SPECIAL FEATURES OF VESICLE TRAFFICKING IN SKELETAL MUSCLE CELLS

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Abstract
Skeletal muscles are composed of long, multinucleated cells called myofibers, which are highly differentiated cells and therefore unique in structure. In the present study the organization of the endocytic and exocytic pathways in isolated rat skeletal myofibers was defined with confocal and electron microscopic methods.

In isolated myofibers the I band areas were shown to be active in endocytosis. The sorting endosomes were distributed in a cross-striated fashion while the recycling and late endosomal compartments were located to perinuclear areas and interfibrillar spaces, where they followed the course of microtubules.

Protein trafficking in the different stages of muscle cell differentiation was also analyzed. The studies with L6 myoblasts and myotubes showed that during myogenesis varying fractions of different viral glycoproteins were sorted from the endoplasmic reticulum (ER) into a specific compartment that did not recycle with the Golgi apparatus. This compartment is suggested to be the sarcoplasmic reticulum (SR).

The studies with living muscle cells showed further changes in vesicle trafficking taking place during myogenesis. With GFP-tagged tsO45G protein, transport containers were detected in 20% of the infected myofibers, while all infected L6 myoblasts or myotubes showed intense movement of corresponding structures. We also detected significant differences between the pre-and post-Golgi traffickings in myofibers.

When the distribution of the ER in adult myofibers was studied, the confocal microscopic data showed that the labeling patterns of the rough endoplasmic reticulum (RER) and the SR markers were different. Blocking of different cargo proteins in the RER revealed two discrete distribution patterns, neither of them identical with the SR. The collected electron microscopic data supported the idea that in mature myofibers there are two separate RER compartments. We suggest that the RER compartment capable of export function located around the myonuclei and on the Z lines, while the non-exporting RER compartment localized to terminal cisternae and probably took care of the synthesis of the SR proteins.

Keywords: endocytosis, enveloped viruses, muscles, protein trafficking, sarcoplasmic reticulum
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Abbreviations

BFA  Brefeldin A
BHK  Baby hamster kidney cells
BiP  Binding protein
BSA  Bovine serum albumin
Ca^{2+}  Calcium
CLQ  Calsequestrin
COPI, II  Coat protein I, II
DAB  Diaminobenzidine
DGC  Dystrophin-associated glycoprotein complex
DHPR  Dihydropyridine receptor
cDNA  Complementary deoxyribonucleic acid
DMEM  Dulbecco's minimal essential medium
DTT  Dithiothreitol
EM  Electron microscopy
Endo H  Endoglycosidase H
ER  Endoplasmic reticulum
FITC  Fluorescein 5-isothiocyanate
FLIP  Fluorescence recovery after photobleaching
FRAP  Fluorescence loss in photobleaching
GA  Glutaraldehyde
GFP  Green fluorescent protein
GLUT4  Glucose transporter 4
GPI  Glycosylphosphatidylinositol
HA  Hemagglutinin
HRP  Horse radish peroxidase
LDL  Low density lipoprotein
mRNA  Messenger ribonucleic acid
mAb  Monoclonal antibody
MTJ  Myotendous junction
NMJ  Neuromuscular junction
OST  Oligosaccharyl transferase
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<tr>
<th>Acronym</th>
<th>Abbreviation</th>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
<td></td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
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<tr>
<td>PDI</td>
<td>Protein disulfide isomerase</td>
<td></td>
</tr>
<tr>
<td>RER</td>
<td>Rough ER</td>
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</tr>
<tr>
<td>SER</td>
<td>Smooth ER</td>
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</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
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</tr>
<tr>
<td>SFV</td>
<td>Semliki Forest virus</td>
<td></td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>SERCA</td>
<td>SR/ER Ca-ATPase</td>
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<tr>
<td>SNAP</td>
<td>Soluble NSF-attachment protein</td>
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<tr>
<td>SNARE</td>
<td>SNAP receptor</td>
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</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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</tr>
<tr>
<td>TfR</td>
<td>Human transferrin receptor</td>
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</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
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</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine</td>
<td></td>
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<tr>
<td>TRAP</td>
<td>Translocon associated protein</td>
<td></td>
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<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
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<tr>
<td>WSN</td>
<td>Influenza A virus, WSN strain</td>
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List of original publications

This thesis is based on the following articles and additional unpublished results. The articles are referred to in the text by Roman numerals:


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References
1 Introduction

Proteins are taken in or transported out of the cell all the time. This protein trafficking is a complicated sequence of several well-controlled steps. The transport processes and the molecular mechanisms behind them have been clarified to some extent in certain mononucleated cultured cell types. However, it is still obscure whether the transport processes are similar in all cell types. A fully differentiated skeletal muscle cell is very different compared to a mononucleated cell.

Skeletal muscle cells, or fibers, form by fusion of mononucleated parent cells, myoblasts. During the differentiation into multinucleated muscle cells the organization of the cellular organelles and the plasma membrane of the myoblasts changes dramatically, and muscle specific organelles such as the sarcolemma, the sarcoplasmic reticulum (SR) and the transverse (T-) tubules are generated. A fully mature skeletal muscle cell is a very large highly organized cell with tens of nuclei and filaments of the contracting system filling up much of the space in the cell. While the surface organization of the muscle has been studied, little is known of the vesicle trafficking pathways, which arise from the distinct surface domains and muscle-specific membrane systems. Understanding these pathways in the muscle fiber is essential for gaining a molecular understanding of the trafficking of functionally important proteins. Therefore the basic facts have to be clarified also in these functionally very specialized cells. This work was carried out to find out how protein transport is arranged in the fully mature skeletal muscle cells.

To be able to characterize cell-biological phenomena in highly differentiated cells a proper cell model system is needed. Besides commercially available myogenic cell lines, we use isolated rat *flexor digitorum brevis* myofibers in our studies. This unique system provides us a view over the whole fiber as it exists in living organisms and facilitates the use of viral vectors.

The literature concerning vesicle trafficking in muscle cell is very scarce. Therefore the following review will first clarify the features of vesicle trafficking, as it is known to exist in mononucleated cells, and then continue by describing the skeletal myofiber.
2 Review of the literature

2.1 General features of protein trafficking

Every cell must communicate with its environment, since cells regulate their developmental and functional programs through their interaction with the external milieu. For that purpose eucaryotic cells have an elaborate internal membrane system that allows them to take up macromolecules by a process called endocytosis. Besides regulated digestion of macromolecules, the internal membrane system provides a means to regulate the delivery of newly synthesized material to the exterior of the cell. The travel of a molecule along this biosynthetic-secretory export pathway ends up to exocytosis, a process in which the material is secreted out of the cell. The trafficking of cargo is achieved by the continuous and regulated capture of cargo and targeting molecules into vesicles that bud from a donor membrane and deliver their contents to a receiving department. The intracellular compartments involved in vesicular trafficking in eucaryotic cells are shown in Figure 1.

It is essential for a multicellular organism and the function of its specialized cell types that the multiple transport and sorting events are highly accurate (Miaczynska & Zerial 2002). They determine the protein and lipid composition of specialized compartments, receptor protein functions and membrane homeostasis (Schu 2001). Trafficking events also regulate various signaling pathways so that within an embryo they reliably orchestrate many developmental decisions (Vincent 2003). Protein sorting into different transport vesicles requires specific interactions between sorting motifs on the cargo molecules and vesicle coat components that recognize these motifs. Cargo concentration and coat formation are linked in order to ensure efficient cargo loading into the assembling vesicles (Kirchhausen et al. 1997). Among the recycled proteins are structural components of the trafficking pathway that must be used repeatedly to sustain transport (Schekman & Orci 1996).
2.1.1 Transport vesicles

The transport vesicles come in several different varieties, which can be conveniently classified by the composition of the protein coat surrounding them. It is generally true that each type of vesicle carries out one specific or several very closely related transport steps between a particular pair of compartments. Vesicles are formed by the deformation of part of a pre-existing membrane into which cargo for transport has been sorted, followed by a scission of the vesicle from the membrane. After formation the vesicle is uncoated and then directed to its correct destination. Finally, the vesicle fuses with its target membrane (Rothman 1994; Evans & Owen 2002). Each membrane compartment must possess specific molecular machineries that enable fusion of different incoming vesicles as well as formation of distinct transport carriers destined to various organelles.

Besides vesicle-mediated transport also a cisternal maturation process has been proposed to take place in the Golgi apparatus. In this model proteins are not packed in transport vesicles, but the whole membrane cisterna, including the proteins to be transported, travels through the Golgi stack (Martinez-Menarguez et al. 2001).

2.1.2 Coat proteins

Vesicle biogenesis is regulated by specific proteins and co-factors that control distinct steps, including budding, transport, docking, and fusion with target membranes. Distinct coat proteins mediate each budding event, serving both to shape the transport vesicle and to select by direct or indirect interaction the desired set of cargo molecules (Schekman and Orci 1996; Cosson & Letourneur 1997). Arranged at high density, these proteins literally coat the membranes and possess an intrinsic ability to generate the forces necessary to bend a relatively flat membrane into a vesicle. (Lederkremer et al. 2001; Aridor & Traub 2002). Over the past few years, the structures of domains of many of these components have been determined. These structures are yielding a detailed understanding of how transport vesicles are generated (Evans & Owen 2002).

The first characterized scaffold protein is clathrin, which is found on different types of coated vesicles (Brodsky et al. 2001). One of the hallmarks of clathrin-coated vesicles is their selectivity. The major proteins that drive clathrin coat formation are the clathrin adaptor protein (AP) complexes (Kirchhausen et al. 1997). Four different basic heterotetrameric AP complexes are known (Boehm & Bonifacino 2002). Clathrin functions in receptor-mediated endocytosis at the plasma membrane when associated with AP2 adaptors (Rapoport et al. 1997), and in transport from the trans-Golgi network (TGN) to endosomes when associated with AP1 adaptors (Lederkremer et al. 2001; Gorelick & Shugrue 2001). Also AP3 interacts with clathrin but AP4 is most likely part of a non-clathrin coat (Boehm & Bonifacino 2002).

Coat systems that do not involve clathrin were found to include coat proteins I or II (COPI, COPII) (Rothman 1994). COPI coats function in transport through the Golgi apparatus and in retrograde transport from the Golgi apparatus to the endoplasmic reticulum (Gaynor et al. 1998). Segregation of anterograde- from retrograde -directed
cargo happens by packing it into distinct sets of COPI-coated vesicles (Orci et al. 1986). COPII coats function in transport from the ER to the Golgi apparatus. Secretory bulk flow has been shown to be COPII dependent (Phillipson et al. 2001). Interactions between cargo and COPII coat directs protein sorting from the ER (Kuehn et al. 1998).

The only transport step the coat protein of which has not been clarified so far is the step from the trans-Golgi network (TGN) to the plasma membrane. While the transport vesicles from TGN to endosomes are clathrin-coated, the coat protein of the vesicles destined to plasma membrane is not known (Pfeffer 2003).

Fig. 1. The intracellular compartments involved in vesicular trafficking in eucaryotic cells. Black arrows show the endocytic pathway and gray arrows the biosynthetic-secretory exocytic pathway. Also utilization of different coats in various steps of the pathways is shown. ER; endoplasmic reticulum, ERGIC; ER-Golgi intermediate compartment, CGN; cis-Golgi network, TGN; trans-Golgi network, SV; secretory vesicle, PM; plasma membrane, EE; early endosome, LE; late endosome, L; lysosome. (modified from Alberts et al. 1994)
2.1.3 Pilot proteins

Specific docking of a transport vesicle at intended acceptor compartment requires that it be endowed with distinct "pilot" proteins to encode its destination. Corresponding receptors must exist on target membranes to capture the vesicles. Membrane integrated cytoplasmically oriented soluble NSF-attachment protein (SNAP) receptors, termed SNAREs, appear to mediate in part the fusion of membranes both on vesicles (v-SNAREs) and on target membranes (t-SNAREs) (Hay et al. 1997). SNAREs are incorporated into a vesicle in the early stages of its formation (Rothman & Wieland 1996; Evans & Owen 2002). SNARE family members are distributed in a compartment-specific fashion. In each compartment two or more subcomplexes form a protein complex. Interactions of these protein components determine the fidelity of vesicle docking (Hay et al. 1997; Clague 1998).

Rab proteins, small GTPases belonging to the Ras superfamily, have been primarily implicated in vesicle docking as regulators of SNARE pairing. Rab GTPases catalyze the accurate association of pairs of targeting molecules located on the surfaces of transport vesicles with their corresponding membrane acceptors (Pfeffer 2003), but some of them seem to have other roles as well in vesicle budding, cargo sorting and motility along cytoskeletal tracks (Novick & Zerial 1997; Seabra et al. 2002). Different Rab proteins may be needed for each step of vesicular transport (Clague 1998).

Transport vesicles are formed by pinching off selected pieces of membrane containing cargo and v-SNAREs. The small GTP-binding proteins Arf1p (in the case of COPI) or Sar1p (for COPII) initiate the coat formation process Local lipid modification occurs and cooperation of v-SNAREs, cargo-proteins and modified lipids leads to forming of a high-affinity coatomer-binding site. Upon binding, coatomer induces curving of membrane, which leads to the release of a coated vesicle (Schekman & Orci 1996). The molecular basis for the heterogeneity in COPI vesicles is not known, but coats contribute to the specificity of cargo packaging (Glick 2001). COPII coat is composed of Sec23/Sec24 and Sec13/Sec31 protein complexes and Sar1. A resident ER membrane protein Sec12 activates Sar1 to recruit first Sec23/Sec24 and then Sec13/Sec31 complex to ER exit sites (Lederkremer et al. 2001; Aridor & Traub 2002). Sec23/Sec24 recognizes sorting motifs on cargo and targeting proteins, marking them for inclusion into vesicles.

2.1.4 Role of cytoskeleton

The routing of transport vesicles throughout the cell is controlled by three classes of proteins: those present on target membranes, those present on transport vesicle membranes, and a series of cytosolic proteins including ATPases and GTPases (Rothman & Wieland 1996). In order to cover the distance between compartments more efficiently, eucaryotic cells take advantage of the cytoskeleton and the molecular motors that move on them. Microtubule and actin filament networks cooperate functionally during a wide variety of processes, acting as tracks along which carrier vesicles can move from one membrane compartment to another (Robinson et al. 1996). Besides their role in
movement of vesicles, the cytoskeletal elements pay a role also in cargo sorting (Aridor & Hannan 2002). The best-known motor proteins are dynein-related and kinesin-related motors, which drive transport of the vesicles towards the minus or plus ends of microtubules, respectively (Goode et al. 2000).

2.1.5 Selection of cargo

The cargo of the transport vesicles is selected during vesicle budding. Both coat proteins and the pilot proteins as well as the proteins attaching vesicles to the cytoskeleton take part in cargo selection. Secretory proteins show characteristic ER-to-Golgi transport velocities (Lodish & Kong 1983), and soluble ER-resident proteins such as binding protein (BiP) are secreted only very slowly from the ER (Pelham 1995). This is most easily explained by postulating that secretory proteins possess positive sorting signals that are recognized by sorting receptors traveling from the ER to the Golgi apparatus with characteristic rates (Herrmann et al. 1999). ER resident proteins, on the other hand, carry retrieval signals which facilitate their receptor-mediated recycling to the ER if they escape from this compartment (Semenza et al. 1990; Aridor & Balch 1999). Most of the proteins that are packaged into COPII vesicles interact specifically with coat subunits and/or cargo receptors, although some proteins seem to exit ER by a "bulk flow" pathway, at least in specialized secretory cells (Aridor & Hannan 2002).

Possible cargo selection proteins belong to the p24 family which appear to be cycled between ER and Golgi in COPI and COPII-coated vesicles. An other possibility are p53/58 lectin-type molecules which may bind to sugar moieties of glycosylated cargo proteins (Kuehn et al. 1998). Cargo receptors may also couple quality control with ER export (Aridor & Traub 2002). The AP-2 adaptor complex, specially its µ2 subunit, positioned between the outer assembled clathrin lattice and the underlying membrane, is functioning as the sorting element of the coat (Rapoport et al. 1997; Aridor & Hannan 2002). Some cytoplasmic C-terminal amino acid motifs, either alone or in conjunction with other transport determinants, have been shown to accelerate ER export of numerous type I and probably polytopic membrane proteins by mediating interaction with COPII coat components (Nufer et al. 2002).

