Molecular Aspects of Membrane Trafficking in *Paramecium*

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Results achieved in the molecular biology of *Paramecium* have shed new light on its elaborate membrane trafficking system. *Paramecium* disposes not only of the standard routes (endoplasmic reticulum → Golgi → lysosomes or secretory vesicles; endo- and phagosomes → lysosomes/digesting vacuoles), but also of some unique features, e.g. and elaborate phagocytic route with the cytoproct and membrane recycling to the cytopharynx, as well as the osmoregulatory system with multiple membrane fusion sites. Exocytosis sites for trichocysts (dense-core secretory vesicles), parasomal sacs (coated pits), and terminal cisternae (early endosomes) display additional regularly arranged predetermined fusion/fission sites, which now can be discussed on a molecular basis. Considering the regular, repetitive arrangements of membrane components, availability of mutants for complementation studies, sensitivity to gene silencing, and so on, *Paramecium* continues to be a valuable model system for analyzing membrane interactions. This review intends to set a new baseline for ongoing work along these lines.
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I. Introduction

Membrane trafficking has manyfold facets. It includes budding of vesicles ("fission"), in contrast to vesicle docking and membrane fusion, and, furthermore, intracellular transport, including membrane recycling (Kirchhausen, 2000). A plethora of details is known, particularly from yeast and mammalian systems, although many important aspects still remain

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to be elucidated, such as the simple sounding question: which molecules form the pore during membrane fusion? Or the more intriguing question: how are specific pathways of membrane trafficking predetermined?

Over the years, ciliated protozoa, such as *Paramecium* and *Tetrahymena*, have served as model systems for many aspects of cell biology (Plattner, 2002; Turkewitz *et al.*, 2002), although molecular information just on membrane trafficking now lags behind other systems. Therefore, frequently only a cursory discussion of certain aspects has been possible in the past, although some specific advantages (outlined in the following paragraph) of these ciliated protozoa make them most appropriate to address some specific questions.

For several reasons, this review concentrates on *Paramecium* as a model system: (i) This cell disposes of a regular "design" (Plattner, 2002); (ii) this marks several routes not so overt in other ciliated protozoa; (iii) many more analyses on membrane traffic have been executed with this cell than in other ciliates; (iv) an international *Paramecium* genomics project (Dessen *et al.*, 2001; Speeling *et al.*, 2002) has delivered new molecular details; (v) while the basic machinery for membrane-to-membrane interactions, i.e., docking and fusion, can now safely be assumed to be the same in *Paramecium* as in "higher" eukaryotes, additional proteins, not previously known from other cells, have been found; (vi) *Paramecium* is also attractive to study molecular diversification inside one cell; and (vii) reliable molecular information on this ciliated protozoan becomes increasingly available. The newly started *Tetrahymena* genome project (Turkewitz *et al.*, 2002) may allow a stimulating comparison with basic findings from the rapidly progressing *Paramecium* project, which steadily develops in the direction of proteomics.

Membrane trafficking can encompass transport, docking, fusion, and fission. The latter is a process forming two compartments from one. Membrane fusion appears much more complicated and requires recognition of the partners to be fused, i.e., specific docking, before final fusion. The different pathways of membrane trafficking occurring in a *Paramecium* cell are outlined in Fig. 1. Molecular details known so far are summarized in Table I.

For different types of membrane fusion in higher eukaryotes, it is now well established that the molecular machinery, including *N*-ethylmaleimide-sensitive factor (NSF)/soluble NSF attachment proteins (SNAP)/SNAP receptor (SNARE) proteins, governs membrane-to-membrane interactions by forming pin-like mechanical links between adjacent membranes to clamp them together and, thus, to prepare them for fusion (Söllner *et al.*, 1993a,b; Rizo and Südhof, 1998; Jahn and Südhof, 1999; Jahn and Grubmüller, 2002; Martin, 2002; Mayer, 2002). NSF in a structure-bound form is an ATPase that acts as a SNARE-specific chaperone (Whiteheart *et al.*, 2001). It thus allows to bring SNAREs in an appropriate position, i.e., to form pins

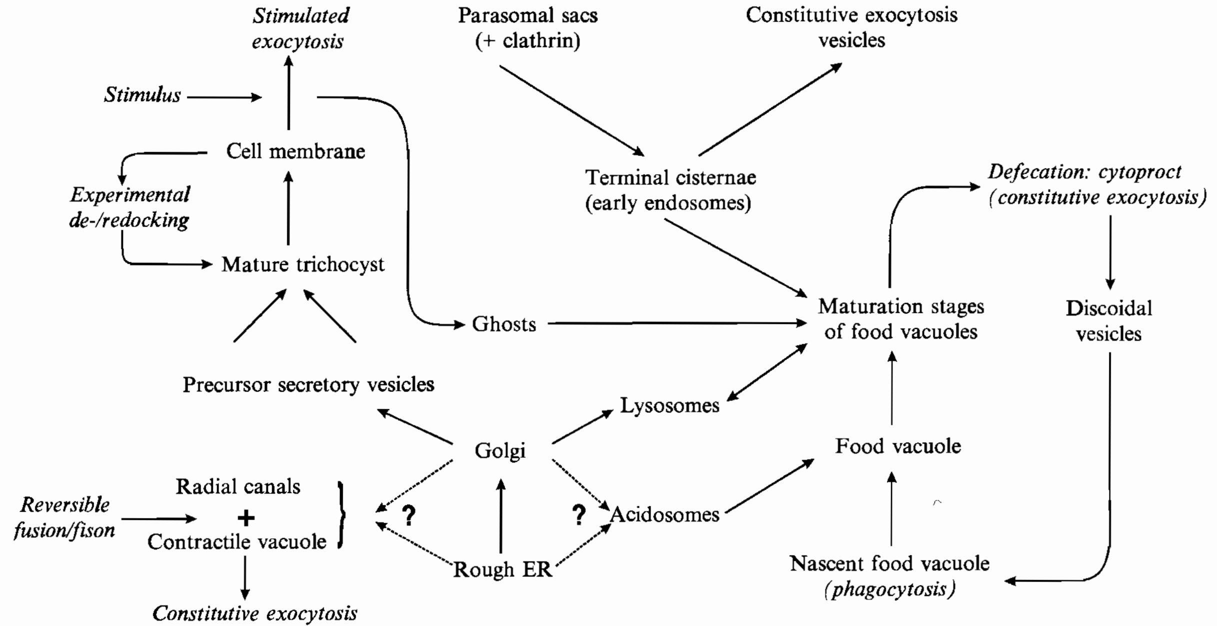

FIG. 1 Scheme of the main membrane trafficking routes in *Paramecium*, including different routes of vesicular transport, vesicle docking, and fusion, as well as vesicle fission. Omitted are minor routes, such as some aspects of the endo/phago/lysosomal system, and aspects of the biogenesis of organelles, such as alveolar sacs. For further comments, see text.

