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The Differentiation of L6 Muscle Cell Line Induces Changes on Trafficking of Vesicular Stomatitis Virus Glycoprotein

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Pro Gradu Thesis

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CONTENTS

Abstract

Protein transport and targeting within a muscle cell is an extensively studied subject. However, the exact mechanisms of transport along the endocytotic pathway are inadequately understood, as well as the relations, origins and integrities of the organelles therein. Although recent advances in cell and molecular biology have shed new light on these subjects, much remains yet to be resolved.

The endocytotic transport pathway consist of endoplasmic reticulum (ER) - and sarcoplasmic reticulum (SR) which is thought to develop from the first; the Golgi apparatus or complex with its many subcompartments; and various transport vesicles. Stable and/or transient physical connections have also been suggested to exist between the structures. A multitude of studies have been conducted on the role of coated intermediate vesicles, microtubules and transient tubular structures in the transport of protein and/or structural elements of the various compartments. Trafficking of a vesicular stomatitis glycoprotein (VSV G) has been succesfully utilized in studying the changes in endocytotic transport pathway.

The targeting of proteins during their transit has also been prone to new knowledge lately. The targeting mechanisms from the ER to the Golgi and at the ER-SR boundary remain, however, elusive. It awaits to be firmly affirmed whether there are signals for exclusion and/or retention of proteins, or should a saturation-based model be used.

The transformation of a mononucleated muscle precursor cell to a part of multinucleated muscle fiber is a complex process termed differentiation. During the differentiation the cells are subjected to numerous changes in architecture, as well as in protein traffic. The deviation of VSV G protein from myoblastic into post-myoblastic transport route has been suggested to demarcate the birth of the SR. The differentiation process can be divided into several distinctive stages, the timing and sequence of which have been a matter of controversy for some years.

In the present study we suggest specifications onto the timetable and sequence of some differentiation steps. We find that differentiation-specific morphological changes in the

architecture of developing L6 myotubes, which have been thought to induce changes in protein traffic, do precede the determination step in the differentiation process. We further define the timing, and confine the spatial location of the partial digression of VSV G protein from the myoblastic transport route to a post-myoblastic one. With L6 cell line, in these conditions, the division of VSV G transport appears approximately one day after the induction of the differentiation process. Furthermore, the unprocessed G protein is concluded not to enter the medial Golgi, based on the results of endoglycosidase H-digestion. Here we also confirm the earlier findings of Rahkila and colleagues (1996), according to which only half of the G protein digresses at the specified timepoint.

1 Skeletal muscle cell

Mammals have four muscle types, one of which is called skeletal muscle. The cells of this muscle have a characteristic striated appearance, and they are responsible for practically all movements that are under voluntary control [Alberts et al 1994, 1171 - 1179]. Functionally, the skeletal muscle can be defined as the muscle cells attached to the skeleton [Berne and Levy 1993, 281 - 291]. The heart muscle cells resemble their skeletal counterpart in that they, too, have a striated appearance: these two cell types are consequtively called striated muscle cells. The other two muscle cell types are the smooth muscle cells and epithelia-residing myoepithelial cells which, in contrast, do not show striation [Alberts et al 1994, 1171 - 1179].

1.1 Myofiber

The size of a differentiated skeletal muscle cell can be as large as 2 or 3 cm in length, and 100 μm in diameter. Each fiber is surrounded by an endomysium, underneath of which lies a thin, elastic membrane called sarcolemma; this, in turn, consist of plasma membrane and basement membrane between which reside the satellite cells responsible for the growth and regeneration of the muscle [McArdle et al 1996, 315 - 317]. Other fiber constituents include the sarcoplasmic reticulum and tranverse tubules; mitokondria; protein transport machinery; subsarcolemmal cytoplasm; and myofibrils, that make up the structure responsible for contraction. Each skeletal muscle fiber is a syncytium, containing numerous nuclei within a single cytoplasm, as opposed to non-muscle and undifferentiated muscle cells. [Berne and Levy 1993, 281 - 291]. The structure of the multinucleated skeletal muscle cell is presented in *Figure 1*.

Formation of myofibers

The numerous changes in muscle cells during differentiation include fusion of mononucleated myoblasts to form multinucleated myotubes; reorganization of subcellular organelles, such as the Golgi apparatus [Tassin et al 1985]; and the assembly of new membrane systems, such as the sarcoplasmic reticulum (SR) and the tranverse (T-) tubule system [Flucher et al 1991]. During further development *in vivo*, nuclei migrate to the periphery of the cell, whereas newly formed myofibrils fill the center of the cell [Kelly and

Zacks 1969]. Multinucleated muscle cells are often referred to as muscle fibers due to their elongated appearance [Berne and Levy 1993, 281 - 291].

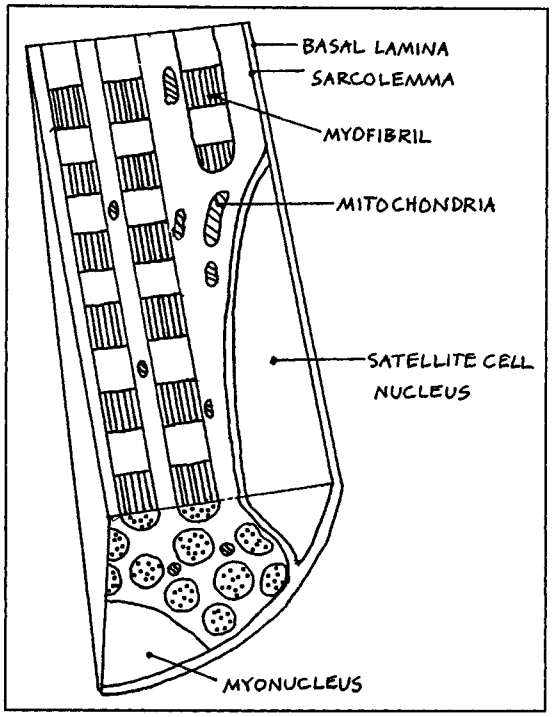


FIGURE 1: Intracellular structure of a differentiated skeletal muscle cell. The majority of the cytoplasm is filled with myofibrils, comprising the contracrile apparatus. Myonuclei are located in the periphery under the cell membrane sarcolemma. Satellite cells are situated between the sarcolemma and basal lamina that surrounds the cell. The figure is modified from Neville et al (1998).

1.1.1 Myofibril

The main component in muscle fiber cytoplasm is myofibril, the structure responsible for contraction. The characteristic striation pattern of a muscle fiber arises from the repeating pattern of these structures. Myofibrils are composed of thick filaments, which consist mainly of myosin, and thin filaments, whose main components are actin and tropomyosin. The thick and thin filament arrays form an overlapping lattice system, and the change in the architecture of repeating units called sarcomeres causes muscle contraction. [Berne and Levy 1993, 281 - 291]

1.1.2 Sarcoplasmic reticulum and transverse tubules.

Sarcoplasmic reticulum

The muscle specific sarcoplasmic reticulum (SR) is composed of structurally and biochemically distinct compartments that are involved in different aspects of calcium regulation. The longitudinal SR is responsible for calcium uptake into the SR whereas the

terminal cisternae are the calcium storage and release compartments forming junctions with the transverse tubules. [Flucher 1992]

Models of development for the SR

Two models of development have been proposed for the SR: it could gradually differentiate from the multifunctional endoplasmic reticulum (ER) [Boland et al 1974] or alternatively, the SR could be formed by the outgrowth of a distinct membrane structure from the ER [MacLennan et al 1978]. Both models for the biogenesis of the SR presume a continuity between the SR and the biosynthetically active portions of the ER [Flucher 1992]. This conclusion has regained support from other authors, as the SR seems to share components with the ER [Volpe et al 1992,Villa et al 1993]. It has, however been concluded that the ER and the SR are not identical [Rahkila et al 1996].

T-Tubules

The tranverse (T-) tubules are specialized invaginations of the plasma membrane, and they serve in the propagation of the action potential into the interior of the muscle fiber. T-tubules form so called triad junctions with the SR: these are the structures underlying the coupling of excitation and calcium release in skeletal muscle. T-tubules form simultaneously with the SR, but the mechanism of their formation remains unclear. [Flucher 1992]

1.2 Protein transport machinery

The transport of proteins from the ER to the Golgi and beyond has been subject to close scrutiny since the pioneering studies of Palade (1975), and it is thought to involve vesicular and/or tubular transport of proteins as well as transport of membrane constituents back to their organelles of origins. The precise mechanism of transport remains partly unresolved, but its various steps have been shown to involve coat proteins and/or transient tubular structures, microtubules and molecular engines. [Kreis et al 1995]

1.2.1 Endoplasmic reticulum

All eukaryotic cells have an endoplasmic reticulum (ER). Morphologically, the ER appears as an extensive membrane bound organelle composed of a network of branching tubules

and flattened sacs extending throughout the cytosol. The ER is the largest endomembrane system within eukaryotic cells and performs a wide variety of functions including calcium uptake and release, lipid and protein synthesis, protein translocation, folding, glycosylation, concentration and export to the Golgi complex. [Rose and Doms 1988, Hurtley and Helenius 1989, Sitia and Meldolesi 1992]

Compartments of ER

Traditionally, the ER is recoqnized to be composed of three morphologically distinct subcompartments; rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) are defined according to the presence of ribosomes, or lack of them, on the membrane region; and the nuclear envelope [Hobman et al 1998]. It has been proposed to further divide the ER into subcompartments that are distinct in terms of their protein constituents and/or morphological appearance [Sitia and Meldolesi 1992].

1.2.2 Transitional elements

Transitional elements of the ER [Palade 1975] can be identified by their characteristic part-rough/part-smooth appearance. This region of the ER is involved in the formation of transport vesicles that relocate cargo to the Golgi complex and can be further divided into two subdomains: 1) a domain enriched in protein components of COPII vesicles [Barlowe et al 1994]; and 2) a region with components of COPI (also called coatomer) vesicles [Orci et al 1991, 1994]. These vesicles play a crucial part in intracellular protein trafficking [Hobman et al 1998].

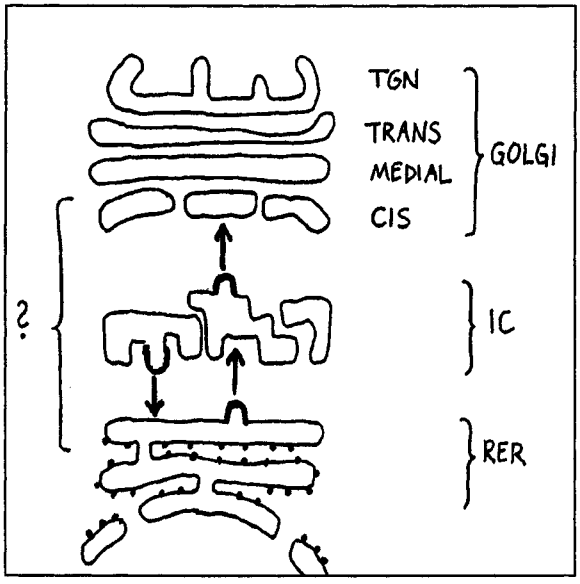


Figure 2: A schematic model of the relationship of the ER - transitional elements to rough ER (RER) and the Golgi. In this model, the transitional elements are a physically separated membrane structure, and two vesicular steps are required to transport proteins from the ER to the Golgi. Transitional elements are demarcated by a question mark, intermediate compartment indicated by IC and trans Golgi network by TGN. Figure modified from Hauri and Schweitzer (1992).

Naming of transitional elements

The precise distinction has been difficult to draw between the transitional elements (Palade 1975) and the so-called ER-Golgi intermediate compartment (ERGIC or IC; Saraste and Kuismanen 1984) or vesicular-tubular cluster (VTC; Balch et al 1994) located between the ER and Golgi apparatus. Other names have been suggested for this structure, among them the salvage compartment [Warren 1987] and SEREC (smooth ER exit compartment) [Hobman et al 1998]. Moreover, if one defines the VTCs as the first acceptor compartment for the vesicles budding from the ER, then there is little to distinguish this organelle from a definition of the *cis* elements of the Golgi complex. [Teasdale and Jackson 1996] Earlier, Mellman and Simons (1992) postulated that the VTCs found in the perinuclear region of the cell would form the *cis* Golgi network (CGN). Here, we use the terms VTC or IC, as these are the names most commonly attached to this structure in the literature.