In polarized epithelial cells also membrane rafts, dynamic laterally organized lipid assemblies, play a role in the apical-basolateral sorting, which takes place in the Golgi apparatus. Raft-associated proteins are preferentially transported to the apical membrane compartments. The raft-mediated sorting might play an important role in the protein transport also in highly differentiated muscle cells.

2.1.6 Membrane rafts

Lipid raft is a region or domain of a membrane enriched in cholesterol and sphingolipids. The budding and fusion events clearly involve significant alterations in membrane
structure and it is likely that transformations of lipid components either accompany or help to induce these processes. Enzymes that modify membrane lipids function in the regulation of constitutive membrane traffic (Roth & Sternweis 1997).

The apical plasma membrane of epithelial cells is greatly enriched in glycosphingolipids. The existence of lateral assemblies, or rafts, of glycosphingolipids and cholesterol in the lipid bilayer was first proposed by Simons and co-workers (Simons & Ikonen 1997). At present these lipid rafts, formed in the membranes of the Golgi apparatus, are considered as a part of the machinery ensuring correct intracellular trafficking of proteins and lipids. The most apparent roles of lipid rafts are in sorting and vesicle formation, but they seem to have roles also in vesicle movement, cytoskeletal connections and vesicle docking and fusion. It is possible that lipid rafts do have a role even in the formation of the T-tubule system of striated muscle cells (Harder & Simons 1997; Parton et al. 1997; Scheiffele et al. 1997; Ikonen 2001).

Plasma membrane proteins with long enough membrane-spanning segments preferentially partition into the lipid rafts and thus become sorted into apically directed transport vesicles, while other proteins are excluded from them (Scheiffele et al. 1997; Kurzchalia & Parton 1999). The apically directed functionally diverse set of cell surface proteins, including receptors, adhesion molecules, and enzymes, become insoluble to extraction in the detergent Triton X-100 as they traverse the secretory pathway and are delivered to the plasma membrane. This acquisition of resistance to detergent extraction is thought to correspond to assembly into lipid rafts (Nichols et al. 2001). The detergent-insoluble glycolipid-enriched domains (DIGs) can be isolated by sucrose gradient centrifugation because of their high lipid content, and easily separated from other Triton X-100-insoluble material such as cytoskeletal assemblies (Parton & Simons 1995; Harder & Simons 1997). Influenza hemagglutinin (HA) is a well-studied raft-associated transmembrane protein that is found in the DIG fraction (Skibbens et al. 1989). The fractions also typically contain glycosylphosphatidylinositol (GPI)-attached proteins (Brown & Rose 1992).

Raft associated proteins can provide analytical landmarks for rafts but do not serve as general markers. A good example is caveolin, a cholesterol-binding protein that associates with only a subset of rafts, namely caveolae. The caveolin gene family consists of caveolin-1, -2, and -3. Caveolae are spherical or flask-shaped invaginations of the plasma membrane found in most cell types. When the caveolin gene is not expressed, caveolae are not seen. Rafts can exist independently of caveolae, but they must exist prior to formation of caveolae for proper insertion of caveolin into membranes (Smart et al. 1999; Ikonen 2001). Both rafts and caveolae play pivotal roles in intracellular signal transduction functioning as pre-assembled signaling complexes, message centers, or chemical switchboards for integrating signal transduction (Smart et al. 1999). Endothelial caveolae are also suggested to be involved in transcytosis (Stan 2002).
2.2 Endocytic pathway

The plasma membrane effectively separates the inside of the cell from the outside. Yet every eucaryotic cell is constantly taking up samples of the outside world by a variety of different mechanisms and targeting them to specific organelles within the cytoplasm. These processes are collectively termed endocytosis. Endocytic mechanisms serve many important cellular functions including the uptake of extracellular nutrients, regulation of cell-surface receptor expression, maintenance of cell polarity, and antigen presentation. Aberrations in endocytic processes play a role in several diseases (Seabra et al. 2002). Also viruses, toxins and symbiotic micro-organisms use endocytic pathways to gain entry into cells (Mukherjee et al. 1997; Clague 1998).

In the endocytic pathway, internalized molecules are delivered to early endosomes, where efficient sorting occurs. Although some molecules, in particular recycling receptors, are rapidly recycled back to the plasma membrane for reutilization, others, including downregulated receptors, are transported to late endosomes and lysosomes for degradation.

Two main types of endocytosis are distinguished on the basis of the size of the endocytic vesicles formed. Phagocytosis (or "cellular eating") involves the uptake of large particles (>250 nm), and pinocytosis (or "cellular drinking") involves the ingestion of fluid and solutes via small vesicles (<200 nm). In mammalian cells, cargo can be endocytosed via clathrin-coated pits by receptor-mediated endocytosis, via clathrin-independent pathway or via caveolae. All these routes are forms of pinocytosis.

In addition to the receptors and ligands that participate in endocytosis, there are molecules that are responsible of the unique characteristics of each endocytic compartment. These factors include the molecules that regulate internal pH, which is essential for the dissociation and degradation processes (Mellman 1996; Clague 1998). They also include the molecules that control organelle shape, distribution and translocation, and the molecules that regulate vesicle docking, fusion and budding (Mellman 1996). Dynamin (Orth & McNiven 2003) is known to interact with endocytic machinery and the actin cytoskeleton thus enabling the trafficking of endocytic vesicles along actin cytoskeleton (Qualmann et al. 2000).

2.2.1 Receptor-mediated endocytosis via coated pits

The best-characterized mechanism for gaining entry into the cell is the clathrin-coated vesicle, which in most animal cell types and under normal conditions represents the major portal of entry into the cell interior. In this process the macromolecules bind to complementary cell-surface receptors, accumulate selectively in coated pits, and enter the cell as receptor-macromolecule complexes in clathrin-coated vesicles (Robinson et al. 1996; Aridor & Traub 2002). This receptor-mediated endocytosis takes place in all nucleated vertebrate cells and plays an important role in many physiological processes (Dautry-Varsat & Lodish 1984; Mukherjee et al. 1997).
Clathrin-coated pits are 150 nm invaginated structures on the plasma membrane that occupy about 2% of the plasma membrane surface (Mukherjee et al. 1997). Clathrin heavy and light chains organize to form a basketlike polyhedral lattice (Musacchio et al. 1999) on the cytoplasmic side of the plasma membrane. The pit pinches off as a clathrin-coated vesicle (Brody et al. 2001; Kirchhausen et al. 1997).

Some membrane proteins, like LDL, are significantly concentrated in coated pits, various glycolipids and phospholipids show a random incorporation in these structures, and influenza HA is an example of proteins that are selectively excluded from coated pits (Mukherjee et al. 1997). Also some receptors, like LDL receptor, are constitutively concentrated in coated pits, but others become concentrated upon ligand binding. Most receptors are targeted to coated pits when recognition or internalization motifs in their cytoplasmic domains interact with intracellular assembly or adaptor proteins that form part of the clathrin coat.

2.2.2 Endocytic compartments

The endocytic organelles can be broadly divided into early and late compartments. Early endosomes (EE) include all the intracellular organelles that are on the major receptor-recycling pathway. Late endosomes (LE) are compartments that are involved in the breakdown of internalized cargo. Both the shape and the localization of endosomal compartments are major determinants of their functions. Rab proteins have been shown to act as membrane domain organizers and phosphoinositide metabolism could provide a basis for the communication between these domains required for the completion of a trafficking step between EE and LE (Miaczynska & Zerial 2002). In addition ubiquitination of the endosomal cargo mediates sorting in EE-LE transport (Bache et al. 2003).

All internalization pathways lead to the appearance of cargo in the peripheral EE. Coated vesicles must be at least partially uncoated to fuse with EE, which then undergo homotypic fusion. From EE cargo is sorted back to the plasma membrane, to the recycling compartment or to LE by means of carrier vesicles or through a “maturation” process, which means a change in biochemical composition and morphology. Carrier vesicles and late endosomes often display a typical morphology of multivesicular bodies, which contain internal membrane structures. These structures appear to be formed by invagination of the endosomal membrane (Mukherjee et al. 1997; Clague 1998). EE seem to serve as a common sorting site for a variety of transport steps in both specialized and non-specialized cells. Another function of the endosomal system is to regulate the cellular response to signaling molecules. Endosomes have been shown to down-regulate ligand-receptor complexes and provide a platform for the interaction between signaling molecules (Ceresa & Schmid 2000; Di Fiore & De Camilli 2001).

The EE system can be divided into sorting and recycling compartments. Tubulovesicular sorting endosomes contain recycling molecules such as transferrin as well as ligands that will be degraded such as LDL. Recycling endocytic compartment, judged by optical and electron microscopy and the different pH values it maintains, is a
physically separate compartment. It contains recycling molecules but lacks molecules that will be degraded (Robinson et al. 1996). The recycling pathway plays a complex role in membrane trafficking and it has sorting capabilities of its own. It not only returns material to the plasma membrane, but can also send membrane components back to sorting endosomes in a retrograde manner. While the sorting endosomes are spread throughout the cortical cytoplasm of the cell, the recycling compartment in many cell types is densely concentrated in the pericentriolar region, juxtaposed to the Golgi stacks and the TGN. (Hopkins 1983; Yamashiro et al. 1984)

LE are similar in size to sorting endosomes, but they appear to have more internal vesicular profiles, for which reason they are also called multivesicular bodies. In general LE are nearer to the center of the cell and have the microtubule minus-end directed motor dynein associated to them. LE as well as EE can send to and receive cargo from the TGN (Press et al. 1998; Juuti-Uusitalo et al. 2000). LE either mature into lysosomes or transfer cargo to lysosomes through vesicular intermediates. Lysosomes can only be identified at the molecular level by the fact that they lack certain proteins found in late endosomes. Both compartments might represent separate elements of a common dynamic network, involved in sorting and degradation, respectively (Mukherjee et al. 1997).

### 2.2.3 Endocytosis in polarized cells

In contrast to fibroblast-like non-polarized cell, cells in tissues are polarized and have two, and sometimes more, distinct plasma membrane domains to which different types of vesicles must be directed. In a typical epithelial cell, the cell surface is divided into two functionally and biochemically distinct, but physically continuous areas. The apical domain, which faces the lumen, often has special features such as cilia or brush border of microvilli. The basolateral domain covers the rest of the cell. The domains are separated by a ring of tight junctions, which prevent proteins and lipids from diffusing between the domains.

In addition to polarized delivery of proteins, there must be appropriate targeting of endocytosed material that is returned to the plasma membrane. Polarized epithelial cells exhibit coated-pit endocytosis from both the apical and basolateral surfaces. Recycled material can either return to the membrane from which they were internalized, or they can cross the cell and be delivered to the opposite membrane in a process called transcytosis. Morphological observations using fluid-phase tracers have indicated that two separate and functionally distinct types of endosomes underlie the apical and basolateral plasma membrane domains (Parton et al. 1989; Robinson et al. 1996).

### 2.2.4 Endocytosis via non-coated pits

Phagocytosis refers to the internalization of large particles that must bind to specific plasma membrane receptors capable of triggering their own uptake, usually by causing
the formation of F-actin driven pseudopods that envelop the bound particle. Under most circumstances, phagosomes rapidly fuse with endosomes and/or lysosomes exposing their contents to hydrolytic enzymes (Mellman 1996). Pinocytosis refers to the constitutive formation of smaller vesicles also carrying extracellular fluid and macromolecules that are nonspecifically bound to the plasma membrane. These processes are more difficult to study, since they may not be concentrative and lack specific markers. The best evidence comes from the observation that bulk membrane internalization and the uptake of fluid-phase markers like HRP continues even after cells have been specifically treated to inhibit clathrin-mediated internalization (Mukherjee et al. 1997). At present, it is clear that non-clathrin-coated pit uptake does occur in many cell types, but it is difficult to estimate the relative volume or membrane surface area taken up by these mechanisms.

One possible route for non-clathrin-coated pit endocytosis is via caveolae, which are uniform omega- or flask-shaped membrane invaginations that are considerably smaller than clathrin-coated pits. Proteins and lipids associated with caveolae have been reported to be specifically excluded from the coated pits and to be endocytosed by alternate mechanisms. Two caveolae-mediated endocytic pathways has been shown to exist that target either the Golgi apparatus or the ER from the plasma membrane (Le & Nabi 2003). However caveolae cannot account for all clathrin-independent endocytosis (Robinson et al. 1996; Mukherjee et al. 1997). The prevailing view is that clathrin-mediated and non-clathrin-mediated endocytosis normally occur in parallel, the clathrin-independent pathways being activated at least when the clathrin-dependent pathway is inactivated.

### 2.3 Exocytic pathway

All eucaryotic cells secrete proteins. Most of the secreted proteins are carried to the plasma membrane via the biosynthetic pathway and become modified by glycosyltransferases, glycosidases, proteases, or other enzymes as they travel through the cell (Kuehn et al. 1998). All eucaryotic cells also contain numerous membrane-bound compartments with specialized functions and therefore unique protein composition. Compartmentalization requires that the cell transport macromolecules from their site of synthesis to the appropriate specialized compartment.

Newly synthesized proteins enter the biosynthetic-secretory pathway in the ER by crossing the ER from the cytosol. Subsequent transport, from the ER to the Golgi apparatus and from the Golgi apparatus to the cell surface or other destinations, is mediated by transport vesicles or containers, and at each successive step, specific sorting decisions can be made on the basis of transport signals and retention signals (Kim & Arvan 1998). To travel along the secretory pathway and eventually reach their appropriate cellular destinations, newly synthesized secreted and membrane proteins must fold and assemble correctly. Failure to do so results in their retention in the ER and eventual degradation. The proper conformational maturation of nascent secretory pathway proteins is both aided and monitored by a number of ER chaperones and folding enzymes in a complex process termed ER quality control (Hammond & Helenius 1994).
2.3.1 Endoplasmic reticulum

2.3.1.1 Structure of the ER

The ER is the largest intracellular organelle, with an extensive array of interconnecting membrane tubules and cisternae that extend throughout the cell including the nuclear envelope. Many of the functions of the ER are confined to distinct ER subregions rather than homogeneously distributed throughout the organelle. The fine structure of the ER and the extent of its development in any given cell type depends on which of these functions predominates (Baumann & Walz 2001). The lumen of the ER seems to be continuous according to GFP diffusion studies (Dayel et al. 1999), and some ER cisternae maintain a close spatial relationship with other cellular membranes, indicating a molecular linkage of the two membrane systems. For instance the terminal cisternae of the SR are juxtaposed to the cell surface T-tubules to form the triad in skeletal muscle. The gap is of constant width and bridged by electron-dense structures called feet, which are cytoplasmic domains of the ryanodine receptor (RyR) Ca$^{2+}$ channels (Takekura et al. 2001).

The role of microtubules in the organization of the ER is clear. Disruption of the microtubule system by pharmacological agents, like colchicine or nocodazole results in a collapse of the ER, which forms an aggregate of membranes around the nucleus. This effect is reversible. ER uses the microtubule cytoskeleton as a framework for extending and maintaining its reticular organization (Terasaki et al. 1986). Microtubules and actin cytoskeleton might also have a role in ER compartmentalization. Motile ER uses motor proteins like kinesin to glide along microtubules (Baumann & Walz 2001).

ER membranes are differentiated into rough and smooth regions (RER and SER, respectively), depending on whether ribosomes are associated with their cytoplasmic surfaces. SER is prominent in certain cell types, such as liver, muscle and neurons. The two domains share most proteins, but RER is enriched in some membrane proteins by an unknown mechanism (Rolls et al. 2002). In cells with high secretory activity, the RER is very prominent and often consists of flattened cisternae arranged in stacks that are densely occupied by polysomes. In other cells, the RER forms a loose network of tubular cisternae that are only sparsely studded with polysomes (Baumann & Walz 2001).

For luminal ER proteins, which contain KDEL motif (Farquhar & Palade 1998), and for type I transmembrane proteins carrying a dilysine motif, specific retrieval mechanisms have been identified. However, most ER membrane proteins do not contain easily identifiable retrieval motifs, but they may be localized to this organelle by mechanisms that involve retention, retrieval, or a combination of both (Fu & Kreibich 2000). Retrieval of ER membrane proteins may be initiated from multiple positions along the exocytic pathway (Jackson et al. 1993). ER membrane proteins with the ability to bind to distinct cellular structures, e.g. cytoskeletal components, may diffuse from their site of synthesis through the ER network and be retained on ER cisternae within reach of this cellular structure (Semenza et al. 1990; Farquhar & Palade 1998; Martinez-Menarguez et al. 2001).
The regulation of intracellular calcium, the synthesis and quality control of membrane and secretory proteins, as well as the synthesis of phospholipids, cholesterol, and steroids, are listed among the main functions of the ER. The lumen of the ER is recognized as a key compartment of the cell in which multiple functions are carried out by resident, non-membrane proteins (Meldolesi & Pozzan 1998a). It is a major calcium storage site in the cell, calcium release from ER stores being important for many cellular responses.