TABLE I

Gene Products, Relevant for Membrane Trafficking in *Paramecium*, for which Genes Are Known with EMBL Data Bank Accession Numbers Indicated[a]

Gene product	Trafficking steps	Availability (accession #)
Adaptor proteins		
AP1	TGN → lysosomal budding	Partial sequences, confidential (available soon)
AP2	Endocytosis	Confidential (available soon)
AP3	Endosomes	PT006K15R
AP4	TGN/endosomes/lysosomes	PT017M05R
ARF[b]/SAR (monomeric G-protein)	Golgi, etc. (brefeldin A target)	Several sequences, confidential (available soon)
Calcineurin (PP2B)	Endocytosis	
A-SU[c] (catalytic)		AF014922, AJ567906 and confidential sequences (available soon)
B-SU (regulatory)		AJ554047, AJ554048
Calmodulin	Different steps	M34540, further sequences confidential (available soon)
Clathrin, heavy chain	Different steps, including endocytosis and lysosome budding	PT008K05R, PT014115U
Coatamer proteins	ER → Golgi, intra-Golgi	
α-COP		PT017G21U
Other COPs		Confidential sequences (available soon)
Regulators of monomeric	Different steps of trafficking	
G-proteins		
GAP (guanine nucleotide activation proteins)		AF129515, several confidential sequences
GEF (guanine nucleotide exchange factors)		Several sequences, confidential (available soon)

H^+-ATPase		
V_0 proteolipid part	Potential fusion mediator	PT001A13R, AL446850, AJ566616 to 566619
a-SU (V_0 associated)	Integration of catalytic SU	PT007I19U*, AJ538330* (available soon)
nd2	Stimulated exocytosis	AJ437480
nd7	Stimulated exocytosis	Y07803
nd9	Stimulated exocytosis	AJ293945
NSF	General membrane docking	AJ347751*, AJ347752*, PT009A07R
Rab-type monomeric		
G-proteins	Different potential steps	Numerous sequences, mainly depostied by D. Fraga and B. Hinrichsen; see EMBL data bank for details. Further sequences still confidential
Rho-type monomeric G-proteins	Different potential steps	U03615, U03617, further sequences confidential (available soon)
SNAREs		
Synaptobrevin	Docking/fusion	PT018K05U, PT018009R, AJ566298 to 566301*
Synaptojanin	Endocytosis	PT020M01R

[a] "Confidential" means sequences from this and other laboratories, including the International *Paramecium* Genome Project and GDRE (information available via the internet address indicated in the text), deposited in the EMBL data bank at this time, to be disclosed at the latest publication of this review. Not included are sequences from components of the cytoskeleton and motor proteins, from signaling components, ion channels and a variety of ion pumps, which would be of indirect relevance for membrane trafficking. PT signatures are mainly partial sequences; sequences established in this laboratory are labeled by asterisk, with all sequences being available soon.

[b] Adenosine ribosylation factor.

[c] Subunit.

between adjacent membranes. NSF is an AAA-type ATPase (Neuwald *et al.*, 1999), which is released upon ATP hydrolysis (Littleton *et al.*, 2001). Once established, SNARE pins are resistant to disruption by NSF and the SNAP protein, type αSNAP (Weber *et al.*, 2000). SNAREs are considered relevant actors not only in docking, but, by some authors, also in membrane fusion (Nickel *et al.*, 1999). Alternatively work has assigned this function to secretory carrier membrane proteins (SCAMPS) (Brand and Castle, 1993; Liu *et al.*, 2002). Some time ago, there was indirect evidence of the relevance of the lipoprotein basepiece, V_0 (cf. Wilkens, 2001), of a H^+-ATPase in neurotransmitter release (Morel *et al.*, 1991). Latest developments envisage this molecular component as a fusogen when present without the H^+-pumping catalytic part so that matching pairs in adjacent membranes could closely approach each other and serve this function (Mayer, 2001; Peters *et al.*, 2001). Also, in yeast, V_0 and SNAREs are connected via a Vtc protein (Müller *et al.*, 2002).

At this time, actual fusogenic proteins are not yet established with any precision in most systems and, therefore, are still a matter of intense debate (Zimmerberg, 2001; Jahn and Grubmüller, 2002). Nevertheless, the involvement of NSF, SNAPs, and SNAREs in mediating membrane-to-membrane contacts is widely accepted. All models operate with molecules arranged side by side, in both of the membranes to interact. In the case of SNAREs, they are designated as v or t type, e.g., during exocytosis, depending on whether they occur on the vesicle membrane or on the the cell membrane as the target membrane (Söllner *et al.*, 1993a,b; Söllner and Rothman, 1994). Synaptobrevin and cellubrevin are v-SNAREs, SNAP-25 and syntaxin are t-SNAREs. Monomeric "small" GTP-binding proteins (G-proteins) also participate in vesicle docking (Rothman and Söllner, 1997; Takai *et al.*, 2001).

Exocytosis sites in *Paramecium* are ultrastructurally clearly defined microdomains (Plattner, 1987, 2002; Vayssié *et al.*, 2000). A concept envisaging the microdomain arrangement of SNAREs at exocytosis sites slowly emerges also in mammalian systems (Chamberlain *et al.*, 2001; Lang *et al.*, 2001). While synaptotagmin is an established Ca^{2+} sensor during stimulated exocytosis (Sugita *et al.*, 2002; Tucker and Chapman, 2002), it also participates in some internal membrane fusion processes that it modulates (Grimberg *et al.*, 2003).

When vesicles pinch off, a molecular machinery that is rather different from the NSF/SNAP/SNARE complex is engaged (Section IV,B,1). Different "coat protein complexes" help collect the proper components in vesicles to be formed (Nickel and Wieland, 1997; Kirchhausen, 2000; Malkus *et al.*, 2002; Yang *et al.*, 2002; Gundelfinger *et al.*, 2003). Pinching off is mediated by the monomeric G-protein, dynamin to which different auxiliary proteins are coassembled. These aspects are discussed in more detail in the respective sections.

II. *Paramecium*: A Cell with Elaborate Routes of Intense Membrane Traffic

A. Value as a Model System

Functional and (ultra)structural features of membrane trafficking and some of the membrane-to-membrane interaction sites in *Paramecium* have been analyzed thoroughly (Allen, 1988; Görtz, 1988; Plattner, 1993) before molecular biology became available as a tool for this organism. Some aspects, like the organization of trichocyst docking sites, are so prominent that some time ago, in connection with classical genetic (Beisson *et al.*, 1976) and dynamic ultrastructural studies (Knoll *et al.*, 1991), this system delivered novel hints to important determinants for vesicle docking and membrane fusion. It then served to establish new models of these basic phenomena (Plattner, 1987, 2002; Plattner and Kissmehl, 2003). Another aspect of membrane trafficking with impact on work with "higher" eukaryotes dealt with the digestive cycle (Allen and Fok, 1993a, 2000). This work also revealed novel aspects, such as phagosome acidification by dedicated organelles, the acidosomes (Allen and Fok, 1983). Nonetheless, molecular details governing membrane interactions have remained elusive with this system until recently, while a number of laboratories analyzing mainly yeast and mammalian cells achieved great progress at the molecular level.

B. Recent Molecular Approaches to the Study of Membrane Trafficking

Until recently, information on molecular details was available for *Paramecium* only to a limited extent (Kung *et al.*, 2000), although some additional molecules previously unknown from mammalian cells had been identified as determinants of some aspects of membrane trafficking (Vayssié *et al.*, 2000). On the basis of an indexed genomic library with ~60,000 macronuclear DNA clones (Keller and Cohen, 2000), the group of Jean Cohen (CNRS, Gif-sur-Yvette) initiated an international *Paramecium* genome project (Dessen *et al.*, 2001), which delivers a steadily increasing number of sequences, including some relevant for basic aspects of membrane trafficking (Table I). It allows us to design appropriate primers and to clone the respective genes.