Nature of the VTCs

Biochemically, the VTCs have been characterized as the site of N-acetylgalactosamine (GalNac) addition and palmitylation of proteins; and morphologically, they have been suggested to be identifiable by localization of the proteins p53, p58, and rab [Hauri and Schweizer 1992]. Based on morphological observations as well [Saraste and Kuismanen 1984, Bannykh et al 1996], it has been suggested that the VTCs lack continuity with the ER and may thus be a transient structure composed of transport vesicles fused with each other [Balch et al 1994]. According to Bannykh and Balch (1997), the reportings of continuity between the VTCs and the ER [Hobman et al 1992, Krinjse-Locker et al 1994] are conditional to viral infection of the cell. These authors further postulate that the markers that can be defined as resident proteins, are yet to be found, and emphasize that VTCs are a highly dynamic structure the composition of which remains to be firmly established.

Models for formation of the VTCs

At least two opposing models have been created for the formation and consumption of the VTCs. One model suggests that the VTCs are an unique compartment that contain a core of resident proteins distinct from those found in the ER or the Golgi. In the anterograde transport, according to this model, ER-derived COPII vesicles undergo heterotypic fusion

with the VTC [Bannykh and Balch 1997]. According to another model, tubular elements could form *de novo* from the homotypic fusion of ER-derived COPII vesicles. In this model, the VTCs would then move to the central Golgi region, where they would undergo further homotypic fusion with elements from other peripheral sites to form the CGN [Mellman and Simons 1992, Balch et al 1994]. During the transit to the central Golgi, COPI vesicles would direct recycling components back to the ER, thereby maintaining a steady-state balance between input and output of membrane. However, key evidence that COPII vesicles or the VTCs undergo homotypic fusion is missing. [Bannykh and Balch 1997]

1.2.3 Golgi apparatus

The Golgi apparatus or complex has been identified in mononucleated eukaryotic cell as several membrane-enclosed flattened cisternae held together as parallel stacks [Farquhar 1985]. Later, these structures have been found enriched in glycoprotein and glycolipid processing enzymes, with vesicles and tubules associated with the rims of stacks [Mellman and Simons 1992]. More refinedly, two tubular networks of membranes at either end of Golgi stacks have been identified; The *cis* Golgi network (CGN; Rambourg and Clermont 1990) functions as an acceptor compartment of newly synthesized material from the ER; and the *trans* Golgi network (TGN; Griffiths and Simons 1986), is recoqnized as the exit site for Golgi [Cole and Lippincott-Schwartz 1995]. Different regions of the Golgi can be identified by compartment-specific enzyme markers [Davidson and Balch 1992]. During myogenesis, the arrangement of the Golgi elements changes profoundly. [Holleran and Holzbaur 1998]

Lipids of the Golgi membrane

The Golgi is an intermediate between the ER and plasma membrane in terms of lipid composition. Because all models of intra-Golgi membrane traffic involve transient membrane continuities and contain an anterograde and a retrograde component [Mironov et al 1997, Pelham 1998], it has been concluded that the thin plasma membrane of the ER matures into the thick plasma membrane of the Golgi. [Van Meer 1998]

Functions of Golgi

The Golgi complex is responsible for the transport of proteins and lipids from the ER to downstream compartments (including lysosomes and the plasma membrane) and recycling of membrane components back to the ER. It is also involved in various biochemical processes (i.e. glycosylation of proteins and biosynthesis of lipids) that enable the cell to tailor its biosynthetic and secretory products for specific needs [Sciazy et al 1997]. However, there is no reliable way to predict the precise type of glycosylation that will be done onto a given glykoconjugate by the Golgi apparatus of given cell type [Van Meer 1998]. Numerous factors can dramatically alter the glycosylation potential of the Golgi [Varki 1998].

2 Myogenesis

New skeletal muscle cells form by the fusion of mononucleated precursor muscle cells called myoblasts. After having proliferated into sufficient confluence, the myoblasts fuse with one another or onto an existing structure, as is the case in a regenerating muscle, and undergo a dramatic change in phenotype to become multinucleated muscle cell [Olson 1992]. During the fusion process, intracellular reorganization occurs: the myofibrillar contractile apparatus develops together with an extensive intracellular membrane network consisting of the sarcoplasmic reticulum (SR) and the transverse (T-) tubulus system [Flucher 1992]. Furthermore, these formerly longitudinally orientated tubulovesicular structures are organized into a transverse cross-striated structures [Rahkila et al 1996], and the organizations of both the Golgi complex as well as the centrosome changes [Tassin et al 1985].

The complex event described above has been termed differentiation, or myogenesis. It is regulated by a battery of muscle specific genes, guided by a fibroblast-formed framework of connective tissue, involves cell-cell adhesion molecules and requires a matrix for the myoblasts to attach. Once fusion has occurred, the myoblasts never again enter the cell cycle. [Olson 1992] It has, however, been shown [Okazaki and Holtzer 1966] that cultured myoblasts prevented from fusing are able to express muscle specific genes and achieved some degree of sarcomeric organization.

2.1 Stages of differentiation

According to Andres and Walsh (1996), myogenic cell differentiation can be divided into at least four different stages: 1) entry of myoblasts into the differentiation pathway, termed as determination, as indicated by the expression of myogenin and/or MyoD; 2) irreversible cell cycle withdrawal, as indicated by the induction of cell cycle inhibitor p21; 3) phenotypic differentiation, as indicated by the appearance of the contractile machinery or, more precisely, the expression of MHC; and 4) cell fusion, as indicated by the presence of multiple nuclei in a skeletal muscle cell. A schematic model of differentiation process of cultured cells is presented in *Figure 3* (next page).

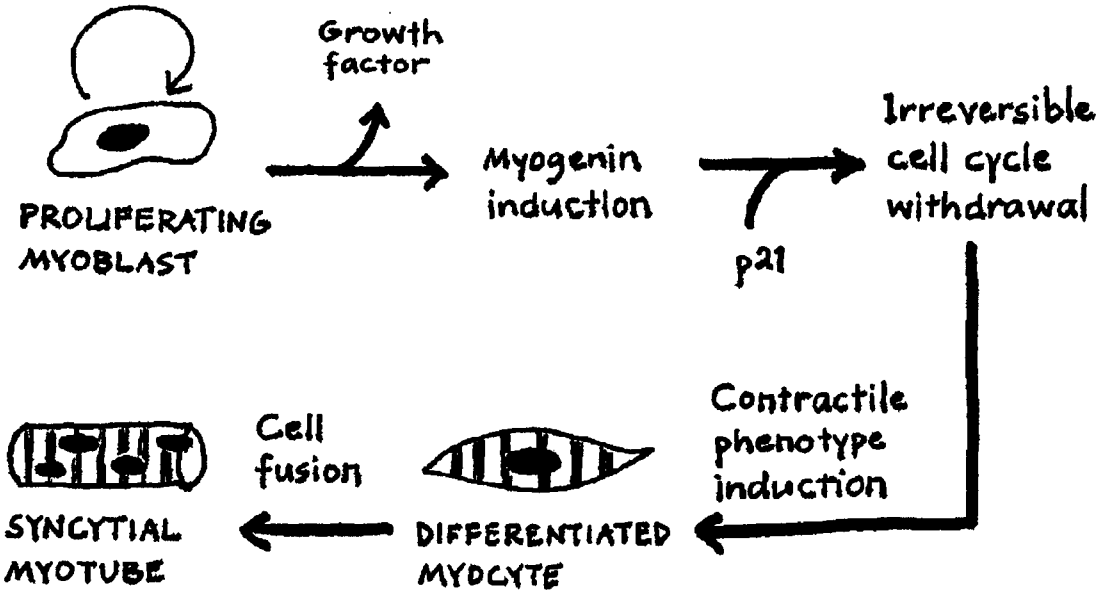


Figure 3: Model for myogenic differentiation of cultured cells. This model shows the temporal relationship between various events in differentiation, but does not imply any causal relationships. Myoblasts asyncronously induce myogenin expression after removal of growth factors, yet remain capable of replicationg DNA. Subsequently, myogenin-expressing myocytes withdraw from the cell cycle, then phenotypically differentiate, and finally fuse into syncytial myotubes. The different myogenic events are described using regular script and cell phenotypes in capital letters. Figure modified from Andres and Walsh (1996).

Reasons for cells to differentiate

The differentiation process can be invoked by muscle trauma or stretch and subsequent activation of the quiescent satellite cells; genetic coordination program in embryo [Pinney and Emerson 1992]; or by artificial manipulation of cell culture conditions *in vitro* [Rosenthal 1989]. Consecutively, differentiation can be examined *in vitro* either with embryonic primary cells, satellite cells or myogenic cell lines [Adams 1990].

Differentiation genes

Under the differentiation process, the myofiber is in a dynamic state of adaptation, coordinated by hormones, mechanical activity and neuronal factors that activate different genes [Muscat et al 1995]. The determination of myoblasts is accomplished through the expression of various muscle regulatory factors (MRFs), for instance MyoD [Davis et al 1987], myogenin [Wright et al 1989] and myf-6 [Braun et al 1990]. These genes can even induce myogenesis in cell lines not committed to myogenic lineage [Pinney et al 1988].

2.2 Markers of differentiation

The degree of differentiation of muscle cultures can be demonstrated morphologically, and by the expression of muscle specific proteins and enzymes. Multinucleated myotubes exhibit cross-striations and later, spontaneous contractions [Yasin et al 1977]. The proportion of nuclei in multinucleated cells in cell culture acts as an additional morphohological indicator. Creatine kinase (CK), glycogen phosphorylase and their muscle specific isoenzymes increase several fold in differentiating myotubes [Martinuzzi et al 1986]. Furthermore, creatine kinase activity have been found to parallel the degree of fusion and the content of total DNA in differentiating muscle cell [Woods et al 1997]. The differentiation event can also be seen in that the amounts of acetylcholine receptors, alfa-actin and tropomyosin increase [Blau and Webster 1981].

Neonatal and adult fast muscle myosin as well as slow myosin are expressed in differentiating muscle cells [Ecob-Prince et al 1989]. The expression of other muscle-specific proteins, such as tetranectin [Wewer et al 1998), intracellular urokinase [Le Moigne et al 1990] and carboxylic anhydrase III [Gunning et al 1987] have been used successfully as indicators of differentiation. Also, the members of MyoD gene family have been utilized. For instance, the expression of myogenin has been detected in all differentiated myocytes, in all muscle cell types [Braun et al 1990], and it has thereby been suggested that the proportion of myogenin-positive nuclei would be relevant as an indicator for terminal differentiation. Andres and Walsh (1996) did, however, prove that myogenin expression alone is not sufficient to promote cell cycle arrest, but cell cycle inhibitor p21 is required. In spite of this, myogenin can still be used as a marker of determination.

2.3 Timetable

The timing and sequence of various differentiation events have been widely discussed, but the complete temporal relationship among the events that govern differentiation remains controversial. Although it has been suggested that the postmitotic state is not acquired until myocytes fuse [Devlin and Konigsberg 1983], more recent findings have plausibly revealed the sequence of consecutive steps in skeletal muscle myogenesis. [Andres and Walsh 1996]

Temporal relationship of expression of MRFs and morphological changes

The timing of the expression of various myogenic regulatory factors of differentiation is fairly well known [Cusella-De Angelis et al 1992, Rudnicki et al 1993, Yablonka-Reuveni and Rivera 1994, Andres and Walsh 1996]. The sequence of the expression of muscle specific regulatory genes has been shown to be dependent on various factors [Maley et al 1994], but MyoD and/or myogenin are nevertheless demonstrated to be among the first proteins expressed [Yablonko-Reuveni and Rivera 1994, Cornelison and Wold 1997]. However, myogenin-positive cells remain capable of replicating DNA, and it is not until the subsequent expression of p21 when the postmitotic state is achieved. This, in turn, precede the expression of MHC that is subsequently followed by the fusion of the cells. [Andres and Walsh 1996] The timing of morphological changes in cell seems to be dependent on the cell type and on extracellular conditions [Barjot et al 1995, Andres and Walsh 1996].