RER must be present in all cells because it is the site where proteins made on membrane-bound polysomes are inserted into, or translocated across the limiting membrane. Individual translocon complexes are relatively mobile, but when they are engaged in cotranslational translocation and assembled into polysomes, they exhibit significantly reduced mobility (Nikonov et al. 2002). Newly synthesized proteins destined for non-ER membrane systems or secretion are packaged into vesicular carriers. This vesicular budding is not randomly distributed over the entire ER surface but is restricted to specialized areas, the exit sites or transitional elements, which have an increased level of organization compared with the surrounding ER membranes. Exit sites are small and highly dynamic, making it difficult to determine how their molecular composition differs from other ER subregions. The number of exit sites varies between cell types, they are prominent in the neighborhood of the Golgi apparatus but also found at the nuclear envelope and the cell periphery (Bannykh et al. 1996).

Whereas the RER is the site of cotranslational membrane insertion of proteins, the SER is thought to be the site of lipid biosynthesis, detoxification and calcium regulation. Pumps for Ca uptake (SERCAs), Ca binding proteins, and channels for Ca release (IP3, RyR) are associated with this membrane network (Meldolesi & Pozzan 1998b). Also RER seems to be organized into functional subregions that are specialized with respect to mRNA translation and/or posttranslational protein processing. Segregation of particular mRNAs to distinct areas seems to be especially prominent in highly polarized cells, like neurons and muscle cells (Racca et al. 1997). This mRNA segregation may provide unique environments for the biogenesis of substrates. Translation of mRNAs on distinct ER subregions might also be a mechanism for targeting and confining the encoded proteins to different cell regions or surface domains, and thus of supporting cell polarity (Baumann & Walz 2001). Different ER domains may represent stably organized subcompartments or be dynamic parts of a single continuous ER system (Lippincott-Schwartz et al. 2000).

Both the ER lumen and the ER membrane are often densely packed with proteins, some of which are incompletely folded or unassembled components of multisubunit complexes. The protein concentration in the lumen of the ER may be as high as 100mg/ml in the mononucleated cells. Newly synthesized proteins are inserted in the ER in a highly unfolded state. During and after the membrane passage an elaborate system of chaperones and modifying enzymes assist in the maturation process. Mature proteins are selectively exported from the ER in a folded form through the activity of the COPII coat system, except for molecules that are permanent residents of the ER itself. Folding of proteins in living cells within the ER compartment occurs under highly restrictive conditions unique to this microenvironment. Secretory proteins are translocated into the
ER as they are being translated; thus the NH$_2$ termini of secretory proteins routinely begin to fold in the ER before the COOH termini have even been synthesized. Specific interactions between recruited coat subunits and short peptide sequences (transport motifs) on cargo proteins direct the incorporation of cargo into budded vesicles (Kim & Arvan 1998).

The core of the translocon is the Sec61p complex, which is thought to make a tight seal with the ribosome and thereby provide a physically protected path for the nascent polypeptide chain. Several other proteins have been shown to be involved in the translocation process: translocation associated protein (TRAM), the small ribosome-associated protein 4 (RAMP4), and the oligosaccharyl transferase (OST) complex containing ribophorins I and II. Some of these proteins, such as the ribophorins, have been shown to be restricted to the RER by forming a stable supramolecular network. Other translocon components, on the other hand, may be relatively mobile within the ER and be recruited to the translocon complex when needed. Sec61p has been shown to localize also to the ER-Golgi intermediate compartment (Greenfield & High 1999).

The ER contains mechanisms designed to differentiate normal and abnormal forms of a wide variety of exportable proteins, though the quality control monitoring of the secretory pathway does not occur exclusively in the ER. Co- and posttranslational protein folding and maturation are assisted by molecular chaperones and other factors present in the ER lumen. The chaperones themselves remain localized within the ER, because they contain retention and retrieval signals (Hammond & Helenius 1994). The chaperones presumably recognize structural signals that are enriched in misfolded and incompletely folded molecules, and interactions with them typically begin as growing nascent chains enter the lumen through the translocon complex and continue until the newly synthesized proteins are folded, assembled, and ready to leave the ER. Proteins interact differently with the molecular chaperones (Hammond & Helenius 1994; Molinari & Helenius 2000). Chaperones prevent the formation of misfolded protein structures, both under normal conditions and when cells are exposed to stresses such as high temperature (Hartl 1996).

Prominent examples of ER-resident chaperones are binding protein (BiP), endoplasmin, calnexin (Bergeron et al. 1994), and calreticulin each having a specific function. In addition to these chaperones, there are folding catalysts like luminal protein disulfide isomerase (PDI). Most ER-resident luminal proteins have functions related to protein folding and maturation. Despite their presence in the entire ER, chaperones may not be uniformly concentrated throughout the organelle and may be enriched in the RER. Although chaperones are generally regarded as markers of the entire ER, exemptions to this rule have been detected. Chaperones may be absent or at least be highly diluted in ER exit sites, or they are excluded from spaces occupied by insoluble protein aggregates. Several molecular chaperones seem to escape ER retention and be present along the entire secretory pathway (Baumann & Walz 2001).

Several methods of quality control have been suggested. The ER chaperones may aggregate the misfolded proteins or have a role in making the exportable proteins recognizable to cargo receptors. Alternatively, if anterograde traffic requires escape from ER chaperones, it is possible that unfolded proteins fail to bury chaperone recognition sites (Hammond & Helenius 1994). Proteins that fail to achieve functionality are identified by ER signaling receptors and degraded to prevent their accumulation. On the other hand, transient protein aggregation enables the ER machinery to cope with a heavy
or unbalanced protein synthesis load. Such aggregation allows temporary sorting and storage of specific proteins without the induction of protein degradation or the programmed death of cells (Aridor & Balch 2000).

When proteins fail to achieve transport competent conformation and leave the ER, they are often diverted to a specific degradation pathway for selective removal. This process is called ER associated degradation (ERAD). In order to become accessible to the degradating proteasome of this system substrates must first be retrogradely transported from the ER into the cytosol, in a process termed dislocation (Jarosch et al. 2002). The exact mechanisms by which proteins are selected for degradation are unknown. For some cargo proteins degradation is inefficient, leading to their accumulation in the ER. Loss of function of the mutant protein and/or its accumulation in the ER leads in many cases to the development of disease (Aridor & Hannan 2002; Rutishauser & Spiess 2002). Some chaperones have been shown to co-operate with the glycosylation machinery in ERAD. It has been shown that BiP has an important function in keeping unfolded proteins in solution, which is obviously an indispensable prerequisite for their dislocation. Also members of the PDI family are involved in ERAD.

2.3.1.3 ER to Golgi transport

The intermediate compartment between the ER and the Golgi apparatus is a dynamic structure that captures cargo released from the ER in COPII coated vesicular carriers and promotes recycling by COPI coated vesicular carriers (Bannykh et al. 1998). The small GTPase Sar1 that is included in the COPII coat seems to have a role in linking cargo selection with ER morphogenesis through the generation of transitional tubular ER exit sites (Aridor & Traub 2002). Further, binding of specific ER exit motifs on transmembrane proteins to the sec23/sec24 subcomplex of the COPII coat appears to play a role in cargo selection (Belden & Barlowe 2001; Votsmeier & Gallwitz 2001). The transmembrane protein p55/58 that recycles between ER and Golgi elements has been suggested to be required for the coupled exchange of COPII for COPI coats during segregation of anterogradely and retrogradely transported proteins (Tisdale et al. 1997). Secretory proteins undergo a main concentration step between the ER and the cis-Golgi network (Oprins et al. 2001). All correctly folded proteins are transferred from the lumen and membrane of the ER to the cis-Golgi network, but the resident ER proteins are returned. The KDEL receptor binds to soluble ER resident proteins bearing KDEL or related sequences (KKXX or XXRR) and mediates their COPI-dependent retrograde transport from vesicular tubular clusters (VTCs) and Golgi apparatus to the ER (Semenza et al. 1990; Farquhar & Palade 1998; Martinez-Menarguez et al. 2001).
2.3.2 Golgi apparatus

The Golgi apparatus is a polarized network of stacked membrane containing one or more stacks of disc-shaped cisternae, which are organized as a series of at least three biochemically and functionally distinct compartments, termed cis-, medial- and trans-cisternae. The Golgi apparatus functions at the crossroads of the secretory pathway, playing a key role in the processing and transport of secretory and lysosomal proteins as well as in the biogenesis and traffic of membranes (Tassin et al. 1985). In the Golgi apparatus the products of the ER are covalently modified, and then distributed to various final destinations. In addition to that, selected components are returned back to the ER. Besides serving as a sorting and dispatching station, the Golgi apparatus serves as a major site of carbohydrate synthesis as well as the site where the synthesis of complex sphingolipids is completed (van Meer 1989; Lippincott-Schwartz 1998).

Two models have been launched to explain the movement of newly synthesized proteins through the Golgi stack. The vesicle shuttle model implies that small vesicles, which form at the cisternal rims mediate the forward transport of cargo proteins to the next cisterna. In this model each of the more or less stationary Golgi cisternae contains a specific set of polarized enzymes, which explains the polarized distribution of these proteins over the stack (Rothman 1994; Schekman & Orci 1996). The cisternal maturation model proposes that import of ER-derived membranes at the cis-Golgi side together with the formation of transport vesicles at the trans side of the Golgi apparatus results in the progression of the entire cisterna from the cis- to trans side (Martinez-Menarguez et al. 2001). Cisternal progression takes place substantially more slowly than most protein transport. Therefore it appears unlikely that cisternal maturation model is the predominant mechanism of anterograde movement (Orci et al. 2000; Martinez-Menarguez et al. 2001).

The Golgi apparatus, unlike the ER, contains many sugar nucleotides, which are used by a variety of glycosyl transferase enzymes to carry out glycosylation reactions on lipid and protein molecules as they pass through the Golgi apparatus. N-linked oligosaccharides, for example, which are added to proteins in the ER, are often initially trimmed by removal of mannoses, and then additional sugars are added. In addition, the Golgi apparatus is the site where O-linked glycosylation occurs and where glycosaminoglycan chains are added to core proteins to form proteoglycans (Alberts et al. 1994).

2.3.2.1 Role of cytoskeleton in Golgi positioning

Selective and effective delivery of protein and lipid species to diverse cellular sites requires intimate association of the Golgi apparatus with the cytoskeleton. Transport intermediates that move into or out of the Golgi apparatus often travel significant distances through the cytoplasm. In mononucleated mammalian cells, the Golgi apparatus is centered at the microtubule-organizing center and is actively maintained there by associations with microtubule motors. Transport intermediates in the form of vesicles and
tubulovesicular intermediates mediate forward and retrograde transport between the ER, Golgi apparatus and plasma membrane. They track plus- or minus-end directed along microtubules that emanate out of the microtubule organizing center. In the TGN to plasma membrane traffic cargo has even been found to be capable of switching tracks (Toomre et al. 1999).

An abundance of actin and actin binding proteins may facilitate the Golgi's spatial control of membrane traffic (Lippincott-Schwartz 1998). Spectrin-ankyrin skeleton is also required for Golgi integrity and anterograde protein trafficking. Yet the organization of spectrin on the Golgi apparatus is uncertain. The highly dynamic nature of the Golgi apparatus probably relies on a wide array of molecular motors and microtubule-anchoring proteins. Regulation of these opposing but complementary activities would allow the Golgi apparatus to maintain its steady-state localization while constantly receiving and generating membranes (Rios & Bornens 2003). An interesting question is how the transport along microtubules is directed in multinucleated myofibers, since during myogenesis the microtubule-organizing center disappears and the microtubules are arranged in a totally new fashion. Also the Golgi element disperses to smaller stacks during myogenesis (Ralston et al. 1999).

2.3.2.2 Sorting in the TGN

The TGN is the major sorting center of the secretory pathway (Griffiths et al. 1984). It modifies and directs proteins and lipids to the appropriate cellular destinations. One route, the constitutive or default pathway, delivers proteins to the cell surface while another, selective pathway sorts proteins to the intracellular endosomal membrane system. In polarized cells cytoplasmic sorting signals guide membrane proteins selectively to the basolateral plasma membrane. The apical sorting determinants are at present poorly understood. Lipid rafts that form in the TGN may mediate sorting of GPI anchored proteins and glycosphingolipids to the apical plasma membrane (Matter & Mellman 1994; Traub & Kornfeld 1997). In some, but not all cases N-or O-glycans provide sorting information, and also accessory proteins such as VIP17/MAL may be involved in the process. (Sceiffele et al. 1995; Ikonen 2001). Distinct apical-and basolateral-destined pathways are not a unique property of polarized cells. Related apical and basolateral trafficking routes might exist in all cells with the default itinerary varying according to the cell type (Musch et al. 1996). Also myosin II seems to have a role in transport from the TGN (Musch et al. 1996; Lippincott-Schwartz 1998), probably regulating actin filaments involved in the process.

Proteins to be exocytosed are carried to the cell surface in vesicular or tubular structures, which have unique proteins in their membrane, though the coat proteins have not been identified (Toomre et al. 1999). Proteins are often proteolytically processed during the formation of secretory vesicles. While vesicles containing material for constitutive release fuse with the plasma membrane once they arrive there, secretory vesicles in the regulated pathway wait at the membrane until the cell receives a signal to secrete and then fuse (Alberts et al. 1994).
2.4 Studying protein trafficking

Protein trafficking has been studied mainly with cultured mononucleated fibroblast-like cells or in a few cases in epithelial cells (Rodriguez-Boulan 1983; Kreitzer et al. 2003), or neurons (Dotti & Simons 1990). The only multinucleated exception is osteoclasts, bone resorbing cells, in which protein trafficking has been studied by Salo (1997), and Mulari (2003), with their coworkers.

Enveloped viruses are handy tools in studying protein transport. They enter the cell via coated pits and exploit the intracellular trafficking machinery for their replication. In infected cells viral glycoproteins are subjected to the same posttranslational controls and processing as cellular glycoproteins, but they are expressed in much higher amounts than endogenous proteins and are thus easier to locate. In studies on apical/basolateral protein sorting the fact that different enveloped viruses bud from different cellular membranes has been used (Rodriguez-Boulan 1983; Musch et al. 1996). Influenza virus is known to bud only from apical plasma membrane while SFV or VSV only from the basolateral plasma membrane. Mutant viruses have also been prepared. A classic example of those is much used tsO45, a temperature sensitive VSV mutant, the transport of which can be controlled by changing incubation temperature. Also expression of cloned sequences using viral carrier vectors has been used since 1980s (Olkkonen et al. 1994).

Recombinant viruses are a convenient way to express modified or exogenous proteins in cells that are resistant to transfection methods - like the adult myofibers.

Certain drugs that have well characterized effects on cells are convenient in studying vesicle transport. One of the most common is BFA. BFA is a fungal antibiotic, which reversibly blocks the secretory pathway by disrupting the Golgi apparatus (Doms et al. 1989). It prevents the assembly of the coats that are required for budding of the transport vesicles and inhibits the transport from the ER to the Golgi complex without interfering with folding and oligomerization. In BFA treated cells Golgi disappears and its proteins end up in the ER. When the drug is removed, the normal Golgi apparatus reforms and the Golgi proteins return to their proper Golgi compartments. Other common examples are drugs that disrupt different components of the cytoskeleton like nocodazole (microtubules), or cytochalasin D (actin filaments) (Alberts et al. 1994).

GFP-fusion proteins have opened totally new possibilities for studying intracellular transport. When green fluorescent protein (GFP) is attached by genetic engineering techniques to the protein of interest, the whereabouts of the resulting fusion protein can be followed in living cells. When a cDNA encoding such a fusion protein is expressed in a cell, the protein is readily visible in a fluorescence microscope. GFP techniques are widely used to study the location and movement of proteins in cells. As a classic example the VSVG-GFP should be mentioned. It has been used for example in kinetic analysis of secretory protein traffic in living cells (Hirschberg et al. 1998). Subcellular localization of translocon complex core protein sec61 has also been studied with GFP (Greenfield & High 1999), as well as the continuity of ER lumen with KDEL-GFP (Dayel et al. 1999), or recycling of lipid raft markers (Nichols et al. 2001). The study of GFP fusion proteins is often combined with fluorescence recovery after photobleaching (FRAP), and fluorescence loss in photobleaching (FLIP), techniques, in
which the GFP in selected regions of the cells is bleached by strong laser light (Lippincott-Schwartz et al. 2000).

