the arsenal of SNAREs, actin, H^+ -ATPase principal SUs, as determined for *Paramecium*, already complete? As to other potential key players, an involvement of Rab-proteins and their regulators in regulating membrane trafficking is very poorly elaborated in ciliates, so that small GTPases require extensive

exploration in ciliates. Which is the role of paralogs from a most recent whole genome duplication (“ohnologs”)? Are they all merely for gene amplification (Aury et al., 2006; Duret et al., 2008), that is, for enhancement of identical effects at identical sites? What is the consequence of the finding of only one Qb/c- and of only two Qc-type SNARE genes, in the absence of any known Qb gene, in *P. tetraurelia*? What is the role of “pseudogenes” of R-/Qa-type SNARE-like sequences and of those without a membrane anchor? Gaps also exist on many details about ciliate motor proteins, notably kinesins. Though this is a long list already for future research, many more could be enumerated. Also note that up to now molecular informations come only punctually, either from *Paramecium* or from *Tetrahymena*.

On the positive side of our balance we can note some aspects exceeding, or supplementing informations from “higher” eukaryotic systems. Secretory organelles, such as trichocysts, need not necessarily be acidic to go to the stimulated pathway of exocytosis. Nevertheless, presence of an H^+ -ATPase is required for contents processing and docking. The latter follows an inherent organelle polarity, tip first, unexpectedly from the plus- to the minus-end of microtubules. Secretory contents release by trichocysts evidently reverts or inhibits another vectorial signal, as “ghosts” go to the phagocytotic pathway, while this does not occur after “frustrated exocytosis” (transient membrane fusion without contents release). Exocytosis and endocytosis use regularly installed sites (parasomal sacs) which are epigenetically determined, as is the formation of the contractile vacuole system. Unforeseeably, this organelle undergoes intense “silent” membrane trafficking, with a constantly ongoing rearrangement and/or delivery of membrane materials. NSF gene silencing in *Paramecium* has manifested many of such cryptic membrane interaction sites which normally would not be seen. Such putative exo-endocytosis sites await more reliable and specific identification. Ciliated protozoa also have an elaborate system of membrane recycling along distinct routes. A multitude of posttranslational modifications of tubulin and a plethora of actin isoforms in *Paramecium* suggest specific interactions of the cytoskeleton at different sites of vesicle trafficking. Some of the molecular components of vesicles display an unforeseen complexity of paralogs at distinct subcellular sites. In particular, the phagolysosomal system is most elaborate in ciliates, with many fusions and fissions, involving an exchange of specific SNAREs, H^+ -ATPase SUs, and of actin isoforms. The diversification of vesicle trafficking in the context of phagocytotic food recruitment and processing may be the reason why the number of SNAREs and of other key players of vesicle trafficking resembles that seen on the highest level of evolution. Definitely the number of SNAREs in *Paramecium*, for instance, exceeds by far that extrapolated for the ur-eukaryote, and to some extent that of the urmetazoan, thus suggesting a parallel evolution of subcellular complexity.

Finally, one should not overlook the role as a paradigmatic guide provided by early work with *Paramecium*. This concerned the elaboration

of protein-based membrane interactions, including the “focal fusion” concept. Early on, outside the mainstream, this has suggested an important role for membrane proteins in membrane interaction and fusion. The recent establishment of genome databases, in conjunction with international cooperations, revive and support the significance of ciliates as model systems, also for membrane trafficking.

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