Cloning of the *Paramecium* NSF gene, in collaboration with the Cohen group, was a pivotal step (Froissard *et al.*, 2002; Kissmehl *et al.*, 2002). Because NSF is a chaperone for SNAREs and because these interact with additional components, this now opens the door to many other components of the docking/fusion machinery. Such work allows us to predict

immunogenic sites and, with the monospecific antibodies (ABs) thus produced, we can proceed to the proteomics aspect.

(Ultra)structural localization studies depend greatly on technical achievements. For NSF, this is particularly intriguing because it is bound only transiently to sites of membrane interaction and then released. For LM fluorescence, we have therefore carefully permeabilized cells in the presence of NEM and the nonhydrolyzable ATP analogue, ATP-γ-S (which keeps NSF in place) under conditions allowing the cells to survive before they were exposed to anti-NSF ABs (Kissmehl *et al.*, 2002). Gene silencing involved macronuclear injection of a number of open reading frames (ORFs) of the NSF gene, followed by single cell processing for EM analysis (Froissard *et al.*, 2002).

We now see that in many molecules of interest, sequence homologies may be due to widely scattered amino acids rather than to large motifs. Therefore, in retrospect, we now understand why, using ABs available from other systems, previous attempts to identify proteins relevant to membrane trafficking have consistently failed. With exceptions, this also requires adequate scepticism toward localization studies using ABs against heterologous proteins in a variety of ciliated protozoa, unless specificity has been documented with particular care. Molecular biology has turned out to be a mandatory prerequisite for any further functional and structural work.

C. Established Membrane Trafficking Routes

Cotranslational sequestration of proteins in the rough endoplasmic reticulum (ER) for transport to the Golgi complex and subsequent delivery to lysosomes or to "clear secretory vesicles" for constitutive (nonstimulated) exocytosis belong to the standard repertoire of eukaryotic cells, including *Paramecium* (Allen, 1988; Görtz, 1988) and other protists (Plattner, 1993). In addition, these cells also produce dense-core secretory vesicles ("trichocysts") for stimulated exocytosis (Adoutte, 1988; Plattner *et al.*, 1993). A rather intriguing vesicle trafficking system is represented by the digestive cycle, including the formation of phagosomes ("food vacuoles") and their further processing (Fok and Allen, 1993; Allen and Fok, 2000). Finally, the osmoregulatory system, normally present in two copies, involves membrane trafficking "on the spot," i.e., repeated multiple membrane fusions and fissions without any large-scale movements (Allen, 2000; Allen and Naitoh, 2002).

Many of these membrane-to-membrane interaction sites are predetermined structurally in *Paramecium*. This is true for the sites of phagosome formation in the "cytostome"/"cytopharynx" area (Allen, 1988; Fok and Allen, 1993; Allen and Fok, 2000), the release site for spent phagosomes at

the "cytoproct" (Allen and Wolf, 1974), for the reversible attachment sites of radial canals to the two "contractile vacuoles" and for their outlets at the cell surface (Tominaga *et al.*, 1998), as well as for the constitutive exo/endocytosis sites ("parasomal sacs") located close to ciliary origins (Allen *et al.*, 1992), as well as for the docking/release sites of trichcocysts (Pape and Plattner, 1995; Pouphile *et al.*, 1986; Vayssié *et al.*, 2000).

III. Standard Route: Endoplasmic Reticulum to Golgi Complex and Further On

A wealth of molecular details is known on membrane trafficking in higher eukaryotes, including integral and membrane-associated proteins, as well as temporary coats for specific budding processes (Kirchhausen, 2000). Only circumstantial evidence is available in *Paramecium* for this obvious route. The presence of a 61-kDa protein with immunoreactivity with ABs against mammalian calreticulin-ABs in the ER of *Paramecium* (Plattner *et al.*, 1997b) suggests a Ca^{2+}- and glycosylation-dependent chaperone function, based on data from other cells (Leach *et al.*, 2002; Schrag *et al.*, 2003). The same holds for a calnexin homolog and for some disulfide isomerases whose genes have been cloned (D. Geissinger, R. Kissmehl, H. Plattner, AJ567915, AJ567916). Glycosylphosphatidylinositol (GPI)-anchored variant surface antigens (svAGs) are abundantly synthesized in *Paramecium* (Capdeville, 2000), a process known to take place also in the ER (Ferguson, 1999). Other prominent ER products comprise precursor proteins of trichocyst content, the pretrichynins (Gautier *et al.*, 1994, 1996), not to speak of any other components generally derived from ER.

Different proteins are probably transported to the Golgi complex by vesicles coated with coatamer proteins (COPs) as molecular filters, notably "coat protein complex II," COPII (Nickel and Wieland, 1997; Malkus *et al.*, 2002). Although basic details are not known in *Paramecium*, the occurrence of COPs is quite certain because of the EM appearance of such coats (see later) and because at least one fragmentary gene sequence of one type of COP is known (Table I).

A *Paramecium* cell contains a large number, probably several hundred, of Golgi fields, each of small size (Estève, 1972; Allen, 1988). The same holds true of *Tetrahymena* (Kurz and Tiedtke, 1993). This may explain why the rather simple task to identify these organelles by specific fluorescent labeling has not been accomplished to any satisfactory extent. Using ABs against the Golgi-specific (clathrin-)adaptor protein type AP1 (Traub *et al.*, 1995), we saw numerous punctate internal labeling sites, which disappeared with brefeldin A, although at relatively high concentration (M. Momayezi and

H. Plattner, unpublished observations). This agent causes Golgi breakdown (Nebenführ *et al.*, 2002) by interfering with binding of the monomeric G-protein, type ARF, which normally mediates vesicle delivery. Partial sequences of ARF are available from *Paramecium* (Table I). In addition, in mammalian cells, AP1 is engaged in vesicle traffic from the *trans*-Golgi network (TGN) to endocytotic vesicles and back to the TGN (Hinners and Tooze, 2003). COPI-type proteins would be additional markers (Nickel and Wieland, 1997; Yang *et al.*, 2002), whereas "standard" Golgi markers gave no signal (unpublished observation). For AP1 and COPs, see Table I. Definitely more molecular work is needed to identify the Golgi complex on the light microscope level in future studies on membrane dynamics. This is also mandatory to understand the budding of primary lysosomes in *Paramecium* on the basis of the respective auxiliary molecules, such as AP1 and COPI. Considering detailed knowledge available from other eukaryotes (Urbé *et al.*, 1997; Avran and Castle, 1998), much remains to be analyzed with ciliates.