2.5 Skeletal muscle cell

2.5.1 Muscle types

All three muscle types, skeletal, cardiac and smooth, are composed of elongated cells specialized for contraction. Smooth muscles, as the name implies, are non-striated, mainly found in vascular system, digestive tract and in uterus. The non-striated muscle cells are mononucleated and actin and myosin are less ordered than in striated muscle cells. The contraction of smooth muscle cells is slower though of greater extent and can be sustained longer. Cross-striated cardiac muscle is confined to the heart and is rhythmically contractile. Cardiac muscle cells have one nucleus but otherwise the ultrastructure reminds more that of skeletal muscle cells. The rate of the heart beating is under nervous control but many individual cells do not receive direct innervation. The other cross-striated muscle type, skeletal muscle, forms the major muscle component of the body. It is organized into muscles that are responsible for the gross and fine movements of limbs and the maintenance of body position and posture (Tortora & Grabowski 2003).

Skeletal muscles are organs specialized for rapid force production. They consist of a heterogeneous population of multinucleated, striated myofibers held together by connective tissue. The connective tissue that surrounds both individual myofibers and bundles of them, and in which a rich blood supply and a rich supply of nerves travel, is essential for force transduction. At the end of the muscle, the connective tissue continues as a tendon or some other arrangement of collagenous fibers that attaches the muscle to tissues such as those of the skeleton, forming a myotendinous junction (MTJ). Skeletal muscle cells are coordinated directly or indirectly by the nervous system and capable of highly complex actions. One or more nerves supply each skeletal muscle, while each myofiber receives a single terminal branch of an alpha efferent axon, which ends at a neuromuscular junction (NMJ) (Williams et al. 1989; Tortora & Grabowski 2003).

2.5.2 Myogenesis

Skeletal muscle fibers are highly differentiated cells, which are formed in various regions of the body. Skeletal muscle development is a multistep pathway, in which mesodermal precursor cells are selected to form myoblasts that are withdrawn from normal cell cycle and subsequently differentiate (Buckingham 1994)(Fig. 2.). When skeletal muscle cells differentiate, thousands of structural and regulatory molecules assemble into the semicrystalline sarcomeric contractile units. As a consequence of this precise assembly,
many different classes of proteins function together to convert the molecular interactions of actin and myosin efficiently into the macroscopic movements of contractile activity (Gregorio & Antin 2000).

Myoblasts fuse to form long multinucleated myotubes in which the assembly of myofilaments, as well as the production of muscle-specific proteins, begins. During the differentiation the organization of the cellular organelles and the plasma membrane of the myoblasts changes dramatically, with the consequent formation of a single functional unit. This process involves extensive reorganization of the constituents of the cytoskeleton, the microtubule organizing sites being relocalized at the surface of the nuclei in myotubes, in marked contrast with the classical pericentriolar localization (Lu et al. 2001). Also new muscle specific organelles such as the sarclemma, the sarcoplasmic reticulum (SR) and the transverse (T-) tubules are generated. (Tassin et al. 1985). Apparently also novel membrane trafficking pathways are needed to communicate with the new organelles and membrane domains. One example of profound reorganization of the existing membrane compartments during myotube formation is reorientation of the Golgi elements from polarized juxtanuclear to perinuclear and interfibrillar distribution (Tassin et al. 1985; Rahkila et al. 1996). Also ER seems to diffuse to a common network between the myonuclei. Simultaneously part of the ER differentiates into SR by the gradual displacement of generic ER proteins by SR-specific proteins. In parallel to this, formation of the T tubules takes place (Flucher et al. 1993) assisted by amphiphysin and caveolin-3 (Lee et al. 2002). In adult myofibers SR forms a prominent membrane compartment that is morphologically strictly organized in a cross-striated fashion. However the localization of the RER, as well as the relationship between SR and ER, have remained obscure until our work (III).

2.5.3 Satellite cells-repair units

About 5 percent of all the myonuclei seen in the light microscope at the periphery of myofibers belong to the satellite cells, a cell population distinct from the myoblasts (Schultz 1989). Satellite cells attach to the surface of the myotubes and are thus situated between the basal lamina and the plasma membrane of the mature myofiber. Satellite cells are responsible for muscle maintenance and the extensive repair and regeneration that skeletal muscles are capable of after injury. They are quiescent myogenic precursor cells, which become activated following disruption of the sarcolemma and muscle necrosis in muscle injury. Satellite cells proliferate and differentiate into myoblasts. As long as the basal lamina remains intact in the injury, the myoblasts fuse with each other to form myotubes, which then mature into a new fiber or fuse with an existing one (Schultz 1989; Russell et al. 1992). The regeneration process is identical to myogenesis during fetal development and is similar irrespective of the injury. In contrast, disruption of the basal lamina results in fibroblast repair of the injured site with scar tissue formation (Bodine-Fowler 1994).
Satellite cells can be induced to proliferate and make up an enriched source of myoblasts that can be extracted from the muscle tissue and propagated in culture. Numerous cell lines have been established from these stem cell populations. Satellite cell derived myoblasts can be cultured to mimic different aspects of myogenesis, from proliferation to withdrawal from the cell cycle, fusion into myotubes, and expression of a gene subset encoding contractile proteins, although the individual events are much slower than during in vivo differentiation (Gregorio & Antin 2000).

Studies on membrane trafficking and other cellular events in muscle cells have mainly been carried out with myoblasts. Established muscle cell lines like the rat L6 or mouse C2C12 cell lines are commercially available. These mononucleated cells are much-used tools, because they are relatively easy to cultivate, and they can be induced to fuse to form multinucleated myotubes on culture dishes. Their differentiation stops, though, at a
certain point, and there are aspects of myogenesis, such as the full recapitulation of contractile function and generation of different fiber types or establishment of subcellular domains within the mature myotube, that cannot be achieved in muscle cell cultures (Neville et al. 1997). Expression of desmin is the first sign of muscle differentiation. Subsequently, other proteins taking part in myofibrillogenesis appear in precise order and timing of expression. In cultured myotubes the typical array, but not all, of muscle specific proteins are subsequently expressed (Yablonka-Reuveni & Rivera 1994). The main disadvantage of the use of myotubes, though, is the big differences that exist between the cell lines and those that depend on culture conditions. Since the differentiation is far from complete, the situation in myotubes cannot totally correspond to that in mature myofibers. Mature myofibers have been used in the studies on vesicle trafficking and other cellular events very seldom because their cultivation is rather difficult. In some studies myofibers have been mechanically stripped from muscle tissue but such myofibers cannot survive in culture. In our laboratory we have utilized a method to isolate mature myofibers and culture them on dishes originally developed by Bekoff and Betz (1977). My aim was to use mainly these mature, fully differentiated muscle cells in my studies.

**Fig. 3.** Structure of a skeletal muscle fiber. Sarcomeres compose myofibrils that are surrounded by the sarcoplasmic reticulum (SR). Near the A-I junctions terminal cisternae and T tubules form triads, which regulate the Ca$^{2+}$ release and transport. (Adapted from Rogers 1983)
The cellular units of skeletal muscle are the myofibers, each a long cylindrical structure surrounded by a plasma membrane, the sarcolemma. The subcellular architecture of skeletal muscle, shown in Figure 3, is very different from that of mononucleated cells. Muscle fibers are 10 to 100 µm in diameter and from a few millimeters to several centimeters long, and contain up to several thousand nuclei derived from the fusion of myoblasts in fetal and postnatal life. Most of the myofiber nuclei are located peripherally beneath the sarcolemma. The fibers are further composed of myofibrils, membranes, and cytoskeletal network, which anchor the contractile fibrils to the sarcolemma. Myofibrils are composed of repeating contractile units known as sarcomeres, perhaps the most highly ordered macromolecular structures in eucaryotic cells (Gregorio & Antin 2000).

Each sarcomere consists of thick and thin filaments whose arrangement is largely responsible for the cross-striated banding pattern observed under light and electron microscopy. Sarcomeres are delineated at their ends to Z-lines where thin actin filaments of opposite directions are linked together by α-actinin dimers (Luther 2000). Z-line is located in the middle of the I-band, which appears lighter in a light microscope and contains mainly actin filaments. Polymers of myosin molecules form the darker A-band. The A band is bisected by a light region called the H band, the major component of which is creatinine kinase. Running through the midline of H band is the M line in which the thick filaments are anchored by several myosin-binding proteins. Thick filaments are connected to giant titin molecules expanding to half of a sarcomere, from Z-line to M-line. Titin is thought to function as a spring and a ruler defining sarcomere length after muscle contraction (Gautel et al. 1999), which happens when actin filaments interact with the myosin filaments so that the thin filaments move past the thick filaments toward the center of the sarcomere thus shortening it.

Although all skeletal myofibers have the same basic sarcomeric organization, a number of distinct types have been described according to structural, physiological and biochemical criteria. Skeletal muscle fibers can be generally classified as fast or slow twitch, based on their contractile and metabolic properties and associated patterns of gene expression (Hughes 1998). Red slow twitch oxidative fibers (type I), are involved in sustained, tonic contractile events and maintain intracellular Ca²⁺ concentrations at relatively high levels (100-300nM). In contrast, white fast twitch glycolytic fibers (type II) are used for sudden bursts of contraction and are characterized by brief, high-amplitude Ca²⁺ transients and lower ambient Ca²⁺ levels (<50nM). These properties of skeletal muscle fibers are dependent on the pattern of motor neuron stimulation, so that tonic motor neuron activity promotes the slow fiber phenotype while infrequent motor neuron firing results in fast fibers (Olson & Williams 2000). Structurally, when compared to type II myofibers, type I myofibers tend to be narrower, have thicker Z and M bands, have more glycogen, and their sarcoplasm is rich in mitochondria. The molecular basis for the functional diversity of myofibers is the expression of specific isoforms of most of the proteins involved in muscle contraction and relaxation. Myofiber classification is based on contraction speed and other physiological properties but predominantly according to specific myosin heavy chain (MyHC) isoforms (Schiaffino & Reggiani 1996; Ralston et al. 2001).
Morphological details vary in different muscle fibers. Skeletal muscles respond to changes in physiological demands by remodeling the architecture of individual fibers. Sarcomeres are added or removed when muscles are held at abnormally long or short lengths, and myofilaments are added or removed when muscle fibers function against abnormally heavy or light loads (Trotter 2002). This leads to changes in overall mass of the tissue. Also the spatial relationship among muscle cells and other components of muscle tissue can change and gene expression can be reprogrammed to alter specialized metabolic and contractile properties of myfibers (Olson & Williams 2000).

2.5.5.1 Protein components and membrane skeleton

The primary protein components of skeletal muscle fibrils, myosin and actin, and the tropomyosin and troponins associated with actin, constitute more than 75% of the total protein of the muscle fiber. The remaining proteins, like titin, nebulin, α-actinin or myomesin, are essential in regulating the spacing, attachment, and precise alignment of the myofilaments.

In skeletal and cardiac muscle, sarcolemmal dystrophin associates with various proteins to form a protein complex, which is thought to play a structural role in linking the actin cytoskeleton to the extracellular matrix. Myofiber function and survival are dependent on this link, which stabilizes the sarcolemma during repeated cycles of contraction and relaxation, and transmits force generated in the muscle sarcomeres to the extracellular matrix (Petrof et al. 1993). Integrins (McDonald et al. 1995) and the dystrophin-associated glycoprotein complex (DGC) take part in maintaining this anchorage. The components of the DGC have been described in detail (Ohlendieck 1996). An extracellular component of it, α-dystroglycan, provides a physical link between the extracellular matrix and the intracellular cytoskeleton. The precise distribution of the DGC on the sarcolemma is not known, though dystrophin is concentrated at the sarcolemma of skeletal muscle fibers in longitudinally oriented strands and in costameres, regions of the sarcolemma that overlie Z and M. The most likely candidate to a protein responsible for coordinating the distribution of proteins at the sarcolemma is actin (Williams & Bloch 1999).

Cytoskeletal structures containing intermediate filaments, like vimentin, desmin and nestin, form a physical link to connect the contracting subunits to the sarcolemma in striated muscles. Intermediate filaments may have an important role in cellular organization during myogenesis and in maintaining structural integrity in mature myofibers by redistributing the stress caused by contractile activity (Vaittinen et al. 1999). In mature myofibers vimentin expression is completely down-regulated, whereas nestin is expressed at low levels adjacent to NMJ and MTJ. Desmin expression increases continuously with advancing maturation and it accumulates finally at the margins of Z-lines, anchoring the Z-lines of adjacent myofibrils together and taking part in connecting myofibrils to the plasma membrane (Lazarides 1980; Tokuyasu et al. 1985).
2.5.6 Specific myofiber membrane compartments and their functional significance

The muscle surface membrane consists of the plasma membrane proper (sarcolemma), and the T-tubule system. The sarcolemma and the T-tubule system are continuous but have distinct lipid and protein compositions. The myofiber plasma membrane contains also two kinds of specialized areas, namely the NMJ and the MTJ.

2.5.6.1 Sarcolemma

Treatment with collagenase or other enzymes is needed to strip connective tissue and basal lamina from the sarcolemma. The exposed surface of the muscle fiber is smooth with cross striation pattern that conforms to the underlying myofibrils. The A band and the Z disc are slightly protruded compared to the I bands, and they all are laterally aligned in register. The degree of fiber stretching before and during fixation causes variations in the surface contour.

A notable feature of the sarcolemma is that it is studded with caveolae, which are considered as a specialized type of lipid rafts. In muscle caveolae contain muscle specific caveolin-3. In differentiating skeletal muscle caveolin-3 has been shown to associate with the developing T-tubules, but in mature skeletal muscle caveolin-3 is restricted to sarcolemmal caveolae and is no longer detectable in T-tubules (Parton et al. 1997). Caveolin-3 co-fractionates with dystrophin and DGC proteins (Song et al. 1996). The exact localization of caveolin-3 with respect to defined surface markers has been studied by Rahkila and co-workers (2001). They found out that the entire muscle sarcolemma seems to be an organized array of caveolin-associated raft and nonraft domains comprising a mosaic of t-tubule domains, sarcolemmal caveolae and β-dystroglycan domains.

2.5.6.2 T-tubules and SR

The A-I junctions in the myofibrils are surrounded by T-tubules. T-tubules are regarded as an extension of the plasma membrane forming junctions with the sarcoplasmic reticulum. The best-known function of T-tubules is to rapidly spread changes in membrane potential throughout the muscle fibers, but they might also serve as irradiation channels to provide extracellular fluid direct access to the core of the thick muscle fibers.

T-tubules have been identified as continuous elements running transversely over several myofibrils, which are penetrating to all levels of the myofiber. They are an extensive surface-connected system of membranes, which develop and maintain a protein and lipid composition distinct from the sarcolemma (Flucher 1992; Parton et al. 1997). Some studies have provided evidence for an internal T-tubule compartment, which
subsequently fuses with the sarcolemma (Flucher et al. 1993). In other studies it has been suggested that T-tubules form from the repeated budding of caveolae aided by amphiphysin (Lee et al. 2002). Muscle specific caveolin-3 might be required to generate the unique protein and lipid composition of the T-tubule system (Parton et al. 1997), and amphiphysin II has been found essential for organization and normal morphology of the skeletal muscle T-tubules. Amphiphysin II colocalizes with ankyrin and plays a role in T-tubule branching, in anchoring T tubules to their places, and in organizing protein components of T-tubules (Razzaq et al. 2001). Ankyrin-1 isoform has been found to localize to the M line where it binds to obscurin. This interaction may provide a direct link between the SR and myofibrils (Bagnato et al. 2003).