2.4 Regulation of differentiation

Differentiation is regulated primarily by various mitogenic growth factors and myogenic MRFs. Mitogenic factors are compounds that promote quiescent cells to proliferate, whereas myogenic factors are needed to start the process of differentiation [Bischoff 1990].

Myogenic factors

Myogenic factors are, for instance, the proteins of MyoD gene family [Weintraub 1993]. The MyoD family consist of at least six different proteins, including myogenin and MyoD, that act as muscle-specific transcription factors. They belong to basic helix-loop-helix (bHLH) DNA-binding protein class and can turn various differentiating cell types into muscle. These proteins are expressed exclusively in muscle cells, while in other cells the expression is inhibited by known genes [Weintraub et al 1991].

Mitogenic factors

Examples of the mitogenic compounds are fibroblast growth factor 1 (FGF1) and TGF-beta, which can be synthesized in macrophages as a result of muscle damage [Cantini and Carraro 1995], and have been found to control the expression of single proteins of MyoD gene family [Rantanen et al 1995]. In addition to mitogenic effects, FGF1 has also been

shown to inhibit differentiation and repress the expression of MyoD and myogenin [Weintraub 1993].

Other factors

In addition to members of MyoD family and growth factors, a multitude of other regulatory molecules are known, examples including cyclin dependant kinase 5 (CDK5) [Lazaro et al 1997] and insulin-like growth factor II (IGF II) [Ernst et al 1996]. Also neural signals may regulate differentiation, as suggested by denervation-induced increase in the expression of myogenin [Eftimie et al 1991].

Complexity of the regulation of differentiation

Different MRF genes serve in at least two different developmental functions: myf-5 and MyoD jointly act as determination genes whereas myogenin functions as a differentiation gene [Yun and Wold 1996]. In more recent work, MyoD and myf-5 were found to have distinct, partly contrasting roles in determination events [Kitzmann et al 1998]. In contrast to findings that MyoD is the first differentiation gene expressed [Weintraub 1993], Sassoon with colleagues (1989) demonstrated that in some cases myogenin is expressed earlier than MyoD. Furhermore, some skeletal muscle cell lines do not express MyoD at all [Neville et al 1998]. Many recent works even suggest that there is no absolute requirement for MRFs to execute muscle differentiation under some conditions; also the need for myogenin seems to be conditional. These seemingly contrasting findings underline the complexity of the regulation of differentiation, which can also be seen in *Figure 4* [Yun and Wold 1996].

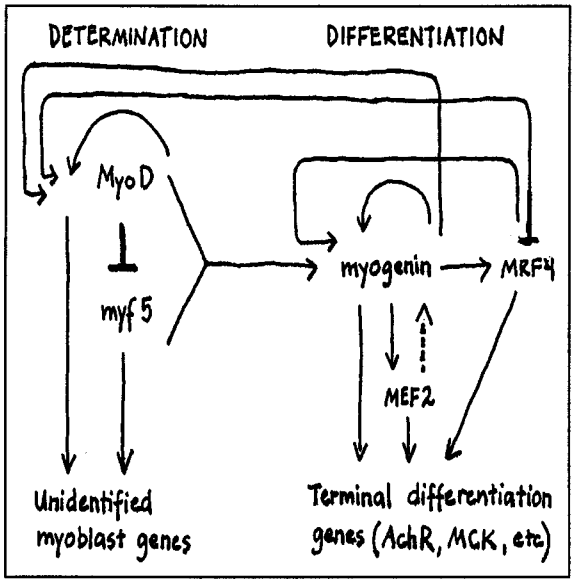


Figure 4: Core skeletal muscle regulatory network and its targets as defined by genetic relationships. The interactions shown are based on gain-of-function and loss-of-function analyses. The dotted arrow indicates co-regulatory activity which in itself is not sufficient to induce changes in function. Activatory relationships are indicated by arrows and inhibitory ones by bars, respectively. Figure modified from Yun and Wold (1996)

2.5 Morphological and functional changes during differentiation

2.5.1 Development mechanism of muscle from myoblasts

During the development the myoblasts fuse to become primary myotubes which are connected by gap junctions and function as a syncytium. The cells express neural adhesion molecules (N CAMs) that affect muscle tissue modeling [Gambke et al 1983]. According to Kelly and Zacks (1969), in developing rat muscle, primary myotubes separate to become independent units of contraction and at the same time, become surrounded by new secondary generation myoblasts. The myoblasts then fuse into secondary myotubes and furthermore, the differentiated primary myotubes become surrounded by differentiating secondary myotubes and undifferentiated cells. Each cell cluster is ensheathed by a basal lamina. Finally, secondary myotubes separate from the walls of primary myotubes to become independent contraction units. The addition of new fibers continues for a period of development and then ceases despite the continued proliferation of myoblasts. The remaining myoblasts fuse to myotubes or become satellite cells. [Moore and Walsh 1985]

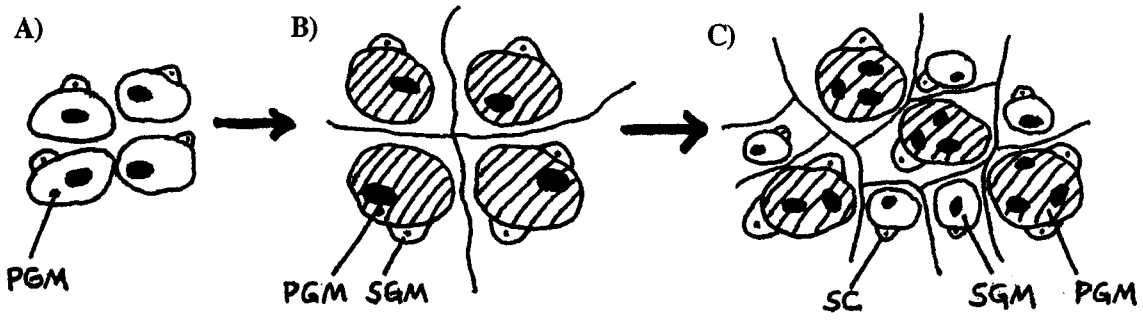


Figure 5: Formation of muscle from differentiating cells according to Kelly and Rubinstein (1986). A) The myoblasts fuse to primary generation myotubes (PGM) with central nuclei. The plasma membranes are closely opposed and some mononuclear myogenic stem cells ensheathe the PGMs. B) At a later stage of development the PGMs are larger and separated from one another. Each PGM is ensheathed by mononucleated cells and by new, secondary generation myotubes (SGM). The SGMs use the walls of PGMs as a scaffolding upon which to differentiate. C) PGMs and SGMs are separeted from each other. Some mononucleated cells become satellite cells, some fuse with existing myotubes.

2.5.2 Regeneration and satellite cells

The mature skeletal muscle is capable of growing and regenerating in response to various stresses, such as muscle trauma (involving fiber death), stretch and compensatory hypertrophy, exercise and denervation [Bischoff 1989]. Because mature muscle cells are unable to divide, new ones can only form by the fusion of myoblasts. For this purpose, a few myoblasts persist as small, inactive cells between the basement membrane and the plasma membrane of a mature muscle cell: these are called satellite cells. [Olson 1992]

As a result of muscle damage, satellite cells are activated to proliferate, followed by differentiation into myoblast, which then fuse to form myotubes and finally mature to new muscle fibers to replace the muscle cells that have been lost. The activation of satellite cells has been shown to be induced by crushed muscle extract [Bischoff 1986], where various growth factors have been identified [Haugk et al 1995]. The proliferation of myoblasts can also be achieved by artificially treating the muscle with exogenous peptide growth factors, which set cells to proliferate and prohibit differentiation by repressing the expression of muscle specific genes. Growth factor withdrawal is then accompanied by a differentiation program similar to the one presented above. [Olson 1992]

2.5.3 Positioning of subcellular organelles

Subcellular organelles in mononucleated cells consist largely of a peripheral endoplasmic reticulum (ER) membrane network that delivers secretory vesicles to the centrally situated Golgi apparatus. The positioning of these organelles depends on the microtubular organization in myoblasts as well as in mature myofibers. A profound reorganization of organelles occurs when mononucleated myoblasts differentiate and fuse to form multinucleated myotubes. The changes in centrosome and Golgi organization during differentiation have been shown to result from the activation of the myogenic program and occur independently of myoblast fusion. [Ralston 1993]

In myoblasts

In the myoblasts, the ER surrounds the nucleus, the Golgi apparatus is next to nucleus (this organization of the Golgi has been referred to as polar), and the centrosome is made of a pair of centrioles surrounded by perinuclear material. [Ralston 1993]

In myotubes

In the myotubal stage the ER assumes peripheric organization and appears as an extended membrane network throughout the multinucleated cells [Metsikkö et al 1992]. Jasmin et al (1989, 1995) have localized the Golgi in a muscle exclusively beneath the neuromuscular junction. By contrast, Ralston (1993) suggested that the Golgi elements are distributed throughout the muscle fibers. Finally, Rahkila and co-workers (1997) have shown with Golgi-targeted mutant viral glycoprotein that extrajunctional Golgi elements do exist. In myotubes, the Golgi apparatus is distributed in a narrow band around the nuclei in the form of perinuclear Golgi elements. Microtubules are devoid of nucleation centers [Tassin et al 1985, Ralston 1993].

In myofibers

In a terminally differentiated skeletal muscle, a rough endoplasmic reticulum is located just beneath the sarcolemma, it surrounds each nucleus and can also be seen between nuclei. The Golgi apparatus is distributed throughout the muscle fiber and is organized like the myotubal Golgi complex. In addition, a smooth-looking membrane system, called the sarcoplasmic reticulum (SR), surrounds the contractile myofilaments throughout the fibers. [Ralston 1993, Rahkila et al 1996, 1997, 1998] It is generally assumed that the SR develops as a specialized ER structure, but how the SR membrane system receives its specific components remains obscure [Flucher 1992, Rahkila et al 1998]. Furthermore, the T-tubules arise from their early intracellular compartment precursors [Flucher et al 1991] simultaneously with the SR [Flucher et al 1993]; In myofibers the T-tubules are continous with the plasma membrane, with their own specific protein and lipid composition [Rosemblatt et al 1981].

In cultured myotubes, the reorganization and synthesis of organelles is reflected by changes in protein transport pathways and maturation processes. For example, in chick embryo muscle cultures, acetylcholinesterare is not secreted as expected, and most of it remains endo H-sensitive, indicating the lack of Golgi processing [Rotundo et al 1989]. Also, in L6 myoblast-derived myotubes a fraction of the Golgi stacks was disassembled and did not process VSV glycoprotein as seen in myoblasts [Kellokumpu et al 1995]. However, although the changes in the Golgi complex seem very dramatic, they do not appear to modify the membrane cycling between the Golgi complex and the ER. [Ralston 1993]

2.6 Mitotic fragmentation of the Golgi

All the membrane trafficking steps studied so far are arrested during mitosis. The absence of newly synthesized vesicular stomatitis virus glycoprotein in the plasma membrane of infected mitotic cell was the first indication that exocytosis ceases in mitosis [Warren et al 1983]. Further studies have revealed that the transport of endogenous proteins from the ER to the Golgi is similarly inhibited [Featherstone et al 1985]. Transport of proteins and lipids through the Golgi complex has also been shown to be inhibited in mitosis both *in vitro* and *in vivo*. In all cases, however, as cells exit mitosis, transport rapidly resumes. [Lowe et al 1998]

The number of Golgi stacks decrease significantly during the mitosis, leaving behind clustered tubular and vesicular elements. These are shown to be derived from the Golgi complex and have been counted to amount approximately 150 per dividing cell. [Lucocq and Warren 1987] An additional presence of variable number of other free vesicles has also been shown, the amount of which ranges from tens to thousands. There is an inverse relationship between the amount of Golgi membranes in the clusters and that of free vesicles, suggesting that clusters shed vesicles into the surrounding cytoplasm [Lucocq 1989]. This also implies that the clusters are fragmentation intermediates and the vesicles the final product and therefore the partitioning unit for the Golgi inheritance [Lowe et al 1998]. These vesicles are suggested to accumulate in mitosis because their fusion is inhibited [Misteli and Warren 1994].