Some of the trichocyst content components are glycosylated, probably including core and peripheral glycosylation (Lüthe *et al.*, 1986; Allen *et al.*, 1988; Glas-Albrecht *et al.*, 1990). For the "mesh-like sheath," a structure connecting the paracrystalline secretory matrix with the membrane in mature trichocysts (Adoutte, 1988), passage through the Golgi complex has been documented more clearly. A monoclonal AB, recognizing a 56/57-kDa component of the mesh-like sheath, also labels the Golgi complex (Momayezi *et al.*, 1993). Finally, ABs against pretrichynins also produce Golgi labeling (Garreau De Loubresse, 1993). The same work, as well as that by Allen *et al.* (1989), shows some vesicles, budding off the Golgi complex which display a smooth cytoplasmic coat, probably of the coatamer type. Such Golgi vesicles do not bind ABs against mammalian COPs (which does not exclude such identity) and side-by-side bristle-coated vesicles are found (Allen and Fok, 1993b, 2000), probably for the transport of lysosomal enzymes. However, neither budding of COP nor of clathrin-coated vesicles has been analyzed in any detail in *Paramecium* as yet. Not only for a COP subunit, but also for the clathrin heavy chain, sequences are available from *Paramecium* (Table I) that should enable one for more detailed work.

In other eukaryotes, lysosomal enzymes may be delivered by clathrin-coated vesicle budding from the TGN (Urbé *et al.*, 1997; Avran and Castle, 1998). According to the ultrastructural details mentioned, one may assume the same for ciliates. In *Tetrahymena*, lysosomal enzymes analyzed so far are glycosylated (Taniguchi *et al.*, 1985), although they lack a mannose-6-phosphate signal (Banno *et al.*, 1993). (Note that not all mammalian lysosomal enzymes carry this tag.)

The occurrence of small G-proteins in *Paramecium* has been derived from Western overlay studies with radioactive GTP-γ-S (Peterson, 1991), but their contribution to any precise step of vesicle trafficking has not been sufficiently

analyzed up to now. Table I summarizes preliminary partial cloning of different types of monomeric G-proteins in *Paramecium*. In addition to the work already published (Fraga and Hinrichsen, 1994), these authors have deposited additional sequences of a variety of monomeric G-proteins (Table I). This aspect would be particularly interesting regarding the interplay between G-proteins and SNAREs at membrane interaction sites, as "throttles and dampers" (Rothman and Söllner, 1997). The different G-proteins are understood as facilitating membrane interactions, while specificity may arise more from SNAREs (Avery *et al.*, 1999; McNew *et al.*, 2000; Scales *et al.*, 2000; Xue and Zhang, 2002). For their function *in vivo*, they require auxiliary proteins of the type guanine nucleotide-activating proteins (GAP) and guanine nucleotide exchange factors (GEF), as they also emerge in the *Paramecium* genome (Table I).

How do Golgi-derived vesicles fuse to form the elaborate trichocyst structures (Adoutte, 1988; Garreau De Loubresse, 1993)? Considering the complex, polar structure of a trichocyst, with a "tip" and a "body" part, it would be interesting to know how the specific components of the secretory contents and the specific membrane components are put together, probably by specific vesicle fusion processes. This also occurs in higher eukaryotes (Tooze *et al.*, 2001), where it depends on the Ca^{2+} sensor synaptotagmin (type III in mast cells; Grimberg *et al.*, 2003), a gene product not known from *Paramecium* as yet. Some glycoproteins may be sorted out when trichocyst contents leave the Golgi complex (Allen *et al.*, 1989). The matrix of the trichocyst body contains paracrystalline trichynin proteins derived from pretrichynins by proteolytic processing (Gautier *et al.*, 1994, 1996). While some components are glycosylated (Glas-Albrecht *et al.*, 1990), differential lectin-binding sites are concentrated in different domains within a trichocyst (Lüthe *et al.*, 1986; Allen *et al.*, 1988), the tip and contains secretory lectins (Haacke-Bell and Plattner, 1987). In total, these studies support a Golgi passage.

Normal trichocysts are not formed in the "trichless" mutant (Pollack, 1974) due to the absence of posttranslational cleavage of pretrichynins, which are then released in the untriggered mode (Gautier *et al.*, 1994). This would be a good model for analyzing the cross-talk between contents and organelle surface with regard to the choice of the export route—the constitutive and the stimulated pathway, respectively (see Section IV,A). A normal trichocyst also contains an inherent polarity signal on its membrane surface, as it normally undergoes saltatory transport along microtubules, in plus → minus direction, with the tip first (Aufderheide, 1977; Plattner *et al.*, 1982; Glas-Albrecht *et al.*, 1991). In contrast, dense-core secretory vesicles ("chromaffin granules") isolated from bovine adrenal medullae, after injection into *Paramecium* cells, all accumulate at the plus end of microtubules, i.e., the direction they go in the cells of origin (Glas-Albrecht *et al.*, 1990). Some mutants cannot deliver their trichocysts, often with aberrant ultrastructure,

to the cell surface and in some other mutants the organellar polarity is disturbed, resulting in nonextrudable trichocysts at the cell membrane (Pouphile *et al.*, 1986). Similar mutants defective on different levels of the secretory pathway have been obtained from *Tetrahymena* (Sauer and Kelly, 1995; Melia *et al.*, 1998). With these cells it was also possible to show that secretory content sorting precedes condensation into a mature form (Turkewitz *et al.*, 1991; Bowman and Turkewitz, 2001).

Another aspect of vesicle trafficking along the Golgi route is the delivery of vsAGs to the cell membrane, as we found in ultrathin section and freeze-fracture immuno gold-labeling studies (Flötenmeyer *et al.*, 1999). After cloning *NSF* genes in *Paramecium* (Kissmehl *et al.*, 2002) in our analysis of the ultrastructural effects of NSF gene silencing, we found circumstantial evidence of involvement of NSF-based molecular machinery on the ER and Golgi level. We found (i) inflated ER cisternae, as if products of ongoing synthetic activity could no longer be delivered further on, (ii) accumulation of small vesicles, labeled with ABs against NSF, amidst ER-rich domains, and (iii) fragmentation or even absence of Golgi areas (Kissmehl *et al.*, 2002). In addition to NSF, another ATPase, p97, in conjunction with additional proteins, serves to establish contacts between internal membrane systems in higher eukaryotes (Uchiyama *et al.*, 2002). No information on this is available from ciliates.

IV. Membrane Trafficking and Vesicle Movement

A. Exocytosis

1. Constitutive Exocytosis

In *Paramecium*, this involves delivery of vsAGs to the cell membrane, probably at the same sites where parasomal sacs are formed during endocytosis (Capdeville *et al.*, 1993; Flötenmeyer *et al.*, 1999). The fact that these sites almost always display the coated pit/coated vesicle aspect, rather than showing delivery of smooth vesicles for constitutive exocytosis, may be explained easily by the longevity of the former, e.g., in metazoans (Marsh and McMahon, 1999; Sankaranarayanan and Ryan, 2000; Rappoport and Simon, 2003). NSF gene silencing (Kissmehl *et al.*, 2002) resulted in a more or less aberrant ultrastructural appearance of these sites, as well as of the nearby early endosomes ("terminal cisternae"; see later).