Calcium must be available for the reaction between actin and myosin for contraction to occur. After contraction, the calcium must be removed. This rapid delivery and removal of calcium is accomplished by the combined work of the SR and the T-tubule system. The SR surrounds myofibrils like a net stocking. One network of SR surrounds the A band, and another network surrounds the I band. Where the two networks meet, at the junction of A and I bands, the SR forms terminal cisternae. The SR controls the level of intracellular Ca\(^{2+}\) in cardiac and skeletal muscles by storing and releasing Ca\(^{2+}\). It is known that the SR initially develops as ER and that, as the muscle cell differentiates, it becomes greatly enriched in SR-specific proteins (Volpe et al. 1992; Villa et al. 1993). Three proteins first purified from the SR are the calcium ATPase (SERCA), calsequestrin (CLQ) and ryanodine receptor (RyR). SERCA is responsible for pumping calcium into the lumen of the SR during relaxation, CLQ is the most prominent of intralumenal calcium-binding protein that greatly increases the SR capacity for calcium, and RyR is responsible for calcium release during muscle activation. The most abundant SR protein outside the SR-T-tubule junction is SERCA, which is normally distributed in tubular elements surrounding the Z line and M lines, as well as in elements aligned with the longitudinal axis of the myofiber (Williams & Bloch 1999). In the lumen of the SR, the major protein is CLQ (Jorgensen et al. 1977), an acidic protein that binds to calcium with moderate affinity and high capacity. CLQ is specifically targeted to the junctional SR by its acidic carboxy-terminal end (Nori et al. 1993). CLQ and RyR are functionally coupled. Triadin is an abundant membrane protein in the junctional SR, where it colocalizes with the RyR. It anchors CLQ to the junctional face membrane and mediates the functional coupling between the RyR and CLQ in the lumen of the SR (Franzini-Armstrong et al. 1987; Guo & Campbell 1995). Conversely, all cells contain SR-like specialized domains but in much smaller amounts. Some non-muscle cells, such as the Purkinje cells of the cerebellum, actually have extensive SR-like domains containing muscle-specific isoforms of RyRs and calsequestrin (Franzini-Armstrong 1999). T tubules are located between adjacent terminal cisternae of the SR forming a triad. SR-T-tubule junctions and their association with myofibrils develop in a series of consecutive steps (Flucher 1992; Flucher et al. 1993). The formation of junctions between the two membrane systems occurs concurrently, initiating molecular changes in both membrane systems (Täkekura et al. 2001).

When a nerve impulse arrives at the muscle membrane, the plasma membrane depolarizes, and there is a rush of Na\(^+\) ions into the muscle cell. The depolarization is transmitted into the depths of the cell along the membranes of the T system. Na\(^+\) ions signal the SR to release Ca\(^{2+}\) into the cytosol, initiating contraction in each myofibril.
This series of events is called excitation-contraction (e-c) coupling. Several proteins that are specifically localized to the SR-T-tubule junction play essential roles in e-c coupling. The T-tubular dihydropyridine receptor (DHPR) senses the voltage across the membrane, and activation of this receptor leads to the release of Ca\(^{2+}\) from the SR (Flucher 1992). The RyR/ Ca\(^{2+}\) release channel is localized in the junctional SR and is responsible for the Ca\(^{2+}\) release from the Ca\(^{2+}\) stores. Both RyR and DHPR are needed for appropriate muscle development though neither of them is needed for T-SR docking or for the targeting and/or association of CLQ and triadin in the junctional SR (Felder & Franzini-Armstrong 2002).

### 2.5.6.3 NMJ

Each myofiber is innervated by an axon terminal and they respond to impulses conducted by motor neurons of the spinal cord or brain stem. A single motor neuron may contact some tens over one thousand myofibers, but each myofiber is innervated by one nerve cell and one axon terminal only. The specialized structure at the contact site between the terminal branches of the axon and muscle is called the motor end plate or the NMJ. At the NMJ the membrane is thrown into deep clefts increasing the receptor area in the myofiber (Ishikawa & Shimada 1982). NMJ is usually located in the middle third of a myofiber. Nerve cells not only serve to instruct the muscle cells to contract but also exert a trophic influence on the muscle cells, which is necessary to maintain their structural integrity. Typically three to five nuclei cluster in the NMJ and protein synthesis as well as many other biochemical processes are very prominent in this junctional area. The hallmark of the NMJ is the high local concentration of the acetylcholine receptor and of several associated proteins, as well as that of mRNAs encoding them (Ralston et al. 1997).

### 2.5.6.4 MTJ

The MTJ is the morphologically distinct interface between muscle and tendon. Ultimately the contractile forces generated by active myofilaments get transmitted to the tendon through this interface. At the MTJ the end of the muscle fiber abruptly tapers. Characteristic of its membrane domain are longitudinal projections and invaginations, which form many finger-like cytoplasmic projections, mingled with the collagen fibrils of the tendon. The morphology of the interface between a muscle fiber and the tendinous connective tissue looks like an adhesive joint. The folds increase the interfacial area by at least an order of magnitude over the cross-sectional area of the muscle fiber. They also ensure that the stresses applied to the interface are experienced mainly as shear stresses (Williams et al. 1989).

Structurally, the MTJ consists of the actin filaments that extend from the last A-band, actin-binding proteins that bundle the actin filaments together, proteins that link the actin filament bundles to the sarcolemma, transmembrane proteins that link to extracellular...
components, the external lamina, and proteins that link the external lamina to the collagen-fibril rich matrix outside it. Current evidence supports the view that skeletal muscle fibers have two parallel systems for linking intracellular and extracellular structural proteins, namely the dystrophin-DPG system (Bao et al. 1993) and the α7β1 integrin system (Trotter 2002). Also desmin is shown to transmit force from myofibrillar force generators to the muscle surface and to the MTJ (Lieber et al. 2002).

2.5.6.5 Membranes of exocytic machinery

In the multinucleated muscle cells the whole exocytic transport machinery appears to be organized differently when compared to the organization of the exocytic machinery in the mononucleated cells (Rahkila et al. 1998). In mononucleated cells ribosomes attached to the surface of RER membranes clearly indicate its location. To locate RER in skeletal myofibers is very difficult, if not impossible, to do by mere morphology.

In ultrathin sections of a myofiber it is difficult to distinguish single ribosomes from single glycogen particles, although they do differ slightly in size and electron density, and glycogen has been shown to locate preferentially at the I bands, along with the glycogenolytic enzymes (Dolken et al. 1975). Horne and Hesketh (1990) found that according to immunostaining of large subunits ribosomes locate mainly to the A bands. But the myosin mRNA localized by in situ hybridization is accumulated primarily at the periphery of muscle cells that are actively involved in myosin synthesis, and even when present in the interior, it shows no preferential association with the A bands. The compartmentalization of myosin isoforms within a muscle cell suggests that myosin might be assembled directly into thick filaments at sites where it is synthesized (Gauthier 1990). In chicken anterior latissimus dorsi (ALD), ribosomes were found to be located between thick filaments, often aligned in rows suggesting that ribosomes are located within the filament lattice to be available for local myosin synthesis (Gauthier & Mason-Savas 1993). It was not shown though which, if any of the ribosomes are involved in the synthesis of new myosin.

During skeletal muscle differentiation, the Golgi complex undergoes a dramatic reorganization (Tassin et al. 1985; Ralston 1993), from the classic, compact juxtanuclear position to dispersed elements that form a belt around each of the myotube nuclei and extend between the nuclei along microtubules like strings of pearls (Rahkila et al. 1997). Each myofiber Golgi complex is made of thousands of small dispersed elements. There is an average of 100 Golgi elements per nucleus, the amount being roughly of the same magnitude as that found in other mammalian cells (Ralston et al. 1999). Golgi distribution at the NMJ seems constant in all fibers and independent of fiber type, but otherwise Golgi distribution is fiber type-specific. In slow-twitch, type I fibers, about 75% of all Golgi elements are located within 1 µm from the sarcolemma, and each nucleus is surrounded by a belt of Golgi elements. In contrast, in the fast twitch type II fibers, most Golgi elements are in the fiber core, and most nuclei only have Golgi elements at their poles. (Ralston et al. 1999; Ralston et al. 2001).
Centrosomal proteins, microtubules, ER exit sites, and Golgi elements are linked and affected by activity. Whether one of them determines the localization of the others is less clear, because very little is known of the link between ER exit sites and microtubules. ER exit sites have been reported to be mostly immobile in HeLa and similar cell types (Hammond & Glick 2000), but their organization changes during muscle differentiation (Lu et al. 2001; Ralston et al. 2001).
3 Aims of the study

The intracellular organization of adult myofibers is very different from that of mononucleated cells. Protein trafficking seems to obey general rules to certain extent, but there also seems to be some muscle specific modifications concerning at least protein targeting and the transport routes.

In this study the purpose was to clarify certain aspects of endocytosis and exocytosis in multinucleated muscle cells. The specific aims can be listed as follows:

1. To study how the endosomal trafficking is organized in multinucleated skeletal muscle cells.
2. To clarify the location of the rough ER and the relationship between the ER and the SR in multinucleated skeletal muscle cells.
3. To characterize the export pathway in skeletal muscle cells.
4 Materials and methods

Table 1. The methods used in original studies (I-III)

<table>
<thead>
<tr>
<th>Method</th>
<th>Used in</th>
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<tbody>
<tr>
<td>Cell cultures</td>
<td></td>
</tr>
<tr>
<td>Culturing L6 myoblasts and myotubes</td>
<td>I, II,</td>
</tr>
<tr>
<td>Isolating and culturing myofibers</td>
<td>I, III,</td>
</tr>
<tr>
<td>Biochemical techniques</td>
<td></td>
</tr>
<tr>
<td>Metabolic labeling</td>
<td>II, III</td>
</tr>
<tr>
<td>Immunoprecipitation</td>
<td>II, III</td>
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<tr>
<td>Endo H digestion</td>
<td>II, III</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>II, III</td>
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<tr>
<td>Viral infections</td>
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<tr>
<td>Protein synthesis by recombinant viruses</td>
<td>I, II, III</td>
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<tr>
<td>Preparation of recombinant viruses</td>
<td>II, III</td>
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<td>Protein analyses</td>
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<tr>
<td>Immunofluorescence labeling</td>
<td>I, II, III</td>
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<tr>
<td>Immunoperoxidase labeling for electron microscopy</td>
<td>I, III</td>
</tr>
<tr>
<td>Nanogold labeling for electron microscopy</td>
<td>III</td>
</tr>
<tr>
<td>Microscopic techniques</td>
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<tr>
<td>Conventional confocal laser scanning microscopy (CLSM)</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Sucrose density gradient centrifugation</td>
<td>II</td>
</tr>
</tbody>
</table>
4.1 Cell culture

4.1.1 L6 myoblasts and myotubes (I, II)

The L6 myogenic cell line was purchased from ATCC. The L6 myoblasts were cultivated on 30 mm diameter dishes in DMEM containing 7% fetal calf serum. The myoblasts were induced to differentiate into multinucleated myotubes by replacing the medium with DMEM containing 1% horse serum and 0.4 U/ml of insulin. Myoblasts were used for the experiments after a two days growth period. Myotubes were used after four days from the induction of differentiation when over 90% of the nuclei located in the multinucleated myotubes. Primary myotube cultures were produced by cultivating rat satellite cells as described by Rahkila and coworkers (1998). Briefly, satellite cells were isolated from rat *flexor digitorum brevis* and plated on Matrigel along myofibers and cultured in Dulbecco's MEM containing 5% horse serum. Satellite cells fused into myotubes within two weeks and were then used for experiments.

4.1.2 Isolation of myofibers (I, III)

Myofibers were isolated from adult female Sprague-Dawley rat footpad muscle *flexor digitorum brevis* as described. Briefly, the muscle was excised and treated with collagenase followed by trituration with a pipette. The isolated myofibers were cultivated on 30 mm diameter dishes coated with Matrigel, in Dulbecco's MEM containing 5% horse serum, in an atmosphere of 5% CO₂. Myofibers were used for experiments after a 24h cultivation period.

4.2 [35S]methionine pulse-chase labeling, immunoprecipitation, and SDS-PAGE. (II, III)

L6 myoblasts, L6 myotubes or mature myofibers infected with the relevant virus were pulse-labeled for 10 min with [35S]methionine (1 mCi/ml; Amersham-Pharmacia Biotech), and then chased for 180 min at the indicated temperature. The cells were then scraped in PBS, pH 7.0, containing 1% Triton X-100, 1% deoxycholate and 1 mM phenylmethylsulfonyl fluoride (500 µl/dish). The lysate was centrifuged to separate the insoluble and soluble fractions. For immunoprecipitations, after an overnight incubation at 0°C with the relevant antibodies, the clear soluble fraction was further incubated with Protein A or Protein G Sepharose (Amersham Pharmacia Biotech), for 1 h at 20°C. After agitation the beads were washed and the attached antigen subjected to SDS-PAGE, either directly or after digestion with Endo-H (Roche Diagnostics). After SDS-PAGE gels were dried and exposed to Kodak Biomax MR film. Radioactivity in the bands was quantitated
with Fujifilm BAS-1800II phosphoimager (Fuji Photofilm Co Ltd) using Fujifilm Science Lab 99 Image Gauge software. For quantitations, virions in the media were pelleted by centrifugation at 50 000g for 60 min at 4°C.

### 4.3 Viral infections. (I, II, III)

**Table 2. List of viruses used in original studies (I-III)**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Source/reference</th>
<th>Used in</th>
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<tbody>
<tr>
<td>Vesicular stomatitis virus (VSV)</td>
<td>Indiana serotype</td>
<td>I, II</td>
</tr>
<tr>
<td>Influenza A, WSN strain</td>
<td>Martin &amp; Helenius (1991)</td>
<td>I, II, III</td>
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<tr>
<td>VSV tsO45</td>
<td>Griffiths et al. (1985)</td>
<td>II, III</td>
</tr>
<tr>
<td>Sendai virus, Z strain</td>
<td>Örvell &amp; Grandien (1982)</td>
<td>II, III</td>
</tr>
<tr>
<td>Semliki Forest virus (SFV)</td>
<td>Dr. Henrik Garoff (Karolinska Institute)</td>
<td>II, III</td>
</tr>
<tr>
<td>Rhesus rotavirus type A (ROTA)</td>
<td>Dr. C.-H. von Bonsdorff (University of Helsinki)</td>
<td>III</td>
</tr>
</tbody>
</table>

**RECOMBINANT VIRUSES**

- recVSVs CT1, CT9, GCC, GGC: John Rose / Schnell et al (1998) II
- recSFV-TfR: Suomalainen & Garoff (1994) I, III
- recSFVss-peroxidaseKDEL: II II, III
- recSFVts-G-GFP: III

Myoblasts, myotubes and myofibers on the cell culture dishes were infected with various viruses. MEM containing 1% BSA and the virus in question (at a multiplicity of 5-10/nucleus, as determined on BHK cell monolayers) was placed on the cells and the virus was allowed to adsorb as indicated. After adsorption the virus was removed and the infection was allowed to proceed further in culture medium or MEM containing 1% horse serum at the relevant temperature.

### 4.4 Recombinant viruses (I, II, III)

**Table 3. Plasmids and pcDNAs used in original publications (II, III)**

<table>
<thead>
<tr>
<th>Plasmids etc</th>
<th>Source/reference</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSFV1</td>
<td>Dr. Henrik Garoff (Karolinska Institute)</td>
<td>II,III</td>
</tr>
<tr>
<td>pcDNA3 encoding Japan strain influenza HA</td>
<td>Dr. Peter Scheiffele (EMBL; Heidelberg)</td>
<td>II</td>
</tr>
<tr>
<td>or a mutant HA (4A511)</td>
<td></td>
<td>II,III</td>
</tr>
<tr>
<td>pRK34 encoding peroxidase-KDEL</td>
<td>Dr. Daniel Cutler/ Norcott et al. (1996)</td>
<td>II,III</td>
</tr>
<tr>
<td>pCB6 encoding ts-G-GFP</td>
<td>Scales et al. (1997)</td>
<td>III</td>
</tr>
<tr>
<td>pSFV-TR</td>
<td>Dr. Henrik Garoff (Karolinska Institute)</td>
<td>I, III</td>
</tr>
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</table>
pSFV1 plasmid was provided by Dr. Henrik Garoff and pcDNA3 plasmids encoding the Japan strain influenza HA or a mutant HA (4A511) have been characterized by Scheiffele et al. (1997). The HA gene was excised from the pcDNA3 by HindIII/BamHI (Amersham Pharmacia Biotech, UK) digestion and after Klenow polymerase reaction cloned into the Smal restriction site in the vector pSFV1. The SpeI restriction site in the insert of the pRK34 plasmid encoding peroxidase-KDEL was removed by using in vitro mutagenesis. The peroxidase-KDEL gene was then excised with BamHI digestion and cloned into the vector pSFV1 like HA gene above. The cDNA encoding ts-G-GFP was excised from the pCB6 vector with BamHI digestion and also cloned into the pSFV1 vector.