Fragmentation pathway of the Golgi components

Two different routes, namely COPI pathway and COPI-independent pathway, have been suggested to share fragmentation of the Golgi components. The mechanisms of a precise and extensive fragmentation of Golgi in animal cells remain unclear: the clusters are partitioned, however, with an accurary far greater than anticipated, so a mechanism more elaborate than simply a stochastic process probably exists. The fragmentation, and later restoration, of the Golgi complex has been used as a model for endogenous membrane trafficking. [Lowe et al 1998]

3 Transport of proteins

The extensive network of intracellular membrane bound organelles allows the eukaryotic cells to carry out a variety of specialized tasks, and greatly increases its surface-to-volume ratio. Furthermore, the proteins charasteristic of each intracellular compartment are transported to their respective destinations via this network. This transit is necessary for the biogenesis of plasma membranes, lysosomes and endosomes; the secretion of intracellular proteins and other proteins from the cell; and the uptake of external molecules by endocytosis. Moreover, the specificity of this transport is harnessed to generate a distinct apical and basal surfaces needed for the polarized function of plasma membranes in most tissues. [Rothman 1994]

The general outlines of the secretory pathway were delineated by Palade and his colleagues in 1975. Nascent proteins are delivered to the lumen of the ER, pass through the Golgi complex for post-translational processing, and are then forwarded to their specific destinations in storage or degradative compartments, in plasma membrane, or outside the cell. The movement of proteins between these compartments occurs by the budding and fusion of transport vesicles [Farquhar 1985].

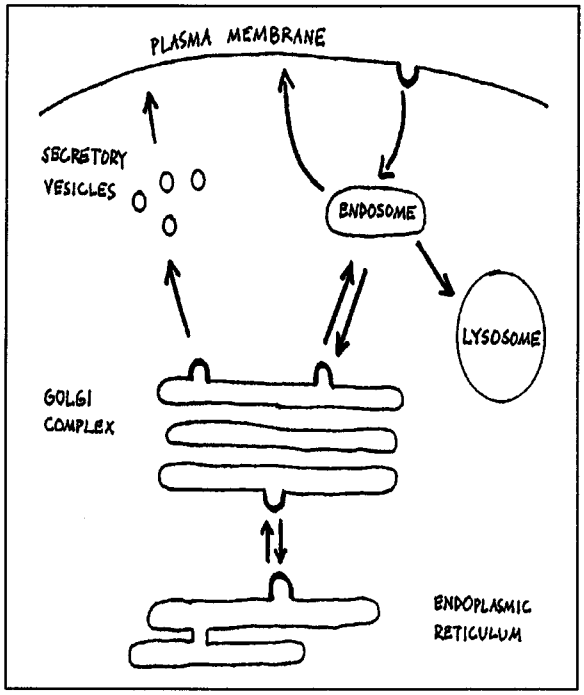


Figure 6: Schematic presentation of membrane and protein traffic in eukaryotic cells. Arrows indicate the direction of transport and bold lines indicate the budding vesicles involved in trafficking. Figure modified from De Camilli et al (1996).

3.1 Vesicular transport

3.1.1 Segregating of cargo to transport vesicles

Although lipids and in some instances cargo may serve to define the site of bud emergence, it is almost certainly through the action of coat proteins that membrane constituents are segregated and the bilayer is mechanically deformed to produce a transport vesicle [Schekman and Orci 1996].

Receptor-mediated concentration of cargo into the vesicles

It has been established that newly synthesized secretory and plasma membrane proteins become five- to tenfolds concentrated as they reach the Golgi apparatus [Teasdale and Jackson 1996]. The findings of for example Balch et al (1994) have led researchers to re-examine the possible existence of cargo receptors that actually target and concentrate proteins into vesicles leaving the ER. The most promising candidate for the role of cargo receptor is ERGIC-53. No experimental data, however, exist to substantiate the role of ERGIC-53 as a cargo receptor. [Teasdale and Jackson 1996]

Hotspots

According to a model by Kuehn and Schekman (1997), there are specialized regions, hotspots, of the ER where cargo and proteins involved in vesicle biogenesis congregate. In this model, gating proteins are postulated to direct transmembrane cargo proteins to privileged sites in the membrane where budding occurs. The gating proteins are also proposed to sieve molecular cargo protein into the budding sites while restricting most of the resident ER proteins. The existence and identity of such gating proteins remain unclear. [Hobman et al 1998]

Golgi-associated spectrin skeleton

There is evidence that Golgi spectrin forms a docking complex that acts prior to the *cis* Golgi, to sequester specific membrane proteins (for instance VSV G) into vesicles transiting between the ER and the Golgi. Furthermore, it has been postulated that the cargo transit within the Golgi stacks and to other membrane apparatus other than the ER, is mediated by this docking complex, possibly involving other isoforms of spectrin and

ankyrin as well. The docking complex is termed as vesicular spectrin-ankyrin adapter-protein trafficking (or tethering) system (SAATS). [Devarajan et al 1997]

3.1.2 Coated vesicles

There is convincing evidence that coated vesicles are involved in vesicular transport from the ER, or intermediate compartment (IC), to the Golgi complex in eukaryotic cells; in transporting steps between successive Golgi stacks; and in post Golgi trafficking. A multitude of different vesicle coats have been suggested to mediate transporting events: these include clathrin in endocytosis, coatomer, also called COPI, in trafficking between the Golgi and the ER, COPII in intra Golgi transport and various others, whose function is not discussed here. [Schekman and Orci 1996]

3.1.3 Proteins and enzymes in vesicular transport

Most of the proteins isolated in connection to vesicular transport appear to be involved in multiple vesicular transport events. Furthermore, N-ethelmaleimide-sensitive factor (NSF; Block and Miller 1992) has been shown to be essential both in transport from the ER to the *cis* Golgi [Beckers et al 1989] and between Golgi cisternae [Wilson et al 1989]. Similarly multi-functional is alfa-SNAP (soluble NSF attachment protein), originally identified as an essential component of transport between the *cis* and medial Golgi [Clary and Rothmann 1990], and homologous to SEC17, a protein required for movement between the ER and Golgi in yeast [Griff et al 1992]. However, the discontinuous nature of the exocytotic pathway suggests that some transport components should only be required at distinct steps, both to maintain the fidelity of various compartments and to ensure vectorial flow. Evidence to support this hypothesis has been provided by Graham and Emr (1991).

3.2 Transport to Golgi

Export complexes at the ER

The export of cargo from the ER occurs through the formation of COPII-coated vesicular carriers. Bannykh et al (1996) found, that ER buds *in vivo* are nonrandomly distributed, being concentrated in regional foci the authors referred to as export complexes. Vesicles within these export complexes contain concentrated cargo molecules. Balch and co-

workers (1994) termed these central compact clusters of pleomorphic elements vesicular-tubular clusters (VTCs) for their morphological appearance.

The juxtaposition of ER-derived buds and central VTCs composes a morphological unit of organization termed an export complex. It is further concluded that budding from the ER through recruitment of COPII is confined to these highly specialized export complexes that topologically restrict anterograde transport to regional foci facilitating thus the efficient coupling to retrograde recycling by COPI. [Bannykh et al 1996] While VTC-containing export complexes are scattered throughout the cytoplasm, VTCs found in the perinuclear region of the cell are believed to form a more extensive array of tubulo-cisternal elements referred to as the *cis*-Golgi network (CGN) [Mellman and Simons 1992]. The model of export utilizing export complexes, as envisioned by Bannykh et al (1996), has regained support from the work of Rahkila and colleagues (1997).

3.3 Through Golgi

Tubule formation in Golgi

The standard view of the intra Golgi trafficking is that it is mediated primarily by vesicles that pinch off from one cisterna and target to and fuse with a different cisterna [Rothman and Wieland 1996]. Both the *cis*- and *trans*-most cisternae of the Golgi complex are composed of extensive membrane tubule networks [Saraste and Kuismanen 1984], and tubular connections between Golgi stacks have been observed in electron micrographs [Rambourg and Clermont 1990]. Tubules can be rapidly generated by Golgi membranes *in vivo* and *in vitro* under various conditions [Sciazy et al 1997], and the tubular processes have been revealed to emerge from the Golgi elements [Cooper et al 1990]. Two models, namely directed maturation and carrier vesicle mediated transport for intra Golgi trafficking have been proposed.

Vesicular carrier mediated transport

In one model of intra Golgi transport, the compartments are discontinous and each is compositionally distinct; intra Golgi trafficking would then necessarily involve transport vesicles. Rothman and Wieland (1996) suggested, that both anterograde and retrograde transport would be mediated by COPI machinery. Contrasting this proposal, Bannykh and Balch (1997) argued that, whereas recycling of transport receptors could be readily

mediated by the COPI machinery, another coat machinery and/or physical contact between the successive stacks is likely to be involved in intra Golgi trafficking [Bannykh and Balch 1997].

Directed maturation model of the Golgi

Because the Golgi stacks have been proven discontinous [Mellman and Simons 1992], transport of cargo through the Golgi will necessarily involve vesicular carriers. In consistency with this postulate is the direct outgrowth of the homotypic assembly model of VTCs. In this view, VTCs, once formed, do not release anterograde-transported cargo into carrier vesicles. Rather, the Golgi stack is composed of a series of progressively maturing, discontinous compartments initially formed by the fusion of COPII vesicular carriers and peripheral VTCs. This concept was named directed maturation as early as 1957 by Grasse, but fusion between VTCs and COPII remains yet to be proven [Bannykh and Balch 1997]

3.4 ER to SR transport

The SR of skeletal muscle fibers contains ER components such as BiP, protein disulfide isomerase and calnexin, strongly suggesting continuity between these two structures [Volpe et al 1992, Villa et al 1993]. Certain SR components, such as Ca^{2+} -dependent ATPase, are presumably transported directly from the ER to the SR, since they have not been found in the Golgi [Jorgensen et al 1977]. In contrast, calsequestrin, a major protein in the SR lumen, has been found in the Golgi, suggesting a transport route through theGolgi for this protein [Thomas et al 1989]. Furthermore, calsequestrin has been shown to travel through the Golgi in transfected mononucleated cells [Reichmann et al 1995], whereas for Ca^{2+}-ATPase this transit did not occur [Karin & Settle 1992]. These results suggest that proteins are translocated from the ER to the SR either directly or via the Golgi [Rahkila et al 1996]. In a later study, Rahkila and colleagues (1997) also suggested a role for the SR membranes in protein translocation, in addition to being active in protein export.

3.5 Retrograde transport from Golgi to ER

A retrograde transport pathway between the Golgi and the ER has long been assumed to exist, based on theoretical grounds, as without it, the continuons flow of membrane lipids from the ER to the Golgi would deplete the ER of membrane [Teasdale and Jackson 1996].

Indeed, COPI vesicles are shown to mediate a cycle of transport including retrograde limb that returns escaped ER proteins and v-SNAREs to the ER, and an anterograde limb that carries the same v-SNAREs and COPI-specific transport factors back to the *cis* Golgi. [Schekman and Orci 1996]. It seems likely that retrieval of di-lysine-bearing proteins occur throughout the Golgi stack [Griffiths et al 1995]. Whether the motif-bearing molecules, which are collected into COPI-coated vesicle in the medial-Golgi, are delivered to an earlier Golgi stack or directly back to ER is unknown [Teasdale and Jackson 1996].

3.6 Post-Golgi trafficking

The TGN has been established as the exit compartment of the Golgi [Rambourg and Clermont 1990], and the trafficking from there on has been shown to be mediated by vesicular carriers. The trafficking of vesicles from the Golgi complex includes a number of distinct steps, starting with their formation and ending with their fusion at the plasma membrane, endosomes or degradative compartments. This process depends on specific molecular machineries responsible for the sorting and targeting of newly synthesized proteins. The essential differences in trafficking between different cell types seem to concern the routes between the Golgi and the plasma membrane. [Lafont and Simons 1996]

3.7 Membrane trafficking

3.7.1 Specificity in vesicle trafficking and SNAREs

Fusion of transport vesicles

Maintaining the identity of membrane-bound compartments requires a fusion mechanisms of great specificity [Palade 1975]. A single polypeptide can catalyze membrane fusion, as shown by the viral envelope fusion proteins [White 1992], and a small number of proteins may well mediate vesicle fusion since targeting is achieved. A vesicle coming from the ER, for example, must fuse with the *cis*-face of the Golgi as opposed to any other target in cytosol in order to maintain compartmentation in the cell. Because NSF and SNAP must promote fusion at multiple locations, there has to be a family of SNAP receptors. This has indeed been shown to be the case, as various different SNAP receptors (SNAREs) have been found. [Rothmann 1994].