Another constitutive exocytotic process is the release of pretrichynins by the "*trichless*" mutant, which cannot transform them by proteolytic cleavage to paracristalline assemblies (Garreau De Loubresse, 1993). This is also

assumed for corresponding mutants in *Tetrahymena* (Bowman and Turkewitz, 2001). Also in *Tetrahymena*, an experimentally inhibited paracrystalline arrangement of mucocyst contents was found not to alter membrane trafficking (Chilcoat *et al.*, 1996), as the organelles still find their preformed sites for stimulated exocytosis. The molecular determinants to divert stimulated from constitutive exocytosis have to be searched in the secretory organelle membrane components, not only in mammalian systems, but also in ciliates (Section IV,A,2). In mammalian cells, both pathways are deviated according to Kelly's "selection and exclusion" hypothesis for the delivery of secretory products into the stimulated and the constitutive pathway, respectively (Kelly, 1985; Urbé *et al.*, 1997).

The ongoing release of lysosomal contents by constitutive exocytosis has been analyzed most thoroughly in *Tetrahymena* (Tiedtke *et al.*, 1993). It appears to be Ca^{2+} dependent. The sites of release and the final function are under debate (Florin-Christensen *et al.*, 1990). Remarkably, some enzymes, such as β-hexosaminidase, are released only partially into the medium, while they are retained partially on the cell surface (Kiy *et al.*, 1993).

As in other systems, NSF-based processes may be involved not only in stimulated (Section IV,A,2), but also in constitutive exocytosis (Avran and Castle, 1998; Gerst, 1999). This has not yet been analyzed with ciliates and it has to remain open, therefore, for a variety of sites where constitutive exocytosis occurs in *Paramecium*. This concerns the two release sites of the contractile vacuoles and that of spent phagosomes ("cytoproct"). For the following reasons, they can be most safely assumed to use NSF and, therefore, by implication, also SNARE proteins. These sites are stained intensely by anti-NSF ABs when NSF detachment is inhibited by blocking its ATPase activity with ATP-γ-S (Kissmehl *et al.*, 2002). From ciliates, no information is available on synaptotagmin involvement in lysosome trafficking, which is in contrast to mast cells, where it (negatively) modulates lysosome exocytosis (Baram *et al.*, 1999). The cytoproct is also distinctly labeled by ABs against some common sequences of annexins (Knochel *et al.*, 1996). These are ABs different from those that label trichocyst docking sites. This finding suggests the involvement of annexins in cytoproct positioning and/or functioning.

2. Stimulated Exocytotic Pathway

Trichocyst docking sites alternate with cilia along longitudinal rows (Allen, 1988). At the emergence of both these organelles, the layer of flat cortical calcium stores ("alveolar sacs"), which are tightly attached to the cell membrane, is interrupted. Within the cell membrane, trichocyst docking sites are delineated by a double row of freeze-fracture particles (proteins) arranged as a "parenthesis" before and as a ~300-nm-wide "ring" after trichocyst docking, respectively (Plattner *et al.*, 1993; Vayssié *et al.*, 2000). Docking of

a trichocyst normally entails the assembly of approximately nine "rosette" particles (proteins) in the center of a ring, i.e., directly in the fusogenic zone (Beisson *et al.*, 1976; Pape and Plattner, 1990), as well as of "connecting material" between the two membranes (Beisson *et al.*, 1980; Plattner *et al.*, 1980). In most strains, the presence of a rosette is an infallible indication of exocytotic membrane fusion competence (Pouphile *et al.*, 1986). In fact, a *nondischarge* mutant of *P. caudatum*, which unexpectedly possesses rosettes (Watanabe and Haga, 1996), was found to perform "silent" membrane fusion without any visible trichocyst release due to a mutated Ca^{2+}-binding component in the secretory contents (Klauke *et al.*, 1998). The only true exception known is strain *nd12*, which, when cultivated at a nonpermissive temperature, possesses rosettes in the absence of any fusion capacity (Mohamed *et al.*, 2002). Rosette assembly is also required for mucocyst secretion in *Tetrahymena* (Orias *et al.*, 1983).

What is the molecular identity of trichocyst docking and fusion machinery, including connecting material, particularly rosette protein particles? In this context, a variety of sequences are available from *Paramecium* (Table I), notably for the following gene products: NSF, synapto-/cellubrevin, calmodulin (CaM), monomeric G-proteins, and several ND gene products identified by rescue experiments and complementation cloning with nondischarge mutants (see later). Considering the many hours-long lifetime of a trichocyst docking site (Plattner *et al.*, 1993), localization by anti-NSF ABs is not feasible because NSF is removed rapidly from adjacent membranes after establishing the SNARE interaction (Section II). Unfortunately, ABs against *Paramecium* SNAREs are also not available as yet. However, it could be shown that the assembly of rosettes, which may require only minutes (Pape and Plattner, 1990; Plattner *et al.*, 1993; Klauke and Plattner, 2000), clearly requires SNAREs for the following reasons. In the *nd9* mutant, a ~2-h-long temperature shift from nonpermissive to permissive conditions (28°C → 18°C) normally causes the assembly of rosettes and induction of exocytosis capacity. NSF gene silencing applied during a 28°C → 18°C transfer inhibits rosette assembly and functional recovery (Froissard *et al.*, 2002).

Another component of trichocyst docking sites is the Ca^{2+}-binding protein, CaM. We localized CaM by ABs against CaM from *Paramecium* precisely at docking sites (Momayezi *et al.*, 1986), which is particularly concise in isolated cell surface complexes ("cortices"). Conversely, Kerboeuf *et al.* (1993) induced rosette assembly in a *cam*-mutant, containing mutated CaM, by transfection with the wild-type *CaM* gene. This was paralleled by acquirement of exocytosis competence. The role of CaM in establishing and/or maintaining trichocyst docking/exocytosis sites in a functional state is supported by the inhibitory effect of "anti-CaM drugs," which, in *Paramecium*, cause the detachment of trichocysts (Klauke and Plattner, 2000). CaM was found to participate in yeast vacuole fusion (Peters and

Mayer, 1998) and in dense-core vesicle exocytosis in mammlians. In this context, CaM may account for a Ca^{2+}-dependent priming of exocytosis, as it binds to a variety of components of the docking/fusion machinery, such as the v-SNARE, synaptobrevin (Quetglas *et al.*, 2002), rab-type monomeric G-protein (Coppola *et al.*, 1999), and the putative Ca^{2+} sensor, synaptotagmin (Tucker and Chapman, 2002). Thus, Ca^{2+} not only mediates exocytotic membrane fusion, but it also primes docking sites (at a preceding stage; Von Rueden and Neher, 1993). Although synaptotagmin—the Ca^{2+} sensor to be expected also in ciliates—has so far remained undetected in *Paramecium*, we presume its occurrence based on a similar requirement of exocytotic membrane fusion for local Ca^{2+} increase (Plattner and Klauke, 2001). This is supported by acceleration of the exo–endocytosis cycle by increasing the extracellular Ca^{2+} concentration (Plattner *et al.*, 1997b), as it also occurs, for instance, in neuronal cells (Palfrey and Artalejo, 1998). Trichocyst exocytosis, which, just like in any other eukaryote, requires a Ca^{2+} signal (Erxleben *et al.*, 1997; Plattner and Klauke, 2001), undoubtedly requires a Ca^{2+} sensor, but CaM is less likely to act as a sensor than synaptotagmin (Sugita *et al.*, 2002). The actual Ca^{2+} sensor in ciliates definitely has to be identified.