4.4.1 Preparation of the recombinant viruses (II, III)

Preparation of the recombinant viruses was performed as described (Olkkonen et al. 1994). The RNA from linearized pSFV vector and the linearized pSFV helper plasmid were co-introduced into BHK-21 cells by electroporation. The SFV structural proteins encoded by the helper form virions that package the recombinant vector RNA and can be used for non-reproductive infection of other cells. Multiplicities of infections of the recSFV stocks were evaluated on BHK-21 cells by immunofluorescence staining. Infections at multiplicity of 1/nucleus were allowed to proceed for 16-20 h before metabolic labeling experiments.
### 4.4.2 Immunofluorescence studies. (I, II, III)

**Table 4. List of antibodies used in original publications (I-III)**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source/ reference</th>
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<td>FicG-conjugated goat anti mouseIgG</td>
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</table>
4.4.3 Conventional confocal microscopy (I, II, III)

Myoblasts, myotubes or myofibers on the cell culture dishes were fixed with 3% paraformaldehyde in PBS for 15 min. Cells were then permeabilized with 0.5% Triton X-100 in PBS. Non-specific binding was blocked with 30 min incubation in 1% BSA and 0.2% bovine gelatin in PBS. Primary antibodies were applied on the myofibers for 60 min at 37°C. After several washes secondary antibodies were applied for another 60 min incubation at 37°C.

In control samples the cells were left uninfected or the primary antibody was omitted. Stained samples were embedded in Mowiol 4-88 (Hoechst) with 2.5% 1,4-diazobicyclooctane as a fading inhibitor. Samples were examined with a Leica (Leica Lasertechnik, I), or Zeiss LSM510 (Carl Zeiss Inc.,II, III) confocal laser scanning microscope. Images were processed with Adobe Photoshop®.

4.4.4 Microscopy of living cells

The conventional method utilizing the temperature-sensitive tsO45-G-GFP was used. Myoblasts, myotubes or myofibers were cultured on glass-bottomed dishes (Willco) and infected with recSFVts-G-GFP. The virus was allowed to adsorb for 2h at 32°C. The infection medium was then replaced with the growth medium and the cells were transferred to 39°C. For blocking the recombinant GFP protein in the TGN the cells were further transferred from 39°C to 20°C for the last 2 hours of infection. L6 cells were monitored alive or fixed for immunofluorescence after a 16 h infection period while myofibers were infected for 24h before experiments.

Time-lapse imaging was done with a Zeiss confocal microscope equipped with an inverted x100 oil objective. The 488 nm line of an argon laser was used. Excitation and emission were monitored using a 505 nm long-pass filter. Samples were line scanned, and power settings were minimized to avoid photobleaching. The fluorescence images of 512x512 pixels were recorded every 4-8 seconds. Final figures were arranged with Adobe Photoshop 6.0.

4.5 Transmission electron microscopy (I, II, III)

In order to detect virally expressed peroxidase activity, myofibers were fixed with 2.5% glutaraldehyde for 1 h and subjected to diaminobenzidine (DAB) and H₂O₂. For immunoperoxidase staining of the TRAP, myofibers were fixed for 15 min with 3% paraformaldehyde in PBS. The cells were permeabilized with 0.5% saponin for 10 min and non-specific binding was blocked with a solution containing 1% BSA, 0.2% bovine gelatin and 0.1% saponin in PBS. Saponin, BSA and gelatin were also present in all subsequent washes and incubations. Incubations with primary and secondary antibodies
lasted 60 min at 37°C. The myofibers were then fixed with 2.5% glutaraldehyde for 60 min at room temperature and peroxidase activity in the cells was visualized by DAB reaction. The cells were postfixed with 1% OsO₄ for 60 min, dehydrated in a graded series of ethanol and embedded in Epon.

For pre-embedding immunogold labeling we modified the method described by Ploug et al. (1998). Accordingly, myofibers were treated as stated above, but aldehyde-groups left by the fixative were also blocked with PBS containing 50 mM glycine and 0.1% saponin. Furthermore 0.2% bovine gelatin in the blocking solution was replaced with 0.1% coldwater fish gelatin. After incubation with anti-peroxidase antibodies the cells were washed properly and incubated overnight at 4°C with anti-rabbit F(ab')₂ IgG conjugated to 1.4nm gold particles. After several 30 min washes, myofibers were fixed with 2.5% glutaraldehyde in PBS for 60 min at room temperature and gold particles in the samples were enhanced for 2 min with HQ Silver (Nanoprobes Inc.). The myofibers were then postfixed with 0.5% OsO₄ for 30 min, dehydrated in a graded series of ethanol and embedded in Epon.

Rotavirus infected myofibers were fixed with 2.5% glutaraldehyde in PBS for 60 min, postfixed with 1% OsO₄, dehydrated in a graded series of ethanol and embedded in Epon.

All EM samples were cut to 100 nm sections, poststained with aqueous lead citrate (rotavirus infected samples also with uranyl acetate), and observed in a Philips CM100 TEM.
5 Results

5.1 Characterization of endocytic pathways (I)

To study endocytosis and endocytic compartments the cultured myofibers were incubated with well-characterized endocytic probes, which can be applied and removed according to the requirements of the experiment. With this method we examined the initial site of internalization, and routing of internalized markers along the endocytic pathway. The organization of the sorting endosomes, recycling endosomes, and lysosomes was also studied with respect to the internal architecture of the muscle fiber and to the microtubule network in the fibers. Visualization of the probes was done either by immunofluorescent staining for confocal microscopy or by DAB reaction for electron microscopy.

5.1.1 Location of the endocytic compartments

The distribution of early sorting endosomes was studied using the well-characterized fluid-phase markers FITC-dextran and horseradish peroxidase (HRP). Fluorescent spots were found just beneath the sarcolemma and in smaller amounts also in the interior of the fibers (I, Fig.1). The fluorescence results were verified by localizing the peroxidase activity in HRP incubated myofibers by electron microscopy. Peroxidase precipitation was seen in 150-180 nm diameter vesicles just beneath the sarcolemma and flanking Z lines. The distribution of the peroxidase-containing structures was restricted within the I band area where they were concentrated at the A-I junctions. In contrast to the distribution of coated pits no labeled objects were seen at the A band area. No peroxidase reaction was seen in caveola structures. In the interior regions peroxidase positive vesicular and tubulovesicular structures, typical of sorting endosomes, were found in the vicinity of T-tubules (I, Fig.1). These findings indicate that early sorting endosomes concentrate at the I band areas of the sarcolemma.

Late endocytic compartments were characterized using the same fluid-phase markers and LDL-bodipy, which is transported to the late endosomal compartments. After
internalization and a 1 h chase period the marker proteins were perinuclearly located in about 70% of the cases, while the rest showed no association with the nuclear localization (I, Fig.2). Fluid phase markers were found to gather around the junctional nuclei in the NMJ as well as in dot-like structures in the MTJ. LDL-bodipy did not penetrate into the T-tubules but was found strictly following the course of microtubules. Lysosome-directed proteins were transported into perinuclear and microtubule contacting interfibrillar dots. Taken together, late endocytic compartments exhibit perinuclear and interfibrillar localization along microtubules.

Transferrin uptake was analyzed to examine the endosomal recycling pathway. The recycling compartment distributed in the perinuclear areas and in the interfibrillar spaces, where it showed longitudinal rows of spots following the course of microtubules. Transferrin also showed partial colocalization with GLUT-4 (I, Fig.5), which is known to be translocated from its intracellular storage compartment to the cell surface. Simultaneous incubation with LDL-bodipy and transferrin showed that early and late compartments are morphologically distinct (I, Fig.6).

5.1.2 Endocytosing areas of myofiber surface (I)

To determine where endocytosis occurred in the myofibers we analyzed the localization of coated pits by electron microscopy. The zone closest to M line was found essentially depleted of coated pits when compared to other zones, indicating that coated pits are distributed in a cross-striated fashion. The I band areas were active in endocytosis along the whole length of the fiber. The concentration of coated pits in myofibers was found to be similar to that in fibroblasts, occupying approximately 1.4% of the surface area. Also immunolocalization of the TfR expressed from a recombinant SFV gave similar results.

5.2 Characterization of the relationship between ER and SR (II, III)

5.2.1 Myogenesis causes differentiation in the efficiency of Golgi processing (II)

5.2.1.1 Differences in processing of viral glycoproteins

Rahkila and coworkers (1998) have reported that during the differentiation of L6 myoblasts into myotubes about half of the VSV G protein was diverted from the export pathway through the Golgi apparatus, but the WSN influenza virus HA did not change its behavior in this respect. We analyzed the behavior of some other glycoproteins during the differentiation of L6 cells. Infected myofibers were pulse-chase labeled and viral glycoproteins in the myofibers analyzed for their Endo H sensitivity. The decrease in
Golgi processing efficiency during the differentiation of L6 myoblasts into myotubes was found to vary according to the marker glycoprotein (II, Table 1).

In the cases of influenza and Sendai virus glycoproteins the transport from the ER to the Golgi apparatus was impaired only slightly during myogenesis. 96% of the Sendai F protein in myoblasts and 82% in myotubes acquired Endo-H resistance. Influenza X-31 HA and Japan strain HA were analyzed since their structures differ from that of the WSN HA. During influenza X-31 infection a major fraction of the HA acquired Endo-H resistance. In myoblasts 84% of the Japan HA acquired Endo-H resistance, whereas in myotubes 75% became resistant (II, Fig.1). The SFV glycoprotein that first appears as a p62/E1 glycoprotein complex in the ER, was also investigated. In myoblasts the E1 subunit acquired almost full Endo-H resistance but only partial (53%) resistance was acquired in myotubes. Furthermore, almost all of the p62 was cleaved in myoblasts but not in myotubes (II, Fig.2). DTT treatment and SDS-PAGE in non-reducing conditions were used to verify that defective folding was not the cause that prevented the arrival of the SFV glycoprotein to the Golgi apparatus.

5.2.1.2 Escape of viral glycoproteins from the Golgi recycling in the myotubes

In order to examine whether the non-processed glycoproteins were in the pathway from the ER to the Golgi apparatus we used pulse-chase labeling in the presence of BFA. In BFA-treated cells Golgi apparatus disappears and its proteins end up in the ER (Doms et al. 1989). The viral glycoproteins that partly lost their Golgi processing during myogenesis did not fully acquire Endo-H resistance in the myotubes even in the presence of BFA (II, Fig. 3A, D), meaning that the non-processed fraction did not remain in the ER, though it did not travel to the Golgi apparatus either.

The mutant VSV tsO45 G protein should be retained in the ER at 39°C due to a folding defect and therefore be fully accessible to the enzymes of the Golgi only in the presence of BFA. In contrast to the situation without BFA, major fraction of the tsO45 G protein did acquire Endo-H resistance upon chasing in the presence of the drug, indicating that processing by the enzymes of the Golgi apparatus really happened (II, Fig. 3B). For comparison, the effect of BFA on a protein that sustains its maturing capability during myogenesis was also tested. The HA of the WSN strain influenza virus is expected to be accessible to the Golgi enzymes also in the myotubes. Accordingly, in the presence of BFA, in WSN infected cells the mobility of the Endo-H treated HA did become gradually smaller during chase both in myoblasts and in myotubes, indicating accessibility to Golgi enzymes (II, Fig. 3C).

We infected myotubes with a recombinant virus, which expressed a glycosylated fusion protein containing peroxidase and the KDEL motif (recSFV-peroxidase-KDEL) to verify that BFA actually recalled the enzymes of the Golgi apparatus to the ER in the multinucleated myotubes. Immunofluorescence studies showed that the virally synthesized peroxidase-KDEL protein colocalized with the endogenous ER marker BiP, and EM studies showed that the synthesized protein localized to the ER (II, Fig. 4). The
pulse-chase labeling experiments showed that the presence of BFA caused changes also in the mobility pattern of the KDEL-tagged peroxidase indicating contact to the Golgi enzymes. No mobility shift occurred in the absence of BFA.

5.2.1.3 Transport block at 20°C reveals a compartment that is distinct from the TGN but colocalizes with calsequestrin

The tsO45 G protein was used to investigate the localization of the G protein that did not reach the Golgi complex in myotubes. A 20°C transport block should retain the synthesized proteins in the TGN. Immunofluorescent staining of infected cells showed a peripheral granular staining pattern that was distinct from the staining pattern representing Golgi stacks and weaker in intensity. This staining component showed a rather poor colocalization with PDI that marks the ER. The results indicate that, during a 20°C transport block, a fraction of the G protein in myotubes was neither retained in the ER nor transported to the Golgi apparatus but remained diffusely distributed (II, Fig. 5A-F).

Since L6 myotubes do not express CLQ that marks the SR in adult myofibers we utilized primary rat myotubes to explore whether the G protein fraction that did not move to the trans-Golgi network showed colocalization with SR proteins. Primary myotubes behaved like L6 myotubes when they were infected with VSV. As with L6 myotubes, immunofluorescence labeling for the G protein indicated prominent spots representing Golgi stacks and a peripheral diffuse labeling that was more intense than in L6 myotubes. Double staining with CLQ and VSVG antibodies indicated considerable colocalization of the two labels (II, Fig 5G-I).

5.2.1.4 Cytoplasmic tail or raft associations are not the reason for changes in transport efficiency in myotubes

The cytoplasmic tail might contain information for the transport modulation occurring during myogenesis. We investigated whether this is the case with recombinant Vesicular stomatitis viruses whose G protein either lacked the cytoplasmic tail (CT1), or possessed a truncated tail with nine proximal amino acids left (CT9). We found that deletion of the cytoplasmic tail did not improve exit from the ER in myotubes. Recombinant VSV G proteins whose cytoplasmic tail (GGC), or the cytoplasmic tail plus the membrane anchor (GCC), were replaced with the corresponding portions of the CD4 protein, were also analyzed. In myoblasts these recombinant G proteins were fully processed within one hour. In myotubes only about half of these G proteins underwent processing, and they thus behaved like the wild type G protein (II, Fig. 6A-B).

Velocity sedimentation studies for the CT1, CT9, and GGC recombinants in sucrose density gradients showed trimeric oligomerization, excluding the possibility that
extensive folding defects inhibited the oligomerization and in that way caused the behavior of these proteins. Furthermore, we investigated whether the ratio between the processed and non-processed forms varied according to the expression level. Both forms were in balance during early infection when endogenous protein synthesis was still going on, although the mature form prevailed after a 3 h infection period. Wild type VSV infection gave similar results (II, Fig 6 C-D).

The WSN HA protein and the Sendai F protein are efficiently processed in the myotube Golgi apparatus. These proteins are apically targeted and presumed to associate with glycolipid-enriched rafts (Simons & Ikonen 1997). Raft association might therefore have a sorting role in myotubes. While about 40% of the wild type HA remained insoluble in Triton X-100 at 4°C in these cells, only 15% of the modified Japan strain HA, that is known to be a non-raft protein (Simons & Ikonen 1997), was insoluble, indicating that rafts were present in the myotubes. We did not find any significant difference between the modified and wild type HA when we compared their processing in the Golgi apparatus during a pulse-chase experiment. The conclusion was that in the myotubes the cytoplasmic tail or raft association did not dictate the efficiency of the transport of the viral glycoproteins from the ER to the Golgi apparatus (II, Fig.7).

5.2.2 Localization of the RER in myofibers (III)

5.2.2.1 ER and SR marker proteins show different labeling patterns

We compared the staining patterns displayed by various ER markers to those of the SR markers CLQ, which locates to the terminal cisternae of the SR, and SERCA, which has a relatively homogeneous distribution in the entire SR. Previous studies have shown that components of the folding machinery of the ER such as BiP or PDI localize to the perinuclear areas and to the interfibrillar spaces. The interfibrillar localization is responsible for the cross-striated staining pattern seen with these markers (Volpe et al. 1992; Rahkila et al. 1997). The staining patterns of the luminal ER markers BiP and PDI as well as integral membrane protein ribI or TRAP distributed over the entire I band areas. The patterns did resemble each other but looked different from those of CLQ or SERCA. CLQ showed regularly arranged double rows of large dots, while the ER markers showed smaller irregularly arranged spots. Also the staining pattern of SERCA essentially differed from those of the ER markers. It showed a thin concentrated line, locating on the Z line, in the middle of fuzzy cross-striations (III, Fig.1).
5.2.2.2 Blocked cargo proteins show different labeling patterns

Several viral proteins that are known to have different exporting efficiencies in L6 myoblasts and myotubes (II) were used as model cargo proteins to locate the folding compartments in myofibers.