The SNARE hypothesis

According to the SNARE hypothesis [Söllner et al 1993], the cognate v-SNAREs (v for vesicle) and t-SNAREs (t for target) function together to affirm correct docking and fusion of vesicles. Speculative extensions of this hypothesis could explain phenomena such as retrograde transport, homotypic fusion and stacking of Golgi cisternae [Rothmann and Warren 1994]. According to a more recent view, the SNAREs are the minimal requirement for the membrane fusion to occur [Weber et al 1998], but do not probably regulate the specificity of the reaction. It has, for instance, been shown that the ER membrane fusion can occur in the absence of appropriate v-SNAREs [Patel et al 1998]. The pairing specificity may instead be mediated by regulatory proteins such as members of Sec1p family and the small GTP-binding proteins [Rothman and Söllner 1997]. Thus, despite intensive studies on SNAREs, their exact function still remains elusive [Schekman and Orci 1996].

3.7.2 Role of microtubules in membrane trafficking

Despite the constant membrane flux, the Golgi has an ability to maintain both its characteristic morphology, perinuclear position and compartmentation. These are maintained by microtubules and microtubule motors, namely dynein and kinesin [Lippincott-Schwartz et al 1995]. In addition to this function, kinesin is required for retrograde, Golgi-to-ER recycling and can also facilitate budding and transport from the Golgi to the surface of the cell in mononucleated cells [Lippincott-Schwartz et al 1995] as well as in myofibers [Rahkila et al 1997].

Nature of microtubules

Microtubules are polar polymers which by polymerizing and depolymerizing mediate the trafficking of cellular organelles and other structures. They provide a directional track for movement, whereas the motor proteins provide the motive force by hydrolyzing adenosine triphosphate. The principal motor proteins, kinesin and dynein, have been shown to attach to intracellular organelles through the action of kinectin and dynactin, respectively. [Vallee and Scheetz 1996]

Changes in microtubule organization

In multinucleated myotubes, microtubules lack the radiation center typical of the mononucleated cells [Tassin et al 1985]. In contrast, perinuclear microtubules, as well as exosarcomeric microtubular lattice, have been demonstrated in adult muscle fibers [Boudriau et al 1993]. Rahkila et al (1997) showed that the markers of the pre-Golgi compartment localize into perinuclear regions and the interfibrillar membranes throughout the myofibers, showing colocalization with microtubules. These findings clearly demonstrate that the Golgi elements are transported along the microtubulus network and moreover, that these elements are distributed along the entire lenght of the fiber, contrasting previous findings of the localization of the Golgi elements at the neuromuscular junctions only [Jasmin 1989, 1995].

3.7.3 Role of lipids in membrane trafficking

A recent report showed that the need for activated ARF could be bypassed by the addition of bacterial phospolipase D (PLD) to the budding reaction [Ktistakis et al 1996]. Schekman and Orci (1996) have suggested that ARF may augment coatomer recruitment through PLD to provide an environment suitable for the formation of a coated vesicle. However, a direct connection between coat assembly and lipid metabolism remains to be established. Based on these findings, it seems likely that also the membrane itself may play a key role in binding and/or stabilizing vesicle coat proteins on the membrane [Seaman 1996]. In addition, also other lipid components have been proposed roles in membrane trafficking [De Camilli et al 1996, Van Meer 1998].

3.7.4 Spectrin network

Recent evidence suggests that Golgi-associated spectrin network can provide a structural framework for this organelle [Beck et al 1997] as well as mediate cargo transport and sorting of selective membrane proteins [Devarajan et al 1997]. This network originates from spectrin and ankyrin isoforms that form a meshwork in the Golgi membrane. An additional role for spectrin-network could be to provide a link between the Golgi and the microtubule cytoskeleton, as suggested by Holleran and Holzbaur (1998).

3.8 Coupling of fusion to budding

Elazar et al (1994) have proposed a vesicle budding-fusion couple hypothesis. According to this view, during the biosynthesis of the vesicle fusion machinery, fusogenic proteins must be concealed by coat proteins to prevent premature fusion among Golgi cisternae. The sequential assembly and disassembly of a coated vesicle ensure that only transported proteins and not entire Golgi compartments are consumed by membrane fusion.

Mechanisms of vesicle formation, budding and fusion

Coatomer and GTP-binding ARF co-assemble on the Golgi surface to form vesicles; their later release constitutes uncoating. In the absence of ARF, the coat forming is stopped and the accumulating v-SNAREs will directly interact with t-SNAREs, leading thus to the uncoupled fusion of donor and acceptor compartments; it is therefore apparent that not only the COP coat drives vesicle budding, but can also prevent fusion prior to the completion of budding [Rothman 1994]. In addition to coatomer and ARF, also fatty acyl-CoA has been shown to be essential for complete coated vesicle formation [Ostermann et al 1993].

Ultimately, the nascent vesicle is released by membrane fission within the bud neck; this step has been shown to require dynamin, a GTP-binding protein that constricts and finally severs the bud neck [Bednarek et al 1995]. Uncoating can be understood as a simple reversal of coat assembly; when ARF hydrolyses its bound GTP, it dissociates from the membrane, and coatomer follows the suit [Rothman 1994]. The fusion of transport vesicles occur via SNARE-dependent mechanism outlined by Söllner and co-workers (1993).

3.10 Transport in polarized cells

Regulation of protein traffic

The plasma membrane of polarized cells is divided into apical and basolateral surfaces, with different compositions. Proteins can be sent directly from the trans Golgi network (TGN) to either surface, or first to one surface and then transcytosed to the other. The glycosyl phosphatidylinositol anchor is a signal for apical targeting [Mostov and Cardone 1994]. Signals in the cytoplasmic domain determine basolateral targeting and retrieval, and

are related to other sorting signals. Since the machinery that mediates basolateral sorting in the TGN has not been identified, it is not known whether one or more vesicular carriers are involved in basolateral transport. [Keller and Simons 1997]

It is likely that there are a number of parallel pathways that mediate the transport from the TGN to the cell surface. Assuming that these pathways can be modulated separately, a cell would have flexibility to react to changing conditions in a specific manner. It has, for instance, been demonstrated that a migrating fibroblast transports 'basolateral' proteins to the leading edge [Peränen et al 1996], whereas in resting cells the same vesicles arrive at the cell surface randomly. It is not known whether apical and basolateral membrane proteins are included into the same vesicle, or whether apical proteins use another mechanisms to reach the basolateral surface. [Keller and Simons 1997]

A relevant example of differential targeting of apically targeted hemagglutin glycoprotein of the influenze virus and the basolateral vesicular stomatitis virus glycoprotein (VSV G) can be seen during myotube formation; in myoblasts, both marker proteins are transported to the cell surface. However, after myotube formation, VSV G is diverted to two intracellular locations, presumably representing the SR and a glucose-transporter-containing organelle. [Metsikkö et al 1992]

4 Targeting of proteins

ER in targeting

The long-standing argument over whether trafficking and sorting of proteins in the secretory pathway rely on discrete retention or transport signals [Rothmann 1987, Klausner 1989] remains to be resolved. Although multiple mechanisms for retention of proteins in the ER have been described, and specific signals for transport remain to be identified, it is probably appropriate to recognize that proteins need to acquire a transport-competent state before they can leave the ER [Teasdale and Jackson 1996]. Indeed, the ER is now recognized as a system of quality control where the proteins immature or not correctly folded are prevented from entering the secretory pathway [Rose and Doms 1988, Hammond and Helenius 1995].

Golgi in targeting

The central role of the Golgi stack in protein targeting is well established. The proteins of the plasma membrane, secretory storage vesicles and lysosomes, for example, have been found to leave the ER and cross the Golgi stack together. It was only during the exit from the last, TGN compartment, when the various precursors were separated according to their destinations. [Griffiths and Simons 1986]

4.1 General sorting mechanism

The initial targeting of proteins to the ER is accomplished using a signal sequence [Blobel and Dobberstein 1975], and export from the ER involves incorporation of proteins and membrane into transport vesicles that are destined to fuse with the next compartment along the pathway. The secretory pathway thereby consists of a series of membrane bound organelles between which proteins and membrane are moved in a vectorial fashion by a coordinated vesicle budding and fusion events. Specific molecular targeting signals and vesicular coat systems segregate proteins to their respective transport pathways. [Rothmann 1994]

4.2 Targeting between the ER and the Golgi

4.2.1 Retention of resident proteins in the ER

Retention by dibasic motif

Each organelle of the secretory pathway is required to selectively allow transit of newly synthesized secretory and plasma membrane proteins, and also to maintain a unique set of resident proteins that define its structural and functional properties. In the case of the ER, for example, residency could be achieved in two ways: 1) through prevention of residents from entering newly forming transport proteins and 2) through retrieval of those residents that escape. Works by Pelham and colleagues (1987, 1990) convincingly demonstrate that soluble molecules tagged with a carboxy-terminal tetrapeptide, KDEL, are exposed to Golgi enzymes and subsequently retrieved back to the ER. This retrieval is established to be mediated by an interaction between complex of cytosolic coat proteins (COPI) and a dibasic motif close to the terminus of the cytoplasmic domain of membrane proteins [Pelham 1990].

Retention by other mechanisms

Many ER resident membrane proteins do not bear dibasic motifs in their cytoplasmic domains. Various alternative targeting mechanisms have thus been suggested. One alternative would be a complex containing protein that possesses a di-lysine protein. The second possibility is one wherein these proteins use alternative targeting motifs, and the third one, for which there is growing evidence, is that molecules may be retained in the ER by exclusion from transport vesicles. [Teasdale and Jackson 1996]

Kin recoqnition

Although the ER-localization of KDEL or di-lysine tagged marker proteins has been established as the result of continuous recycling from post-ER compartments [Connolly et al 1994], it is less certain if the steady-state distribution of endogenous KDEL or di-lysine bearing proteins is totally dependent on retrieval. Various observations [Munro and Pelham 1987, Rose and Doms 1988, Hurtley and Helenius 1989] suggest that many ER proteins never leave this organelle. According to kin recognition model [Nilsson et al 1993],

retention of many ER proteins occurs by the formation of oligomers. In this model, it is proposed that the lumen of the ER is a matrix stabilized by calcium to passively exclude all proteins other than those needed in its formation. [Teasdale and Jackson 1996]

4.2.2 Coat mediated sorting

Figure 7 illustrates the proposed roles of COPI and COPII in vesicle traffic between the ER and *cis* Golgi: COPII vesicles (4) are shown transporting normal cargo molecules, a low number of escaped resident ER proteins, and anterograde targeting membrane proteins (vesicle-bound soluble NSF attachment protein [v-SNARE]) [Aridor et al 1995]. COPI vesicles (1,2,3) are shown mediating a cycle of transport including a retrograde limb that returns escaped ER proteins and v-SNAREs to the ER, and an anterograde limb that carries the same v-SNAREs and COPI-specific transport factors back to the *cis* Golgi. [Schekman and Orci 1996]. It seems likely that retrieval of di-lysine-bearing proteins occur throughout the Golgi stack [Griffiths et al 1995], but whether the motif-bearing molecules, which are collected into COPI-coated vesicle in the medial-Golgi, are delivered to an earlier Golgi stack or directly back to ER is unknown [Teasdale and Jackson 1996].