Currently, we assume that the following components contribute to formation of the "connecting material" described previously: (i) CaM is one of the components; (ii) we conclude from indirect evidence obtained in our NSF studies that SNAREs are additional ones; (iii) furthermore, additional proteins, including monomeric G-proteins may contribute at least transiently; (iv) a synaptotagmin-like Ca^{2+} sensor appears mandatory; and (v) additional components are the ND7 (Skouri and Cohen, 1997) and ND9 gene products (Froissard *et al.*, 2001) and possibly some proteins interacting with ABs against common sequences from mammalian annexins (Knochel *et al.*, 1996).

In a scrutinized discussion on mechanisms of exocytotic membrane fusion in *Paramecium* (Plattner and Kissmehl, 2003), we tried to evaluate the potential relevance of two of the currently most discussed candidates for fusion pore formation: V_0 part of the H^+-ATPase and SNAREs, respectively (Section II). For the availability of V_0 genes and the *a subunit*, see Table I. Based on structural details of trichocyst exocytosis sites and their ultrastructural transformation during membrane fusion (Knoll *et al.*, 1991), we found some arguments in favor of V_0, but unambiguous clarification again requires unequivocal identification by molecular biology. Conversely, is it mere coincidence that approximately nine rosette particles occur at an active trichocyst exocytosis site and that up to nine active fusion complexes in the sea urchin egg are calculated to be required for optimal operation of an exocytotic event (Vogel *et al.*, 1996)? Meanwhile, molecular data on H^+-ATPase components accumulate steadily and, in conjunction with ABs against its subunits and against SNAREs, may finally allow us to analyze their relevance for

membrane fusion during trichocyst exocytosis. Consider that no decision has been reached so far in any other system where dense-core secretory organelle docking/fusion sites are structurally much less distinct.

B. Endocytosis, Phagocytosis, and Lysosomal System

1. Endocytosis

This is considered separate from phagocytosis (see Section IV,B,2) and we differentiate between exocytosis-coupled endocytosis and constitutive endocytosis via parasomal sacs. Considering the very detailed molecular informations on exo–endocytosis coupling in mammalian cells (Gundelfinger *et al.*, 2003), amazingly little is known in *Paramecium*.

All work with *Paramecium* agrees that trichocyst "ghosts" are resealed, fragmented, and internalized without a clathrin coat (Allen and Fok, 1984; Plattner *et al.*, 1985). According to quenched-flow/freeze-fracture analysis, all these steps are accelerated by an increasing extracellular Ca^{2+} concentration (Plattner *et al.*, 1997a). Right after resealing, a "ring + rosette" configuration is transformed into a "filled ring" and then to a "parenthesis" (see Section IV,A,2) as ghosts detach (Knoll *et al.*, 1991; Plattner *et al.*, 1997b). Ghosts are removed completely, depending on the strain analyzed, normally with $t_{1/2}$ ~3 to 9 min (Plattner *et al.*, 1985). This step is also accelerated with an increasing extracellular Ca^{2+} concentration during stimulation (Plattner *et al.*, 1997a), as it also occurs, for instance, in neurons (Palfrey and Artalejo, 1998) where it accelerates the internalization of SNAREs by endocytosis (Sankaranarayanan and Ryan, 2001). Trichocyst docking sites become available again for the insertion of new trichocysts, depending on organelle biogenesis, at a much slower rate (Plattner *et al.*, 1993).

Vesicles arising from exocytosis-coupled endocytosis are of the smooth type (Plattner *et al.*, 1985) and travel far into the cell, even close to the Golgi, complex as shown by peroxidase labeling (Allen and Fok, 1980, 1984), but recycling could not be shown. After a limited extent of exocytosis, the marker goes to secondary lysosomes (Allen and Fok, 1984). Massive exocytosis induction in the presence of a fluid phase marker entails delivery to digesting vacuoles (Lüthe *et al.*, 1986).

Parasomal sacs are established sites of ongoing endocytosis in *Paramecium* and in *Tetrahymena*, as shown by the fluid phase marker horseradish peroxidase (Allen and Fok, 1993a; Allen *et al.*, 1992) and by the application of cationic ferritin (Nilsson and VanDeurs, 1983), respectively. After endocytotic uptake, these markers reach flat compartments that have not been identified so far. Although parasomal sacs display a bristle coat, its identity

with clathrin should now finally be shown because the gene of the heavy chain is now known (Table I). As to physiological functions of these sites, GPI-anchored vsAGs are pressed into these sites, probably by their mere abundance, and thus subjected to endocytosis and delivery to early endosomes (Allen *et al.*, 1992; Allen and Fok, 1993; Fok and Allen, 1993), called "terminal cisternae," and food vacuoles, as derived from immunogold-labeling experiments with *Paramecium* (Flötenmeyer *et al.*, 1999). This transport to digesting vacuoles may serve a permanent turnover (Capdeville *et al.*, 1993).

In higher eukaryotes, coated vesicle formation requires an established set of proteins. Altogether, the molecular machinery required for endocytosis via coated pits is widely different from that for exocytosis. For the availability of gene sequences, see Table I. Depending on the type of endocytosis vesicle, this may include clathrin and its adaptor protein, AP2, dynamin, synaptojanin, and so on (Hinshaw, 2000; Sorkin, 2000), but not SNARE assemblies (Holroyd *et al.*, 2002). There are different types of endocytosis vesicles and not all components are necessarily occurring at the same time, but dynamin is common to most of them (Conner and Schmid, 2003). In fact, dynamin does show up in *Paramecium* (AJ386320, AF351193, AL448655), in addition to the following indirect hints to its occurrence. Dynamin is dephosphorylated during endocytosis by the protein phosphatase 2B (PP2B, calcineurin, CaN) (Marks and McMahon, 1998). PP2B has a catalytic CaM-binding A subunit and a Ca^{2+}-binding regulatory B subunit (Klee *et al.*, 1998). CaN has been isolated from *Paramecium*, and ABs prepared against it (Kissmehl *et al.*, 1997) strongly labeled parasomal sacs on the EM level (Momayezi *et al.*, 2000). Meanwhile, two genes of the A subunit has been cloned in the laboratory of R. D. Hinrichsen (AF 014922), and the B subunit has been cloned also in our laboratory (Table I). No other components are identified in *Paramecium*. This also holds for endosomes themselves.

Actin-binding proteins are also recruited to endocytosis sites (Marsh and McMahon, 1999; Qualman and Kessels, 2002). In a late stage of coated vesicle release from the cell membrane, F-actin assembly, between the plasma membrane and the pinched-off endocytotic vesicle, emerges in mammalian cells (Merrifield *et al.*, 2002; Tse *et al.*, 2003). Although F-actin occurs in the *Paramecium* cortex, its precise localization remains to be elucidated (see Section IV,B,2).

Membrane piecemeals from digesting vacuoles, after defecation, are retrieved from the cytoproct (Allen and Wolf, 1974; Allen and Fok, 1993a, 2000). Discoidal vesicles thus formed serve recycling to the nascent food vacuole. It is not understood whether labeling of the cytoproct by ABs against some common sequences of annexins (Knochel *et al.*, 1996) is to be seen in connection with the exocytotic and/or with the endocytotic activities taking place at this site or merely in conjunction with positioning of the cytoproct.