We infected myofibers with recSFVs encoding variants of the mutant tsO45 G protein that carry GFP fused to their cytoplasmic tail (Scales et al. 1997). Surprisingly we found out that the ts-G-GFP\textsubscript{ct}, expressed from the recSFVs-G-GFP\textsubscript{ct}, marked Z line areas more intensely than the A-I junctions. In contrast the tsO45 G protein at 39°C preferentially localized to the A-I junctional areas, corresponding to the terminal cisternae of the SR. A double staining for CLQ and ts045 G protein indicated partial but not full colocalization (III, Fig. 2). When the temperature was shifted from 39°C to 20°C, at which temperature exported proteins should accumulate in the TGN, both the tsO45 G protein and the ts-G-GFP\textsubscript{ct} displayed a pattern of bright spots around the nuclei and in longitudinal rows, which is a typical Golgi staining pattern.

The transport efficiencies of these two G protein variants were studied at the permissive temperature of 32°C, and Endo H digestion was used to quantitate the amount of the proteins that is accessible to the Golgi enzymes. In the myofibers the processing efficiencies of the two markers were not significantly different. The results obtained by similar analysis using L6 myoblasts and L6 myotubes suggest that ts-G-GFP\textsubscript{ct} fairly well retained its Golgi trafficking during the myogenic differentiation process, but half of the tsO45 G protein was diverted from the route to the Golgi elements. The exporting ER, which was visualized by blocking ts-G-GFP\textsubscript{ct} in the ER, seemed to be flanking the Z lines. We concluded that the protein fraction localizing to the A-I junctions might represent the G protein that is diverted from the Golgi pathway.

The TfRs are normally transported to the sarcolemma. The localization of TfR was determined in the presence of BFA to further examine the localization of the ER elements that undergo recycling with the Golgi apparatus. In myofibers infected with a recSFV-TfR the TfRs were clearly concentrated over the Z lines and around the myonuclei in the presence of BFA. Without the drug, the staining pattern was different, apparently reflecting the entire exocytic pathway (III, Fig. 4A). Sendai virus F glycoprotein is efficiently exported from the ER in L6 muscle cells and normally exocytosed (II). In the presence of BFA Sendai-infected myofibers showed a prominent perinuclear and Z line-flanking staining pattern. Without the drug this labeling pattern was not seen, but the Z line staining disappeared and a typical Golgi staining pattern appeared (III, Fig. 4B). When all the VSV G protein or SFV glycoprotein were retained in the myofiber ER by means of BFA, no prominent Z line component was seen in contrast to the results obtained with TfR or Sendai F protein. The VSV and SFV proteins showed staining distributing over the whole I band areas instead. A perinuclear staining was also seen but it was but rather weak (III, Fig. C-D). The staining pattern of CLQ remained unaltered after a 24 h BFA treatment, indicating that the drug did not affect the integrity of the SR. We did not find any ultrastructural changes in the organization of the SR after BFA treatment either.
5.2.2.3 Distribution of the ER exit site marker sec23

Proteins that are exported efficiently seemed to localize around the myonuclei and over the Z lines when they are blocked in the ER. In mononucleated cells COPII coat complexes specifically mark the exit sites where export from the ER occurs (Bannykh et al. 1996), and in muscle cells the exit sites are known to locate in the vicinity of the Golgi elements (Lu et al. 2001; Ralston et al. 2001). We therefore determined the localization of the COPII component sec23 in myofibers. In addition to the expected Golgi spots we also found distinct cross striations showing a Z line-associated component for sec23. A double staining for sec23 and the SR markers CLQ or SERCA showed very little colocalization and indicated that there are no exit sites in the terminal cisternae (III, Fig. 5).

5.2.2.4 Electron microscopic confirmation of the confocal data

We used electron microscopy to investigate the organization of the ER in myofibers at the ultrastructural level. The electron microscopic data verified the idea that interfibrillar RER structures locate in the terminal cisternae and over the Z lines indicating that the distribution of the classical ER markers in myofibers is different from that of the SR. Again we used viral infections to obtain higher expression levels of the studied proteins and to thus facilitate detection. We determined the effectiveness of the infection with immunofluorescence staining.

Interestingly, rotavirions that are known to bud in the ER were found in membrane-bound structures but not within identifiable SR membranes, suggesting that budding occurred into membranes that were distinct from the SR membranes (Fig 4A). Next, we used ss-peroxidaseKDEL as a marker, which we expected to be retrieved into the supposed exporting ER fraction. The peroxidase was, however, found both within the terminal cisternae as well as in structures that were distinct of the cisternae. It is likely that the ss-peroxidaseKDEL was translocated both into the exporting as well as the non-exporting ER compartments, meaning that we were probably not looking exclusively at the compartment where export sites are situated.

Since myofibers seemed to contain relatively high endogeneous peroxidase activity, we decided to verify the results obtained with the DAB reaction by visualizing the ss-peroxidaseKDEL with nanogold immunolabeling and silver enhancement procedure. In the infected myofibers, both methods revealed positive staining in small tubular structures interestingly intermingled within the longitudinal SR and contacting the terminal cisternae. The structures were concentrated at I band areas (III, Fig. 6A-F; thesis, Fig. 4B,F). BFA did not change the staining pattern. In most cases a clear-cut contact with the SR membranes could not be detected. Those structures that contacted the terminal cisternae of the SR never filled the entire cisternal space and mainly appeared to be facing the limiting border in the cisternal lumen. The staining was specific, since in non-infected myofibers only marginal background of silver-coated particles or cytoplasmic DAB precipitates were occasionally seen.
Experiments to locate the endogeneous TRAP also showed results similar to those obtained with ss-peroxidase<sub>KDEL</sub> (III, Fig. 6G-I; thesis, Fig. 4D). The exported cargo protein ts-G-GFP<sub>ct</sub> which accumulates in the ER at the non-permissive temperature of 39°C, was also studied at the EM level. Anti VSV G antibodies visualized with silver enhanced nanogold showed immunolabeling in structures over Z lines and to a lesser amount over M lines, but the terminal cisternae remained unstained (III, Fig. 6J).

Fig. 4. Examples of the collected data of the electron microscopic localization of the ER in muscle cells. (A) In myofibers rotavirions bud into membranes that are distinct from the SR membranes. (B) Nanogold staining shows ss-peroxidase<sub>KDEL</sub> locating at the terminal cisternae (arrows) or on the Z lines (arrowheads) of a myofiber. Endogenous TRAP locates to the perinuclear area in myoblasts (C) as well as in myofibers (D). The ss-peroxidase<sub>KDEL</sub> visualized by DAB reaction stains ER in myotubes (E) and terminal cisternae (arrows) and Z line flanking structures (arrowheads) in myofibers.
5.3 Characterization of the export pathway

In order to compare membrane trafficking in various developmental stages of skeletal muscle, we infected L6 myoblasts, L6 myotubes and adult rat myofibers with recSFV-ts-G-GFP, which was used as a tool to visualize export pathways in fixed and living muscle cells. In myoblasts and myotubes the trafficking of ts-G-GFP seemed to be similar to what has been reported recently for other cell types. The temperature block worked well and we could detect the GFP-tagged G-protein in the ER at 39°C (Fig.5, A-C), in the Golgi apparatus at 20°C (Fig.5, D-F), and at the sarcolemma in the permissive temperature of 32°C (not shown), as verified with immunostainings of fixed infected cells. In fully differentiated skeletal myofibers, though, protein trafficking on the export pathway seemed to be arranged differently compared to mononucleated cells.

5.3.1 Pre-Golgi trafficking

We followed the pre-Golgi membrane trafficking in living cells by time-lapse imaging after releasing the 39°C temperature block. In the L6 myoblasts and myotubes numerous moving vesicular and tubular carriers were seen. Fig 6 shows examples of the detected traffic. Especially in the myoblasts the carriers moved towards the centrally situated Golgi apparatus. In the myotubes the movement of the carriers was more random. In both myoblasts and myotubes the carriers rapidly moved long distances along linear paths suggesting that microtubules were involved. The carriers regularly merged with the Golgi elements.

In mature living myofibers, however, the trafficking of the GFP-tagged chimera was very weak compared to that in myoblasts or myotubes. In these experiments the confocal plane was adjusted just beneath the plasma membrane since most of the Golgi elements, which are expected to be targets of the postulated transport carriers, resided at this plane. Also the great majority of microtubules have been shown to locate just beneath the sarcolemma (Boudriau et al. 1993; Rahkila et al. 1997). Since transport carriers are transported along the microtubules in other cell types, this is expected to be the case in myofibers as well. We did observe that the Golgi elements in the confocal plane studied rapidly increased in fluorescent intensity. However, moving vesicles or transport containers were seen only in about 20 % of infected cells, and even in these cells the number of detected vesicles was much smaller than in myoblasts or myotubes. Furthermore the direction of the traffic seemed to be preferably transverse. This indicates that in myofibers the transport of proteins from the ER to the Golgi elements seemed not to occur in detectable transport containers along longitudinal microtubules.
Fig. 5. The ts-G-GFP fluorescence overlapped with that of the endogenous ER marker PDI at 39°C. In myoblasts (A), myotubes (B) and myofibers (C) green fluorescence shows that the cells were infected with recSFVts-G-GFP. In the merged images on the right, the yellow color indicates colocalization with PDI. At 20°C ts-G-GFP accumulated in the Golgi apparatus. RecSFVts-G-GFP infected cells were stained with antibodies against the Golgi marker GM130 (red). In myoblasts (D), myotubes (E) and myofibers (F) the GM130 staining overlapped with green GFP spots indicating colocalization. Bars 10 µm
Fig. 6. Examples of detected ts-G-GFP trafficking in muscle cells. Time-lapse imaging of living cells was performed in a Zeiss confocal microscope as indicated in Methods. In A, B and C the temperature was shifted from 39°C to 20°C 5 min before the first image of each row. The images of myoblasts (A) were recorded with a 6 s interval, while the interval between myotube images (B) was 15 s. In myofibers the frequency of detected traffic was less than 1% of the traffic in myoblasts. The pre-Golgi traffic seemed to prefer transverse direction (C) while the post-Golgi traffic, visualized by the temperature change from 20°C to 32°C, seemed to follow longitudinal microtubule tracks more often (D). The interval between the myofiber images shown (C,D) was 6 s.

5.3.2 Post-Golgi trafficking

Post-Golgi trafficking was analyzed in living recSFV-ts-G-GFP infected muscle cells. After the growth period at 39°C the cells were incubated at 20°C for 2h and then the temperature was shifted to 32°C causing the synthesized proteins to travel from the TGN to the plasma membrane. In the myoblasts and the myotubes we found large amounts of migrating tubular and vesicular carriers. Anti VSV antibodies added to the culture
medium clearly stained the plasma membrane verifying that the carriers really ended up to the plasma membrane. Also in the myofibers post-Golgi trafficking seemed to use longitudinal microtubule tracts in contrast to the pre-Golgi trafficking. When the temperature of the infected myofiber culture was raised from the 20°C to 32°C, shuttling transport containers were clearly detected. The destination of these carriers could not be verified in the time period studied. The post-Golgi traffic was more intense than the pre-Golgi traffic but yet sparser than in myoblasts or myotubes.
6 Discussion

In this study we have characterized vesicle trafficking in isolated fully differentiated muscle fibers. The unique experimental system has allowed us to define the organization of the endocytic pathways, to use enveloped viruses as a tool in analyzing protein transport routes, and finally to examine exocytic routes in living fibers. The use of single isolated myofibers has given us the possibility to view the whole fiber from one end to the other. This method also shows us the cells in a situation that resembles "real life" much better than ordinary cell cultures. A possible limitation of the experimental system is, though, that during the isolation procedure the myofibers become denervated. Denervation has been shown to affect muscle cell functions such as glucose uptake (Turinsky et al. 1998), or microtubule and Golgi orientation (Ralston et al. 1999). Myoblast and myotube cultures were mainly used to test and adjust different methods suitable for muscle cells. Experiments with these cells also clearly indicated that certain changes in protein maturation and targeting took place during myogenesis. The results are summarized in the schematic drawing (Fig. 7) and discussed on the following pages.
Fig. 7. Summary of the results. Black arrows show the endocytic pathway from the pits of sarcolemma to sorting endosomes (SE), recycling compartment (RC), and late endosomes (LE), and the location of those compartments in the mature skeletal myofiber. Grey arrows show the exocytic pathway. The stars represent the location of the exporting RER, while the spheres show the location of the un-exporting RER compartment.

6.1 Organization of endocytic and exocytic trafficking in skeletal myofibers

6.1.1 Characterization of endocytosis

The fully differentiated skeletal muscle cells are enormous and apparently much slower or more inactive at least in some respects when compared to mononucleated cells. This was evident also in our studies on endocytosis, since the uptake of solutes and ligands in skeletal myofibers appeared relatively inefficient compared to the satellite cells that usually contaminate the myofiber cultures. Our results show that endocytosis occurs in
the entire muscle fiber. We were able to locate coated pits distributed in cross-striated fashion over the whole length of the myofiber. In spite of high degree of differentiation of the cell, the density of coated pits, like also for example the amount of Golgi elements per nucleus (Ralston et al. 2001), was comparable to that in fibroblasts. We could not verify whether the T-tubules contained coated pits because of the dense filamentous network surrounding them. The location of transferrin receptors (TfR) was found to coincide with that of coated pits occupying I band areas and leaving M lines clear. Interestingly, also in fibroblasts coated pits tended to form discrete sites while excluding others. The spatial regulation of coated pits in fibroblasts is caused by interactions with the actin cytoskeleton (Gaidarov et al. 1999). Therefore also in muscle fibers the membrane skeleton probably dictates the cross-striated distribution of coated pits.

The late endosomal compartments were visualized using both fluid phase markers and LDL-bodipy. The distribution of these markers indicated that the late endosomes and the lysosomes localized predominantly in perinuclear and interfibrillar regions beneath the sarcolemma. Fluid phase markers indicated, however, that parts of these compartments also localized to the interior portions of the myofibers. When we analyzed the early sorting endosome compartment the internalized fluid-phase markers were found mainly just beneath the sarcolemma within the I band areas. The few solute-containing vesicles seen in the interior parts of the fiber had possibly been transported there from the surface of the fiber. Another explanation is that T-tubules mediated internalization, though they are not generally thought as sites of endocytosis. We found that LDL-bodipy, which did not penetrate into T-tubules, was not seen in fiber interior. This finding supports the possibility that endocytosis takes place also in the T-tubules. The fact that amphiphysin II, which very likely functions in the formation endocytic vesicles (Takei & Haucke 2001), is in muscle cells expressed as a peripheral T-tubule membrane protein (Butler et al. 1997), also supports this possibility. Furthermore, GLUT4, which is recruited to and reinternalized via coated pits in adipocytes, localizes to the sarcolemma as well as the T-tubules (Ploug et al. 1998).

The recycling compartment was localized with human transferrin. Our unpublished results show that for some unknown reason transferrin uptake collapses during myogenesis, and this was noticeable also in these experiments. The signal of the fluorescent transferrin tracer remained low, the uptake levels of transferrin being surprisingly low in myofibers compared to satellite cells. Double immunostaining showed that the perinuclear components and dispersed spots of transferrin considerably overlapped with GLUT4, suggesting that the recycling and GLUT4 compartments are partially identical. Partial overlap of GLUT4 and the endogeneous TfR has been reported earlier (Ploug et al. 1998). The data showing TfR distributed in clathrin-coated pits as well as in caveolae, and the kinetic and endosomal distribution data on fluid-phase cargo has been confirmed by Soeiro and coworkers (2002) in cardiac muscle cells.