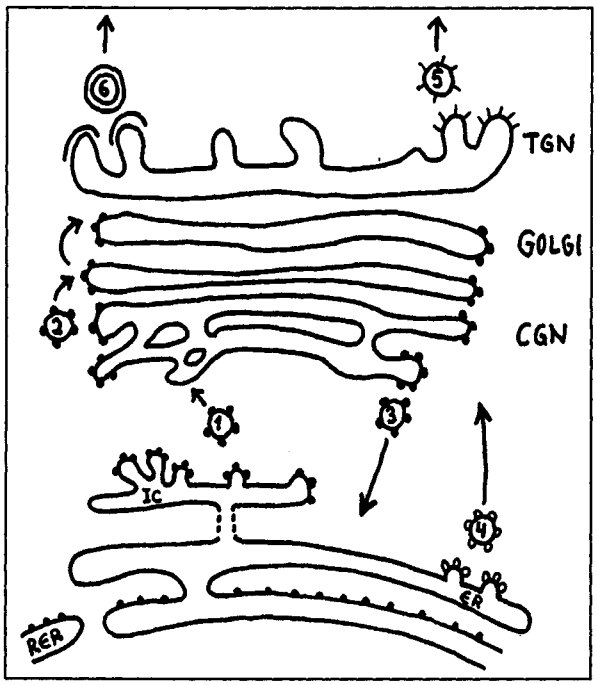


FIGURE 7: Coat mediated sorting of proteins. COPI-coats mediating transport between ER / intermediate compartment (IC) and *cis* Golgi network (1), in intra-Golgi transport (2) and in retrograde trafficking (3) are indicated by black dots. COPII-coats in vesicles involved in ER to Golgi transport (4) are indicated by transparent dots. Clathrin coat (5) is demarcated by spikes and vesicle coat involved in transporting material from the *trans* Golgi network (TGN) to the cell surface (6) is indicated by lines. Arrows indicate the direction of transport. Figure modified from Kreis et al (1995).

4.3 Mechanisms of polarized targeting

Ample evidence exists to support the role for lipids in the protein sorting and budding events [De Camilli et al 1995]. Lipids attached directly to protein cargo may determine the lateral segregation of this class of molecules into transport vesicles. In polarized epithelial cell, a subset of vesicles that bud from the *trans* Golgi compartment are enriched in glycolipid-anchored (glycosylphosphatidylinositol, GPI) proteins that are destined to reside on the apical plasma membrane [Zurzolo et al 1994]. Such anchors may influence protein sorting and vesicle budding directly, or they may do so indirectly through an association with cytoplasmic structural proteins. [Schekman and Orci 1996]

5 Viruses and Vesicular Stomatitis Glycoprotein transport and targeting

5.1 Viruses

In many types of virus infected cells, cellular marcomolecular synthesis is inhibited and replaced by the synthesis of viral macromolecules. Large quantities of viral components are produced, which has greatly simplified the analysis of synthesis, modification, transport and assembly of specific viral nucleic acids and proteins. This has led to the utilization of viral molecules as probes for many aspects of macromolecular biosynthesis, assembly and trafficking. [Compans and Roberts 1994]

Use of viruses in studies of membrane traffic

Cells infected with enveloped viruses that inhibit cellular biosynthesis have been used extensively to study the pathways followed by viral membrane glycoproteins from their site of synthesis in the rough ER, through the Golgi apparatus, to the plasma membrane [Griffiths et al 1985]. Mutant viral glycoproteins have been used to define vesicular transport intermediates [Rothman et al 1984, Rahkila et al 1996, 1997, 1998]. Inves tigations on protein folding and oligomerization as determinants of transport of membrane glycoproteins have also been carried out with viral glycoproteins [Doms et al 1993, Rahkila et al 1998]. Temperature-sensitive viruses are a tool that helps in defining the localization of different macromolecules along the protein transport pathways [Griffiths et al 1985].

The site of the viral assembly process is dependent on virus family. Some viruses are assembled within the cytoplasm and some in the nucleus. Other viruses possess lipid-containing envelopes that are acquired by budding at the cellular membrane. Such viruses, for instance Vesicular Stomatitis virus (VSV), encode one or more membrane glycoproteins: the cellular site of viral glycoprotein accumulation correlates with the site of budding. Thus, the protein components of such viruses provide excellent systems for studies on the mechanisms of targeting of proteins to distinct cellular locations. [Compans and Roberts 1994]

Viral replication cycle

Most studies on virus replication are conducted on cell cultures. In order to achieve the necessary high level of synchrony, single cycle growth conditions are used. Therefore, experimental procedures carried out on the entire culture reflect the replicative event of a single cell. [Celis and Celis 1994]

According to Compans and Roberts (1994), the following steps are common to all viral replication cycles: 1) adsorption, where the virus attaches to specific receptor on the cell surface; 2) penetration/uncoating, where the virus enters the cell by membrane fusion (enveloped viruses) or endocytosis; 3) biosynthesis, where virus-specific proteins are synthezised and viral genome replicated: the precise mechanism to accomplish this varies greatly among virus families; 4) assembly of progeny virions; and 5) release of virions from the cell. For some viruses the assembly and release occur simultaneously.

5.2 Vesicular Stomatitis virus Glycoprotein

Vesicular Stomatitis virus (VSV) is a prototype rhabdovirus, containing a non-segmented single-stranded RNA genome of negative sense enclosed in a host-cell-derived lipid envelope. VSV is highly cytopathogenic for the infected cell: prior to cell death, VSV infection elicits an efficient shut-down of host RNA, DNA and protein synthesis. The G protein is an integral membrane glycoprotein. [Compans and Roberts 1994]

Targeting and transport of VSV G

Vesicular Stomatitis virus glycoprotein (VSV G) buds exclusively at basolateral membrane domains [Roth et al 1985] and can thus be concluded to be targeted to the basolateral membrane. Translocation of VSV G protein into the rough ER has been established to be directed by a hydrophobic N-terminal signal sequence, but targeting mechanisms from the ER on need further inspection.

Dual processing of VSV G

In fibroblasts, the VSV G protein matures during transport through the *trans* Golgi into a form that exhibits reduced mobility in SDS-PAGE [Kornfeld and Kornfeld 1985]; this was

shown to be due to addition of sialic acid in the *trans* Golgi network [Balch and Keller 1986]. In skeletal myofiber, however, as demonstrated in a study by Rahkila and co-workers (1996), only half of VSV G protein was processed in myotubal Golgi, and the processed form became incorporated in the budding viral particles in transverse tubules. It was concluded that in myofibers, processed viral protein is targeted to T-tubules. Furthermore, it was shown that the fraction of the G protein not subjected to reduction in mobility was processed into a slightly more mobile form. This portion of the glycoprotein remained endoglycosidase H-sensitive, suggesting its retention in the ER/SR endomembrane system. Contrasting these findings, however, it has been shown that L6 myoblast-derived myotubes exhibit similar dual processing of the VSV G protein, and both the endo H-resistant and endo H-sensitive forms are transported to the Golgi [Kellokumpu et al 1995].

Destinations of VSV G protein isoforms

The nature of the dual processing of VSV G has been refined, and it has been found out that only about half of the G protein travels through the Golgi and, instead of being externalized, the Golgi-processed form is destined to an intracellular vesicular compartment, which is probably T-tubules. The remaining half of the protein seems to fold, trimerize, and exit the ER, but deviate from the transport pathway leading to the Golgi. The non-processed form does not appear at the cell surface either. In contrast, partial co-localization of VSV G with glucose transporter in vesicular compartments during myogenesis has been revealed. In the same study, it was speculated that the nonprocessed G protein would be transported from the IC into a novel muscle-specific compartment developing in myogenesis. This compartment was suggested to be the sarcoplasmic reticulum. [Rahkila et al 1997, 1998] A hypothetical model for VSV G transport and targeting is presented in *Figure 8.*

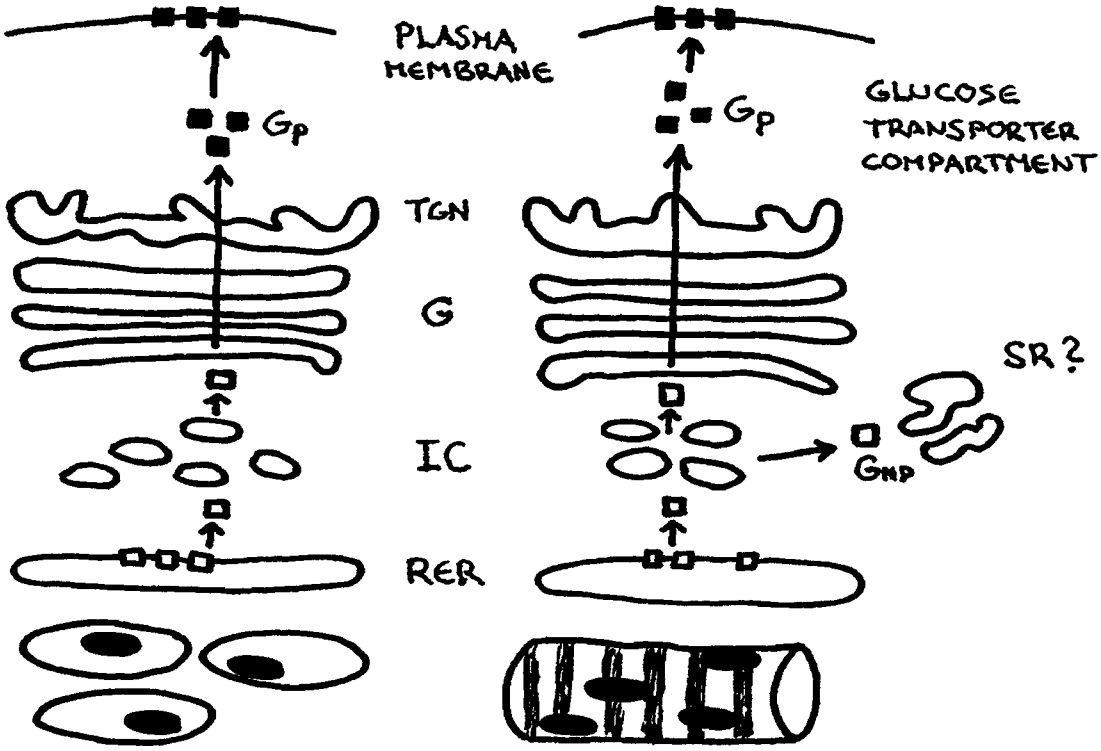


Figure 8: Postulated transport routes for VSV G protein in L6 myoblasts (left) and in multinucleated myotubes (right). In myoblasts, all of the VSV G protein is transported to the cell surface, but in myotubes, it is transported to a pre-Golgi compartment suggested to be the SR, and to a post-Golgi compartment postulated to represent a recruitable glucose transporter compartment. RER indicates rough ER, IC is for intermediate compartment, G in picture indicates Golgi and TGN the trans Golgi network. Gp indicates processed form of G protein and Gnp nonprocessed form. Figure modified from Rahkila et al (1998).

6 Cell culture

Tissue culture has developed into a widespread technique as cultured cells are being used increasingly in the discplines ranging from biochemistry to molecular and cell biology. In general, tissue culture procedures are simple but require care to avoid contamination. Skeletal muscle is relatively easy tissue to culture in large amounts, because it is plentiful and easily available from a wide variety of species. Moreover, the stem cells from which the muscle develops seem to be very resilient; they will survive anoxic conditions within muscle at temperatures between 4°c and 37°c for up to a few days, and are easy to freeze and store in suspension. [Partridge 1989]

6.1 Principles of muscle cell culture

In monolayer muscle cell culture the aim is at regulating extracellular conditions in such a way that they as exactly as possible imitate the conditions *in vivo*. Most cultured cells need support for attachment to grow; this may be a plastic surface of a Petri-dish or bottle, but many cells demand a more physiological surface for attachment, such as a polylysine- or collagen layer. In addition to physical support, cells need nutrition: the contents of artificial medium vary with the specific needs of the cell and the purpose of culturing. Typical medium consists of inorganic salts, glucose, amino acids and vitamins, in addition to distilled water. Usually a quantity of animal serum is added to assure the sufficient gain of different, partly unknown growth factors. Cell culture is performed at physiological temperature and in a CO_2-rich atmosphere in order to maintain the buffering abilities of the medium. [Celis and Celis 1994]

6.2 Cell lines

Whereas culturing with primary cells may more closely reflect the situation *in vivo,* more stable and tolerant cell lines make an attractive alternative. Established cell lines are primary cell-derived mutants and capable of dividing endlessly - a trait primary cells do not exhibit. Other distinct advantages include the fact that these cells are clonal and not contaminated with other non-muscle cell types. Moreover, proliferating myoblasts can be

effectively prevented from spontaneous terminal differentiation, which is then inducible by a relatively short exposure to serum deficient medium or long-term culturing. Finally, the presence and the temporal expression of myogenic regulatory factors and many muscle-specific markers are well characterized in these cells. [Neville et al 1998] Continous lines are available from the rat (L6, L8) and mouse (MM14, G8-1, C2), but not from human origin. It should be kept in mind, however, that these cells do not always retain all of the morphological and biochemical characteristics of primary cultures, and should not be accepted uncritically as complete models of normal myogenesis. [Partridge 1989]

The rat L6 line

The L6 line was derived by Yaffe (1968) from a rat neonatal hindlimb preparation. L6 cells remain as proliferating myoblasts in DME supplemented with 20 % fetal calf serum. Differentiation can be induced upon serum reduction (2 % horse serum) [Neville et al 1998]. L6 cells do not express their own IGFs and are thus dependent on the IGFs present in the medium [Ewton and Florini 1981]; these cells also express a limited number of myogenic cell markers upon differentiation and are characterized by the lack of MyoD in both myoblasts and myotubes [Lathrop et al 1985].