ABs against *Paramecium* NSF also label the cytoproct when the dissociation of NSF is inhibited (Kissmehl *et al.*, 2002). This indicates the involvement of a SNARE machinery, probably in the exocytosis of the spent food vacuole.

2. Phagocytosis and Lysosomal System

Food bacteria are engulfed by ciliates at the cytopharynx, by formation of a nascent food vacuole that is acidified to kill the "prey," before the digesting (food) vacuole migrates by cyclosis through the cell. F-actin contributes to phagosome formation (see later) just as in mammalian cells (Zhang *et al.*, 2002; Tse *et al.*, 2003). Concerning *Paramecium*, we are ignorant about any involvement of rho-type monomeric G-proteins known in other cells (Chimini and Chavrier, 2000), but corresponding sequences are present (Table I). The subsequent pathway involves delivery and retrieval of lysosomal enzymes (Allen and Fok, 1993a, 2000; Fok and Allen, 1993).

In contrast to these scrutinized ultrastructural and functional analyses of the phagolysosomal cycle in *Paramecium*, molecular data are scant—in striking contrast to the wealth of information in mammalian cells (Chimini and Chavrier, 2000; Garin *et al.*, 2001). We know nothing about the occurrence of the standard immuno marker for both, late endosomes and lysosomes, Lamp-2 (Clague, 1998), in ciliates. Again, although ABs against NSF also label the site of phagosome formation in *Paramecium* (Kissmehl *et al.*, 2002), it is not known precisely which process it serves, i.e., phagosome fission, fusion with discoidal vesicles and/or acidosomes. From work with other cells, one may assume that only membrane fusions, but not fission processes, e.g., during phagosome formation, would require a NSF/SNAP/SNARE machinery (Hinshaw, 2000; Sorkin, 2000). However, other work assumes that NSF would participate in phagosome formation (Coppolino *et al.*, 2001), and phagosomal membranes isolated from mouse macrophages were found to contain such components (Garin *et al.*, 2001). Any role of NSF in phagosome formation, if any, remains to be elucidated, but its occurrence may equally well reflect the multiple membrane-to-membrane interactions occurring in the life of a phagosome.

Before a nascent food vacuole is pinched off, two events take place: (i) F-actin is assembled (Allen and Fok, 1983; Kersken *et al.*, 1986) and (ii) a number of acidosomes line up around the surface of a nascent food vacuole with which, after pinching off, they fuse rapidly (Allen and Fok, 1983). Acidosomes are small vesicles, so far of undetermined origin, that transport H^+-ATPase molecules to nascent phagosomes. For sequences available, see Table 1. Whether an equivalent pathway also occurs in mammalian cells is not ascertained. Also, the signal for the rapid, multiple fusion of acidosomes with a food vacuole remains to be elucidated. Also unknown is the involvement, along the digestive cycle, of V_0 subunits of the H^+-ATPase

in the respective fusion processes. If V_0 subunits would ever act as a fusogen, this would most likely occur here, where it abounds so much (Fok *et al.*, 2002), particularly as this function has been derived from analyses with yeast vacuoles (Section II). The binding of CaM to phagosomal membranes along their pathway (Momayezi *et al.*, 1986) suggests a role in membrane interaction, as it is required for such interaction and final fusion in yeast vacuoles (Peters and Mayer, 1998). Accordingly, CaM and Ca^{2+}/CaM-binding proteins are encountered on the surface of phagocytic vacuoles of *T. thermophila* (Gonda *et al.*, 2000).

As outlined in Section IV,A,1, materials ingested by smooth vesicles formed during exocytosis-coupled endocytosis, as well as by coated vesicles, can also be delivered to the phagolysosomal system. In this case, a signal indicating the "empty" state has to mediate this transport route. This is opposite to the findings during "frustrated exocytosis," where a signal indicating the "filled" state allows the reattachment of trichocysts previously detached without preceding contents release (Section IV,C,1).

NSF gene-silencing experiments have resulted in the inhibition of phagocytotic vacuole formation, decreasing cell size, and finally in cell death (Froissard *et al.*, 2002; Kissmehl *et al.*, 2002). Immunofluorescence labeling by anti-NSF ABs was visible most clearly at the beginning and at the end of the digestive cycle, i.e., at the cytopharynx and the cytoproct, respectively. However, at the EM level, the increased delivery of NSF into *bona fide* lysosomes was recognized (Kissmehl *et al.*, 2002). It is difficult to judge whether this simply indicates the disposal of aged NSF molecules in the absence of any further biosynthesis.

Further aspects of membrane trafficking along the phagosomal route, to be elucidated on a molecular scale, are the fusion with lysosomes and the retrieval of lysosomal enzymes at a later stage (Allen and Fok, 1993a, 2000; Fok and Allen, 1993). Ciliates offer the advantage of precise timing of the phagocytic cycle, i.e., one can isolate defined phagosomal stages, as established for *Tetrahymena* (Vosskühler and Tiedtke, 1993). In *Tetrahymena*, some of the spots of two-dimensional electrophoresis gels have been partially microsequenced (Maicher and Tiedtke, 1999)—two of them were novel. Along the phagocytic pathway of *T. thermophila* different monomeric G-proteins are attached sequentially (Meyer *et al.*, 1998). This may reflect selective vesicle interaction steps.

Very strikingly, NSF gene silencing increased the number of autophagosomes considerably (Kissmehl *et al.*, 2002), although their formation begins with closure of a wrapping membrane compartment (ER), i.e., by a membrane fusion process. Our data are compatible with findings in *Saccharomyces cerevisiae* (Ishihara *et al.*, 2001), where this step does not require NSF/SNARE machinery, in contrast to fusion of autophagosomes with the vacuole (lysosome). Inhibition of this NSF-dependent step may be one reason of

the accumulation of autophagosomes in *Paramecium* after NSF gene silencing. The primary reason may be their increased formation due to starvation, as NSF gene silencing shuts down food delivery via phagocytosis (Froissard *et al.*, 2002). Accordingly, in *Tetrahymena*, starvation induces autophagy (Nillson and VanDeurs, 1983).

To summarize, with none of the manifold membrane interactions occurring during the digestive cycle is the molecular basis of membrane interaction and fusion known in any satisfactory detail. However, one may reasonably assume the following details. (i) F-actin, which coats nascent and later food vacuoles (Allen and Fok, 1983; Kersken *et al.*, 1986), may participate in or even mediate cyclosis, and it would have to be organized in a loose form to allow organelle interactions. F-actin contributes to phagosome formation, just like in mammalian cells (Zhang *et al.*, 2002; Tse *et al.*, 2003). (ii) CaM and a NSF-based machinery are probably involved in these membrane interactions. (iii) Apart form SNAREs, V_0 would be available as a candidate for fusion pore formation.

F-actin is generally assumed to be required to drive cyclosis, also in *Paramecium* (Sikora, 1981). Several actin genes have been cloned in *Paramecium* by Díaz-Ramos *et al.* (1998) and in our laboratory. Data available previously have been corrected and supplemented by additional sequences (R. Kissmehl, E. Wagner, J. Mansfeld, and H. Plattner, unpublished observations). The localization and function of the respective gene products have not yet been completed.