We found that the recycling and the late endosomal/lysosomal compartments followed the course of microtubules in skeletal myofibers. In these highly differentiated cells individual microtubules form belts just beneath the sarcolemma at the A-I junctions and microtubule bundles encircle each nucleus and travel longitudinally between the nuclei in selected interfibrillar spaces (Boudriaia et al. 1993). It is likely that also in myofibers the early as well as late endosomes travel along microtubule tracks, since in mononucleated cells dynamic interactions of endosomes with the cytoskeleton have been demonstrated.
6.1.2 Characterization of the export pathway showed differences in vesicle transport appearing during myogenesis

In L6 myoblasts and in partially differentiated L6 myotubes and also in primary rat myotubes the transport of tsG-GFP seemed to be similar to the transport reported in other mononucleated cell types (Presley et al. 1997; Scales et al. 1997; Hirschberg et al. 1998). On the contrary, in mature skeletal myofibers similar trafficking of transport containers could not be detected. In the fully differentiated cells trafficking of vesicular or tubular structures was detected only in 20% of the infected cells, while every infected myoblast or myotube showed intense trafficking of corresponding structures. Besides that, even when observed, the frequency of the traffic was much lower in myofibers than in myoblasts or myotubes. We also detected a clear difference between pre- and post-Golgi trafficking in adult myofibers. Post-Golgi trafficking was a little more prominent and clearly used longitudinal microtubule tracts, while pre-Golgi trafficking was harder to detect and seemed to prefer transverse routing. Since detectable transport containers were seen so seldom in spite of the extensive microtubule network we conclude that in mature myofibers the organization of protein transport must be different from that in other cell types studied.

Myofibers are enormous when compared to mononucleated cells. It can be argued that we did not find the appropriate plane or were not able to keep it well enough. It is true that the size of the cells, but even more the temperature changes, did cause some technical problems. For this reason we used a thicker optical slice (1 µm). We also scanned through every possible plane near the myofiber surface where most of the Golgi elements are situated and the microtubule network is most prominent and easiest to detect (Rahkila et al. 1997), as the export vesicles are expected to travel along the microtubules (Lippincott-Schwartz et al. 2000). Furthermore, we found that muscle cells tolerated well the scanning conditions we used, since when traffic was detected it could easily be followed for at least an hour, although photobleaching did make very long recordings impracticable.

6.2 A novel transport route from the ER into a muscle-specific compartment developing during myogenesis

The results we obtained on the processing of viral glycoproteins showed alterations in the processing of different glycoproteins in the Golgi apparatus when the myoblasts differentiated into multinucleated myotubes. Proteins acquire Endo H resistance when they travel through the Golgi apparatus. The changes in Endo-H resistance showed that
the proteins that we analyzed were processed with differing efficiencies. These results most likely indicate impaired export from the ER to the Golgi apparatus. It can be argued that the use of viral vectors caused overexpression, which led to inefficient transport to the Golgi apparatus because the transport capability was exceeded. However, when we measured the ratio of processed and non-processed protein forms during early and late infections, we did not see significant differences. The wild type and the recombinant VSV G proteins as well as the SFV glycoprotein have also been shown to fold and oligomerize properly, though their transport dramatically dropped during myogenesis (Rahkila et al. 1998).

BFA is a frequently used drug that disrupts the Golgi elements and thus returns all proteins to the ER. We found that in the presence of BFA, VSV G protein was much less accessible to the Golgi enzymes in the myotubes than in the parental myoblasts, in which the G protein became fully processed. On the contrary, a major fraction of the misfolded tsO45 G protein was found to be accessible to the enzymes of the Golgi apparatus in the presence of BFA also in myotubes. The drug also revealed a dramatic decrease that occurred during myogenesis in the Golgi processing of the SFV E1 protein. These findings suggest that a fraction of the VSV and SFV glycoproteins were transported from the ER into a compartment that did not recycle with the Golgi elements. Experiments with the mutant VSV tsO45 G protein during a 20°C block suggested that this new compartment, in which non-processed proteins were targeted, was diffusively distributed but most likely different from the rough ER. Earlier gradient centrifugation studies have shown that during chase both the processed and non-processed G proteins shifted into membrane fractions that have lower density than the rough ER (Kellokumpu et al. 1997). In primary cultured myotubes the non-exporting compartment, which can be identified with the VSV G protein fraction that escapes Golgi processing, showed remarkable colocalization with the SR marker CLQ. Interestingly, it has been shown (Gatti et al. 1997), that CLQ is not transported through the Golgi apparatus in muscle cells. Instead, it was shown to be transported from the ER into separate vacuoles. The recent report concerning ankyrin that is required for targeting of functionally defined proteins also supports this possibility. Ankyrin seems to be somehow involved in the targeting of SR proteins, since ankyrin knockout was found to disrupt SR structure in adult myofibers (Tuvia et al. 1999). While exchange of proteins between the ER and Golgi apparatus is well characterized, in differentiated muscle cells there seems to be a parallel system to transfer proteins from the ER to a muscle-specific compartment. This compartment that appears during the myogenesis of the L6 muscle cells might represent nascent SR but this remains to be resolved.

When we studied the location of the ER in mature myofibers by retarding proteins in the ER with BFA, we found that those protein markers that retained their efficient export after myogenesis (TfR, Sendai F protein), preferentially labeled perinuclear areas and Z lines. On the contrary, the markers that lost their efficient export during the myogenic differentiation process (VSV G protein, SFV glycoproteins) occupied both the Z lines and the A-I junctional areas. It should be noted though, that the marker proteins we have used were not completely exported or retarded. Therefore the patterns observed during blocking conditions just yielded an emphasis of staining to one of the supposed ER components. Also folding defective viral probes, used to locate the ER in mature myofibers, showed two different labeling patterns. These results confirm the idea of two
different ER compartments. Accordingly, in myofibers the ts-G-GFP<sub>ct</sub>, which is relatively efficiently exported, marks the exporting ER elements located to the Z lines and perinuclear regions, while a prominent fraction of the tsO45 G protein located to the A-I junctions, because it is apparently rerouted into a compartment that did not export. Also the finding that CLQ was partially secreted in myoblasts but the secretion decreased considerably during differentiation (Raichman <i>et al.</i> 1995), is compatible with this idea.

The viruses used do not normally infect muscle cells but the viral glycoproteins apparently contain recognizable sorting determinants. We studied which factors caused the diversion from the Golgi pathway in myotubes. The apical proteins we analyzed retained rather efficient passage through the Golgi apparatus during myogenensis, though there were differences between the markers. The apical signal at least in some cases is N-glycan (Benting <i>et al.</i> 1999), and therefore the role of glycans in the transport from the ER to the Golgi apparatus in myotubes cannot be excluded. Although rafts form already in the ER in yeast (Bagnat <i>et al.</i> 2000), the possibility that raft-association in muscle cells determines the efficiency of the ER export was excluded. It should be noted that changing of the entire cytoplasmic tail of the VSV G protein had no effect on ER export, suggesting that the cytoplasmic portion had no role in determining the choice between the Golgi apparatus and the postulated muscle specific route. It is possible that the proteins that are partially shunted to this route do not have specific targeting information for the export pathway in their luminal domain.

### 6.3 Relationship between the RER and the SR in skeletal myofibers

We used confocal and electron microscopic methods to study the localization of the RER and its relationship with the SR in rat fast twitch myofibers. The SR of skeletal muscle cells is regarded as a smooth subcompartment of the ER (Volpe <i>et al.</i> 1992), but the exact relationship between these membrane compartments has remained confused. ER and SR have even been suggested to be totally different organelles in cardiac myofibers. Mesaeli and coworkers (2001) hypothesized that SR Ca<sup>2+</sup> stores could be responsible for control of excitation-contraction coupling whereas the ER compartment may provide Ca<sup>2+</sup> for housekeeping and transcriptional functions.

The real nature of the SR remains a mystery. Is it really comparable to SER? SER is prominent in liver and endocrine cells but in cultured cells the amount of SER is very small. In liver cells the SER has known functions like lipid synthesis, detoxification and calcium storage. But SER is also suggested to be potentially capable of protein synthesis. Results by Black and coworkers (2002) showed that the SER of adrenal cells seems to be capable of binding ribosomes and have high oligosaccharyl transferase (OST) activity. They suggest that this protein synthesis could be directed to synthesis of membrane proteins involved in cholesterol and steroid synthesis that are targeted for particular domains in the ER. However, there is no reported export from the SER in myofibers. Cholesterol has recently been localized primarily to the SR rather than sarcolemmal or T tubule membranes (Clarke <i>et al.</i> 2000). Furthermore, our unpublished results indicated
that the SR fraction of skeletal myofibers was resistant to Triton X 100 extraction, suggesting a raft-like nature of the SR.

6.3.1 Compartmentalization of the ER

The ER of animal cells represents a patchwork of subregions that differ in structure, protein equipment, and motility (Baumann & Walz 2001). However, GFP diffusion studies of several groups (Cole et al. 1996; Subramanian & Meyer 1997; Dayel et al. 1999) indicate a continuous ER lumen. Suggested ER subcompartmentalization mechanisms have been either the interconnection of RER membrane proteins by a filamentous network that allows them to segregate into portions of the ER (Kreibich et al. 1978; Ivesa et al. 1992), or linkage of translocation proteins to the ribosomes, which then anchor them in their places and allow them to be localized (Vogel et al. 1990). Some proteins have been shown to be restricted to the RER, but results of some studies (Hanein et al. 1996; Kalies & Hartmann 1998), indicate that the translocon complex is not a permanent structure but is assembled de novo during the initiation of cotranslational translocation. Also according to Nikonov and coworkers (2002), segregation of the ER into rough and smooth domains involves dynamic association/dissociation of translocation complexes with relatively immobile polysomal arrays.

The subcellular distribution and the structural organization of the exit sites has been examined in detail by Bannykh and coworkers (1996), who found that vesicle budding does not occur randomly over the entire ER surface but is restricted to specialized areas. COP II coated buds localized at the tips of smooth tubules emerging from RER cisternae. Exit sites are prominent near the Golgi but are also found at the nuclear envelope and the cell periphery (Bannykh et al. 1996; Scales et al. 1997). ER exit sites may also determine the course of the microtubules rather than the reverse (Ralston et al. 2001). It is also likely that the static view provided by immunofluorescence of fixed fibers is insufficient to provide a full view of the organization of the highly dynamic microtubules.

The data presented in this study indicate that in myofibers the ER exit sites localize to Z lines but not to the terminal cisternae. It is possible that the RER located to the terminal cisternae and the exit sites located on the Z lines. The finding of two different folding compartments demonstrated by the two VSV G protein variants, however, disagrees with this model. Our results suggest that the ER elements that possess translocation, glycosylation and folding functions, but not export function, locate at the terminal cisternae. Perinuclear and Z line-flanking ER structures also seemed to contain these functions but, besides that, they recycled material with the Golgi apparatus as well.

6.3.2 Location of the RER in myofibers

There are aspects about RER protein localization that are still unresolved, in part because most experiments have been performed with fractionated liver. The question is how far
these findings can be generalized? Are RER proteins targeted to subregions in all cell types or only in some? RER proteins have been observed in ER by immuno EM (Hortsch et al. 1985; Vogel et al. 1990), but in these studies cells were severely perturbed before examination. In several cell types RER and general ER proteins are colocalized, but in neurons RER proteins were concentrated in the cell body, whereas general ER proteins were also found in neurites (Rolls et al. 2002). In these cells RER proteins diffused rapidly within the cell body, indicating that they are not localized by immobilization. Already in 1985 Slade and Severs suggested that it is possible that two categories of RER exist in cardiac myofibers, one concerned with synthesis of SR proteins and thus intercalated within the SR, and another concerned with the manufacture of other membrane proteins, which has no structural continuity with the SR. In this study we provide evidence for the existence of two discrete RER compartments in skeletal myofibers.

It has been considered that in myofibers membrane proteins are synthesized around the myonuclei (Antony et al. 1995), but our laboratory has previously shown that at least some viral glycoproteins are translocated into the interfibrillar membranes (Rahkila et al. 1996), indicating protein synthesis also in the periphery of the cell. These findings agree with the report of Horne and Hesketh (1990), who found that free ribosomes located mainly to the A bands according to immunostaining of the large subunits. Our findings also indicate that typical ER components including translocation, glycosylation, and folding protein markers show localizations that differ from those of the SR markers, such as calsequestrin or SERCA. These findings are compatible with the results published from other laboratories (Volpe et al. 1992; Raichman et al. 1995).

In addition to experiments with different viral probes, which showed different labeling patterns as described above, we used also some other methods to locate the RER. Antibodies against the sec23 protein, a component of the COPII that marks the ER exit sites (Yoshihisa et al. 1993), should not mark the supposed non-exporting ER compartment, which was identified with inefficiently maturing viral glycoproteins. The anti-sec23 antibodies labeled structures which colocalized with a Golgi marker, as is expected on the basis of the results of Ralston and coworkers (1999), who used anti-sec31 antibodies to localize ER exit sites. However, we found sec23 also at perinuclear regions and over the Z lines but not at the A-I junctions. We used also electron microscopy to investigate the organization of the ER and how it is related to the SR. The results verified the confocal data indicating that the distribution of the classical ER markers in myofibers is different from that of the SR. We used ss-peroxidase as a marker, which, against expectations, was translocated both into the exporting as well as the non-exporting ER structures showing staining both in the terminal cisternae and structures distinct from them. Experiments to locate the endogeneous TRAP also showed similar results. These findings are similar to those of Volpe et al. (1992), who found BiP distributed within the longitudinal SR and the terminal cisternae. Continuities with the supposed RER structures and the longitudinal SR were not regularly seen in the sections studied. Resolving this question requires further investigations.

Our finding of two separate RER compartments gives rise to speculations of possible sorting at mRNA level. Also RER seems to be organized into functional subregions that are specialized with respect to mRNA translation and/or posttranslational protein processing. Segregation of particular mRNAs to distinct areas seems to be especially
prominent in highly polarized cells such as muscle cells. This mRNA segregation may provide a mechanism for targeting and confining the encoded proteins to different cell regions or surface domains (Baumann & Walz 2001). In a review of intracellular distribution of mRNA in striated muscle fibers (Russell & Dix 1992) three possible mechanisms for mRNA distribution were suggested, namely, diffusion, cytoskeletal associations, or cotranslational assembly. This means that different mRNA localization patterns over the ER could be established and maintained by utilizing differences in mRNA lifetime, active transport of mRNA subsets to a defined area of the cell, or by specific anchoring of molecules that capture mRNA on the ER membrane (Baumann & Walz 2001). Evidence for the latter possibility has been provided by Wickham and coworkers (1999). They showed that double-stranded RNA-binding protein staufen (Stau), which cross-links cytoskeletal and RNA components, is localized to RER implicating this protein in the targeting of RNA to its site of translation. Stau-mRNA complexes are transported along the microtubules and then anchored to the RER. mRNA targeting has also been shown to take place in plant cells (Choi et al. 2000). Furthermore, it has been shown that in a muscle cell line CLQ accumulated into a pre-Golgi compartment that contained a subset but not all of the enzymes typical of the RER (Gatti et al. 1997). Although vesicular protein trafficking from ER to SR cannot be excluded, it can be hypothesized that the SR-specific proteins are synthesized within the terminal cisternae-associated RER, and then diffuse to the rest of the SR. This model postulates that sorting between exporting ER and SR occurs at the mRNA level. Furthermore, our own unpublished results showed that exchanging the expression vector leads to retargeting of expressed protein, which finding also is in line with the model of protein targeting at the mRNA level.
7 Conclusions

Our experiments were designed to characterize certain special features of vesicle trafficking in skeletal muscle fibers.

When the organization of the endocytic compartment and endocytic processes were characterized, we found that endocytosis occurred in the I band areas along the whole length of the myofibers as well as in the metabolically active junctional areas. Furthermore, while recycling and lysosome-directed pathways were different, they both occupied perinuclear areas and followed the course of microtubules in interfibrillar spaces.

We also provide evidence that during myogenesis different proteins were diverted with different efficiencies from the conventional export pathway through the Golgi apparatus. Our results support the idea that a novel transport route from the ER into a muscle-specific compartment, which is presumed to be SR, developed during the differentiation of myoblasts into myotubes. Furthermore, this study reveals RER structures that are unevenly distributed within the terminal cisternae in addition to structures that are distributed perinuclearly and over the Z lines. We propose that the latter ER structures, located around the myonuclei and over the Z-lines, are responsible for the ER-to-Golgi trafficking in myofibers. This means that exporting and non-exporting RER compartments seem to exist in adult myofibers, the non-exporting one probably feeding the SR.

The characterization of the export pathway showed that also the mode of protein transport changed during myogenesis. In adult skeletal myofibers the arrangement of protein trafficking seemed to be different from that in other cells.

These studies will provide an essential framework for more detailed studies of endocytosis and exocytosis in muscle cells and for example for studies of mechanisms behind diseases affecting muscle structure and function.
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