7 Purpose of study

The purpose of the present study was to confirm and refine the findings [Kellokumpu et al 1995, Rahkila et al 1996, 1997, 1998], according to which the division of VSV G into two differently targeted forms occurs in differentiating muscle cells, and approximately half of the cells acquire endo H-resistance. Additional aims were to chronologically couple this division phenomenon to the known steps of myogenic differentiation process and to further confine the location of the division process.

One specific aim of this study was to define the point of time during the differentiation process, at which the G protein starts to digress from the myoblastic transport route through the Golgi. The change in the trafficking of glycoprotein is suggested to reflect the formation of sarcoplasmic reticulum [Rahkila et al 1996], an event which the result would thus indirectly indicate. In this experiment, metabolic labeling of G protein with radioisotope coupled to electrophoretic detection was utilized. Further, the relative amount of presumably digressing form of G protein was affirmed by densitometry.

We also coupled the point of digression - and thus the proposed formation of the SR - with the known steps in myogenic differentiation, as demarcated by CK-activity and morphological changes in cells. This was done in order to gain new knowledge about the timing and sequence of events in differentiation process.

An additional step was to resolve whether the fractions of G protein enter the medial Golgi during differentiation, thereby confining the location of digression. This knowledge was to be achieved by the results of metabolic labeling connected with endo H-digestion.

8 Materials and Methods

This study was conducted in the Research Laboratory of Sport and Health Sciences at the University of Jyväskylä.

8.1 Materials

Cell culture media and reagents

All media components, if not otherwise stated, were obtained from Gibco BRL (Gaithersburg, MD, USA). For cell culture, growth medium (Dulbecco's minimum essential medium [DMEM] Glutamax, 5 % fetal calf serum [FCS], antibiotics 100 U/ml (penicillin/streptomycin) and differentiation medium (DMEM Glutamax, 1 % inactivated horse serum [HS], insulin lente MC [Novo nordisk, Danmark] 0.4 U/ml, antibiotics as above) were prepared according to instructions by the supplier. As viral infection medium a MEM-based solution was used (MEM, 0.1 % bovine serum albumin (BSA), 2mM glutamine). Phosphate buffered saline (PBS; 150 mM NaCl, 10mM Na/Na_2PO_4, pH 7.4) was diluted from 10 * stock and paraformaldehyde (PFA) (Sigma chemicals, St. Louis, USA) from 8 % solution.

As myogenic cells, rat-derived L6 cell line (ATCC, Maryland, USA) was used. Deattachment of cells was accomplished by trypsin treatment, and as lysis buffer a solution of 10 % deoxycholic acid (Sigma), 10 % Triton X-100 (Fluka biochemica, Neu-Ulm, Germany) in PBS was utilized.

Antibodies

Antibodies against myogenin (polyclonal rabbit [Santa Cruz Biotechnology, Santa Cruz, CA, USA]) and desmin (monoclonal mouse [Zymed laboratories, San Francisco, CA, USA]) were used, together with Hoescht dye 32258 (Hoescht, Frankfurt, Germany) to visualize the nuclear positions. As secondary antibodies donkey-made, FITC-conjugated anti-rabbit and Texas red-conjugated anti-mouse IgG were used; these were obtained from Jackson Immunoresearch. For embedding purposes Mowiol 4-88 (Hoeschst) containing approximately 2.5 % 1,4-diazobicyclooctane (Sigma) was utilized.

Viral infection and radioactive labeling

Vesicular stomatitis virus stock, a generous gift from professor Metsikkö (University of Oulu), was used for viral infection. Radioactive (35S)-methionine (Amersham Corp., Bucks, England) acted as a label for protein synthesis. Unlabeled methionine was purchased from Gibco BRL.

Endo H-digestion

Endoglycosidase H enzyme was purchased from Boehringer Mannheim, Germany.

Equipments

A phase-contrast microscope (Olympus CK2, Germany) and fluorescence microscope (Olympus BX50, Germany) were used in visualizing the samples. Measurements of absorbance were done with a UV/VIS-spectrophotometer (Shimadsu UV160A, Japan). Total protein concentration was estimated with a kit (Bio-Rad, CA, USA), as well as creatine kinase activity (Bio-Rad). Gel electroforesis apparatus was constructed at the Research Laboratory of Sport and Health Sciences (University of Jyväskylä). Quantification of radioactive bands was accomplished using Molecular Mechanics scanner coupled to a computer with ImageQuaNT (Molecular Dynamics, USA) software. For film development Curix 60 – system (Agfa-Gevaert, Germany) and Kodax X-OMAT AR film (Kodak, New York, USA) were utilized. Statistical analysis was done with Microsoft Excel-software (Microsoft, USA).

8.2 Methods

Cell culture

Details of the general cell culture can be found elsewhere [Celis and Celis 1994]. Shortly, the frozen myoblast suspension (1 ml) of L6 cells was thawed into 10 ml culture dishes or bottles with growth medium, and cultured in a 5 % CO_2 atmosphere in growth medium. Upon reaching approximately 50 % confluence, cells were detached using trypsin treatment and divided 1:5.

For differentiation purposes the cells were transferred to 1 ml culture dishes. Cells were allowed to reach 70 - 80 % confluence, and then switched to differentiation medium, fed

daily. Samples were taken immediately at the switching and according to the following post-switching schedule: 6, 12, 18, 24, and 36 hours; 2, 3, 4, 5, and 7 days. Two samples were taken simultaneously, one of which was fixed for immunohistology, and cell mass of the other scraped into ice-cold phosphate buffer (100 µl) and frozen at -80°c.

Spectrophotometry

Frozen samples were thawed and refrozen thrice, centrifuged at 13000 rpm for 5 min, sonicated three times for 15 sec, and finally diluted for analyzation. Absorbances of all samples were measured, and the total protein concentration was estimated with known standards. CK-activity was calculated from absorbance values with the following equation (Bio-Rad):

$$\text{mkat / l (25°c, 340 nm)} = 75.4 * \Delta A / \text{min}$$

Viral infection

Vesicular stomatitis virus stock was diluted 1:15 in infection medium, and applied onto the sample dish (100 µl/dish). Adsorption was performed for 1 hour at 37°c. Subsequently, the sample was incubated for 3 - 5 hours at 37°c.

Metabolic (pulse-chase) labeling

Pulse-chase labeling with [35S]-methionine was performed at 3 - 5 hours post-infection. Samples were subjected to a methionine-negative medium for 15 min. A 15 minute pulse labeling (100 µCi/ml) was done at 37°c, followed by a 90 min chase time in a solution of excess methionine. Samples were taken into lysis buffer on ice at the beginning, at 45 minutes and at the end of chasing period.

Immunofluorescence studies

Cells were fixed in 4 % PFA for 10 min at 37°c, and then permeabilized with a 10 min Triton X-100 (0.5 %) treatment [Louvard 1980]. Immunofluorescence labeling was done using polyclonal anti-myogenin and monoclonal anti-desmin antibodies that were applied to the cells in 1 % BSA at 1:10 (anti-desmin) and 1:50 (anti-myogenin) dilutions, and incubated for 1 hour at 37°c. The cells were then washed thrice for 5 min with PBS, and secondary antibodies labeled with FITC or Texas red were applied at 1:150 dilutions and incubated for 1 hour at 37°c. Nuclei were visualized with Hoeschst 33258 stain.

For morphological observations, cells were fixed as described above and then embedded in Mowiol 4-88. Samples were stored in dark at 4°c and inspected with a fluorescent microscope, no later than four days after having been taken. The samples were then photographed and the fraction of myogenin-positive nuclei was counted manually.

Endoglycosidase H-digestion

For endo H-digestion, samples were incubated in gentle shaking with endoglycosidase H for at least 18 hours at 37°c and subsequently transferred to sample buffer for SDS-PAGE.

Significance of endoglycosidase H digestion

The conversion of glycoprotein from the endo H-sensitive form into the endo H-resistant form is a medial Golgi-specific event [Davidson and Balch 1992]. This finding has been extensively used to investigate whether the target proteins enter the medial Golgi during their transit. The two different forms of glycoprotein can be distinguished by their mobilities in SDS-PAGE gels [Dunphy et al 1985, Balch and Keller 1986]. The movement of proteins from the ER to the Golgi can thereby be assessed by the conversion of VSV G from a totally endogycosidase D (endo D)-resistant form to a species containing one endo D-resistant and one endo D-sensitive oligosaccharide. Similarly, the delivery to the medial cisternae of the Golgi can be measured by the acquisition of complete resistance to endoglycosidase H (endo H) and the delivery to the TGN by the appearance of an endo H-resistant form of VSV G which is sensitive to digestion with neuraminidase and subsequently beta-galactosidase. [Davidson and Balch 1992]

Gel electroforesis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10 % gels as described by Laemmli (1970). Shortly, samples were lysed on ice in lysis buffer and pelleted briefly. Samples were then diluted 1:2 in sample buffer and heated for 3 min at 96°c. Gels were driven with 16 mA or 18 mA until the dye front had reached the bottom of the gel. Gels were fixed in a H_20 solution containing 40 % methanol and 10 % acetic acid by volume, dried and exposed to a film. The presence of unprocessed and fully mature G-protein bands in each fraction was quantified using a computer-aided image analysis program.

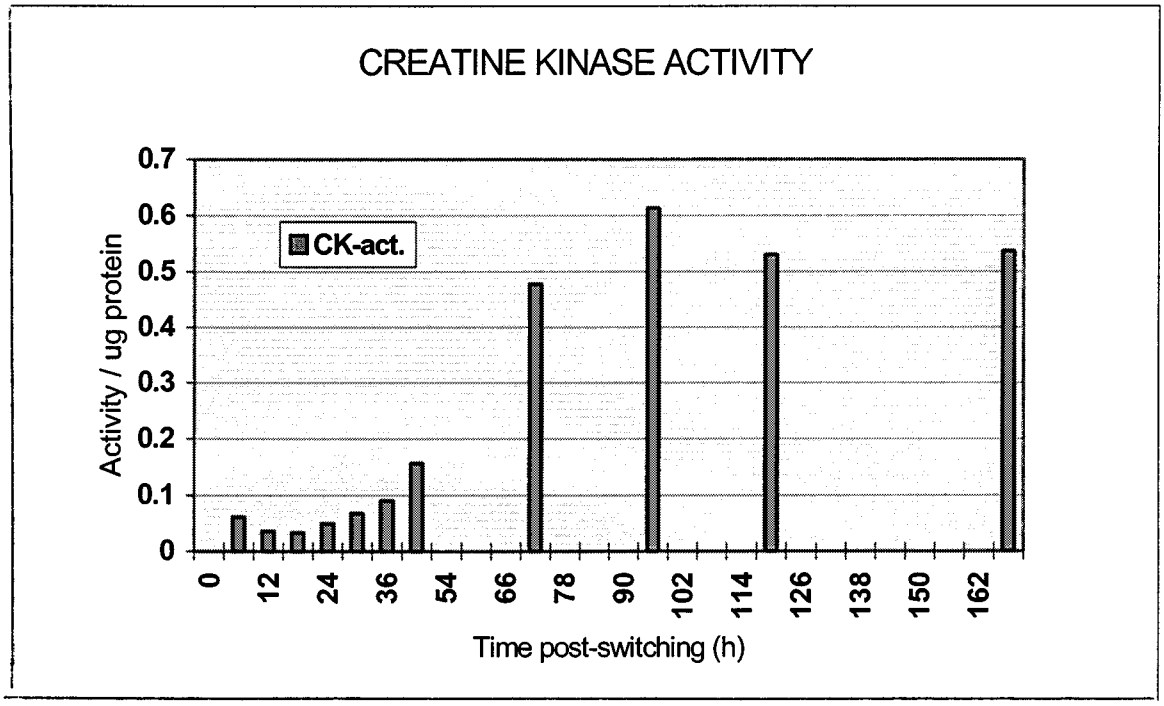
9 Results

9.1 Classic markers of differentiation

Creatine kinase activity

The total amount of protein varied from 3.2 to 18.0 µg per sample; and the changes in absorbance values were between 0.00067 ΔA/min and 0.10533 ΔA/min. The activity of creatine kinase was derived from the mean of two absorbance measurings of a sample. *Graph 1* shows that creatine kinase activity started to rise after 2 days in differentiation medium and reached its peak 3 - 4 days after the beginning of differentiation process. In graphs 1 and 2, legend "time post-switching" refers to the time after the onset of differentiation.