C. Long-Range Vesicle Movements

1. Detachment of Mature Trichocysts from the Cell Surface

The application of "anti-CaM drugs" or of increased Mg^{2+} concentrations in the medium can detach exocytosis-competent trichocysts from the cell membrane (Klauke and Plattner, 2000). The requirement of CaM for establishing trichocyst docking sites has been commented in Section IV,A,2. It is not known whether the enrichment of CaN at and close to docking sites (Momayezi *et al.*, 2000) is relevant directly for exocytosis or exocytosis-coupled endocytosis or indirectly, e.g., for governing Ca^{2+} dynamics. Experimentally detached trichocysts (still containing their secretory contents) can be reinserted and undergo stimulated exocytosis. From this we conclude that (i) trichocysts dispose of a "filled" signal and (ii) that CaM is involved in docking (see also Section IV,A,2). No comparable information is available from any other eukaryotes.

In *Paramecium*, as in mammals, an "empty-or-filled" signal may be represented by transmembrane proteins with a lumenal link to the secretory

contents. On a speculative basis, in *Paramecium*, such a link could be represented by a component of the "mesh-like sheath," notably by a 56-kDa protein (Momayezi *et al.*, 1993) (see Section IV,A,2). One could speculate whether the GP-2 protein in pancreatic zymogen granules could serve a similar function (Kalus *et al.*, 2002). In both these cases, the existence of an "empty/filled" signal and its involvement in directed transport would require much more scrutinized analysis.

2. Microtubules as Long-Range Signals for Vesicle Trafficking

A *Paramecium* cell has a variety of distinct arrays of microtubules (Cohen and Beisson, 1988), among which some are displayed along distinct vesicle transport routes. Microtubules emanate from the cytoproct (Allen and Wolf, 1974) where they may serve the docking of a spent food vacuole. Another population of microtubular "rails" is installed between the cytoproct and the cytopharynx for discoidal vesicle transport (Allen and Fok, 1980). Microtubules also flank the radial canals of the osmoregulatory system (Allen, 2000; Allen and Naitoh, 2002), which they may stabilize. Finally, a much less obvious population originates from ciliary basal bodies in a vertical direction from the cell surface and mediates saltatory docking of trichocysts (Plattner *et al.*, 1982; Glas-Albrecht *et al.*, 1991). The γ–tubulin, associated with ciliary basal bodies (Ruiz *et al.*, 1999), is required to establish basal bodies as microtubule-organizing centers. Microtubules do not participate in membrane transport in the opposite direction, i.e., exocytosis-coupled endocytosis (Plattner *et al.*, 1985).

For versicle trafficking in *Paramecium*, cytoplasmic dynein is the only motor protein that has been functionally characterized up until now (Schroeder *et al.*, 1990). Due to its preference for "+ → –" transport, it may be involved in trichocyst docking. Remarkably, such "+ → –" directed transport to the cell periphery, as established for trichocysts (Plattner *et al.*, 1982; Glas-Albrecht *et al.*, 1991), has also been found in epithelia (Bacallao *et al.*, 1989; Bré *et al.*, 1990).

D. Results Relevant to Membrane Trafficking in *Paramecium*

Table I summarizes the relevant molecular details known so far from *Paramecium*. Many sequences available are fragmentary and, thus, require confirmation and further specification by additional work. With partial sequences it cannot be excluded that they are domains occurring in different proteins. So far it appears that *Paramecium*, on an early stage of evolution, is using, in principle, the same molecular elements as "higher" eukaryotic systems.

Originally, data have been derived from the analysis of the respective proteins. Complementation cloning, with identification of the respective gene and gene product, was another approach (Haynes *et al.*, 1996) that proved highly rewarding in the analysis of membrane trafficking (Skouri and Cohen, 1997; Vayssié *et al.*, 2000; Froissard *et al.*, 2001). In the last few years, the international *Paramecium* genome project (Dessen *et al.*, 2001) has provided important input. This is currently pursued on a European scale as a GDRE project (Groupement de Recherche Européen), headed and administered by Jean Cohen and Linda Sperling (CNRS, Gif-sur-Yvette; France). An internet connection is available at http://paramecium.cgm.cnrs-gif.fr.

With most genes analyzed so far in *Paramecium*, more than one, frequently several, isoforms are found. They are frequently similar to each other, thus facilitating the silencing of different genes in the course of a single gene (ORF) injection (Ruiz *et al.*, 1998; Bastin *et al.*, 2001). It remains to be seen whether this is an alternative to alternative splicing, whether all gene variations are expressed, and whether the corresponding gene products are differentially positioned in the cell. Rapid progress of the *Tetrahymena* genome project (Turkewitz *et al.*, 2002) will be an additional potential source for unraveling molecular details pertinent to membrane trafficking in ciliated protozoa.

V. Conclusions and Perspectives

A vast body of cell-biological work with *Paramecium* provides a solid baseline, which, together with rapid progress in molecular biology, will maintain the status of this organism as an important model system. One of the main questions to solve will be: How is the regular arrangement of the diverse membrane trafficking pathways determined?

In the past, specifically in *Paramecium*, complementation cloning proved useful to identify relevant genes and the function of the respective gene products (Sections II,B and IV,D). Microinjection of appropriate constructs into the macronucleus allows us to obtain transfected clones (Vayssié *et al.*, 2000). This includes the overexpression of GFP–fusion proteins (Hauser *et al.*, 2000a,b). It is hoped that electroporation or "bioballistic" methods (bombardment with DNA-coated gold particles) will allow us to achieve stable transfectants (Boileau *et al.*, 1999). Homology-dependent gene silencing by injection of a large number of ORFs, without flanking regions (Ruiz *et al.*, 1998; Bastin *et al.*, 2001; Galvani and Sperling, 2001), has already proved useful in analyzing some aspects of membrane trafficking in *Paramecium* (Froissard *et al.*, 2001; Kissmehl *et al.*, 2002). Its effect is not fully

understood, but basically it may be due to the binding of complementary nucleotide sequences (Wassengger, 2002). Recent methodological developments may render gene silencing more easily applicable by feeding of transfected bacteria (Galvani and Sperling, 2002). In *Tetrahymena*, "antisense ribosome" systems have been established (Turkewitz *et al.*, 2002). This involves vectors with an antisense 5′ part inserted into ribosomes.

Because membrane traffic can be studied only in the structural context of a cell, the highly regular pattern of a *Paramecium* cell will keep this organism as a focus of interest. This is a realistic goal, particularly as it becomes increasingly amenable to molecular biology.

Note added in proof:

To Section I. The most detailed account available of the *Paramecium* genome project (Sperling *et al.*, 2002, *Eukaryot. Cell* **1**, 341–352) will soon be supplemented by additional sequences. Any new informations can be drawn from the internet address of the colleagues guiding this and the GDRE project, J. Cohen and L. Sperling (http://paramecium.cgm.cnrs-gif.fr).

To Section III. Meanwhile sequences encoding parts of synaptotagmin gene(s) have been identified in our laboratory.

To Section IV,B,1. For partial sequences of dynamin 2 and rab 7 in *Paramecium*, see Surmacz *et al.*, 2003, *Biol. Cell* **95,** 69–74.

To Section IV,B,2. Synaptotagmin has been reported to participate also in internal fusion processess, like formation of some endosomal compartments (Grimberg *et al.*, 2003, *J. Cell Sci.* **116,** 145–154.)

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