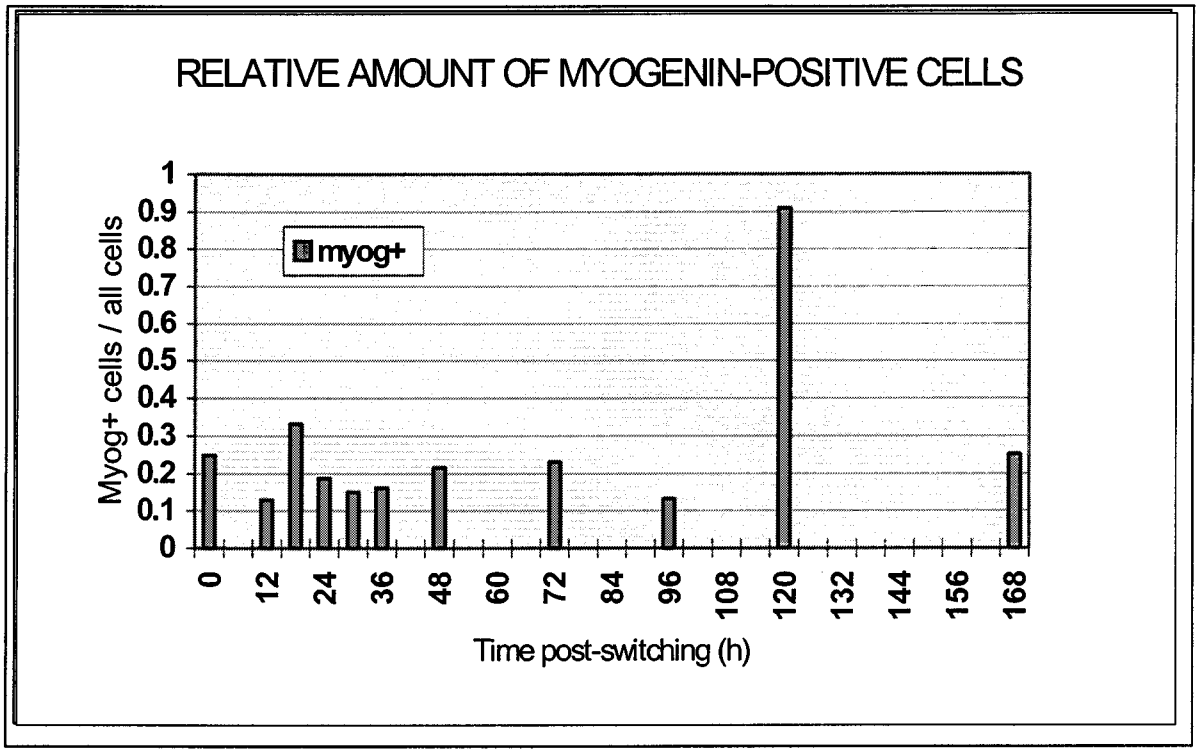
Graph 1



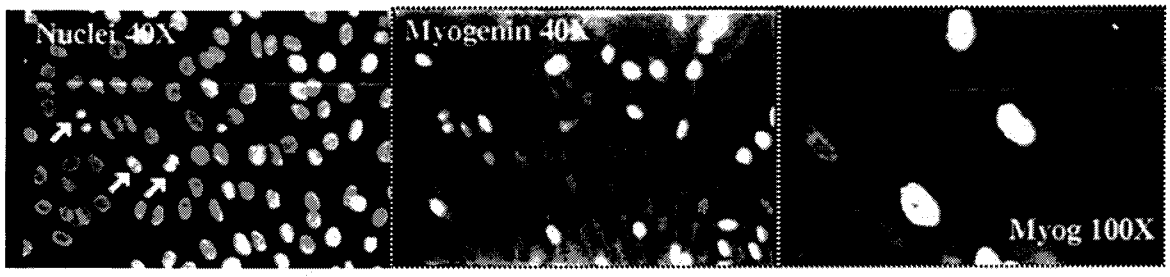
Expression of myogenin

The total amount of nuclei was 242 - 580 / photograph, of which the fraction of myogenin-positive nuclei accounted for 11 - 91 % *(Graph 2)*. Fusion development can be concluded to be complete after 5 days in differentiation medium.

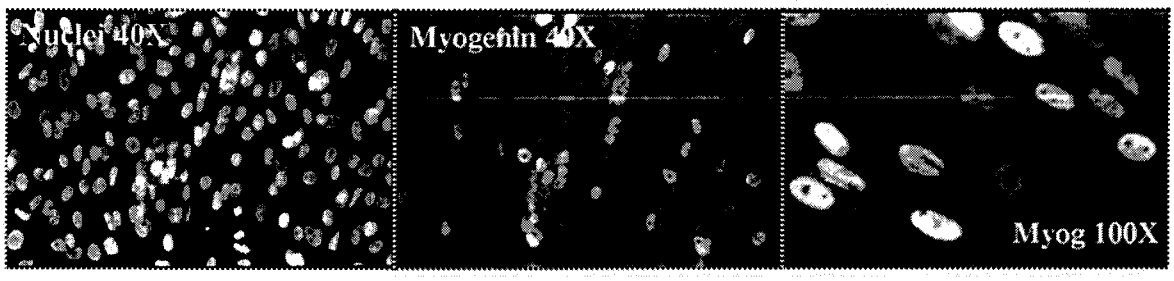
Graph 2



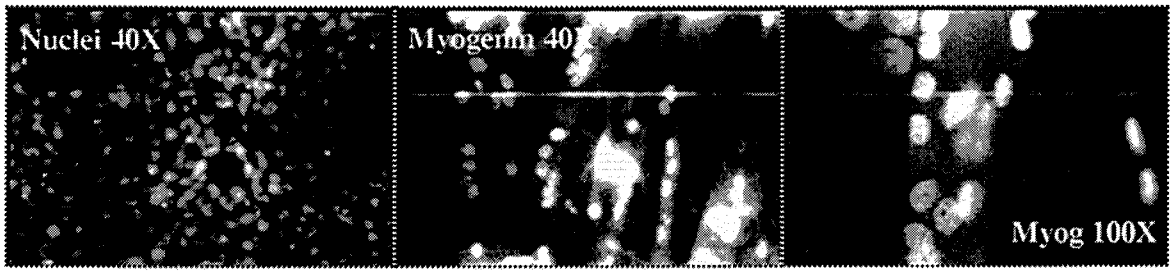
Photographs of differentiating cells



The set of photographs above is of a sample taken at the onset of differentiation of L6 cells. A hoescht-stain *(left)* indicates the nuclei in the sample; anti-myogenin staining *(middle)* indicates myogenin-positive nuclei in the same sample; and finally *(right),* there is a 100X magnification of anti-myogenin stained sample. White arrows point at dividing cells.



The set of photographs is taken of a sample at 36 hours after the onset of differentiation process. The pictures are taken as in the first set. Multinucleated myotubes can be clearly seen in the photo of anti-myogenin staining *(middle)*.



This set of photographs is taken three days after the onset of differentiation. Myotubes are larger than at 36 hours and they contain more nuclei.

9.2 Vesicular stomatitis glycoprotein

9.2.1 Metabolic labeling

In metabolic labeling, the phenomenon of the G protein dividing into two discreetly moving forms can be seen weakly at 24 hours and undisbutedly after 36 hours after switching of the media *(figure 10)*. Furthermore, the densitometric values reveal that approximately half of the viral protein (50 - 65 %) was in faster-moving form after the aforementioned timepoints *(table 1)*.

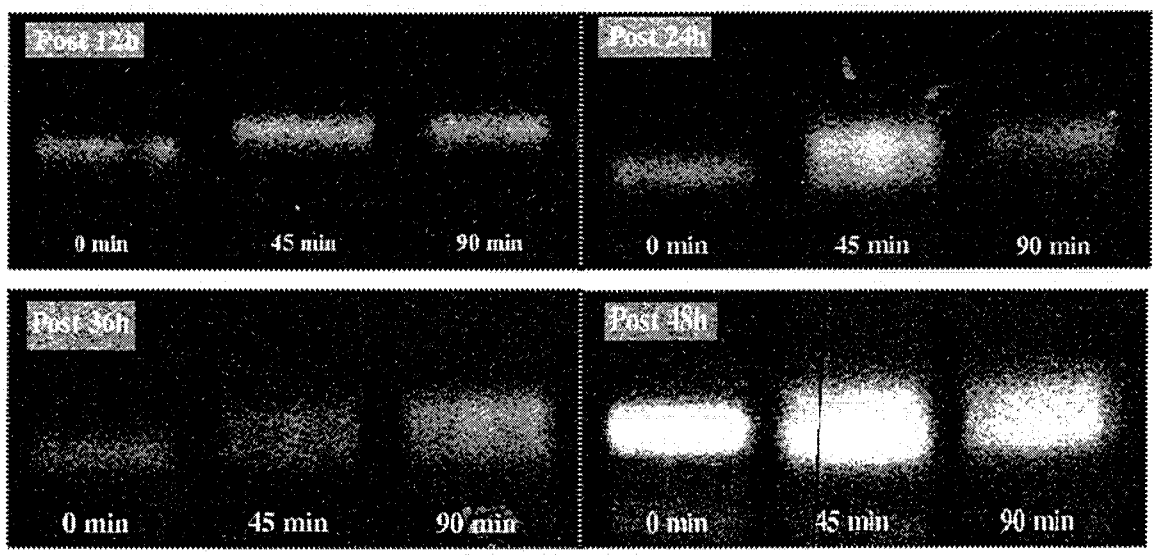


Figure 10: Metabolically labeled samples taken 12, 24, 36 and 48 hours after the onset of differentiation. Chasing times of 0, 45 and 90 minutes were used.

	Sampling time after the onset of differentiation								
Chase time	*0 hours*	*12 hours*	*24 hours*	*36 hours*	*48 hours*	*3 days*	*4 days*	*5 days*	*7 days*
0 min	18	0	0	0	14	19	18	17	14
45 min	42	85	44	44	32	46	48	42	48
90 min	85	85	**62**	**44**	**35**	**50**	**46**	**49**	**49**

Table 1: Densitometric values of metabolically labeled gels. Darkened numbers indicate samples where G protein has divided into two forms with different mobilities.

9.2.2 Endoglycosidase H-digestion

In endoglycosidase H-digested gels, the endo H - sensitive form of VSV G protein can be seen 24 hours (weakly) and 48 hours (clearly) after the onset of differentiation. The division phenomenon occurs after 45 minutes chase *(figure 11)*. Only half of the G protein had acquired endo H - resistance after 48 hours in differentiation medium *(table 2)*.

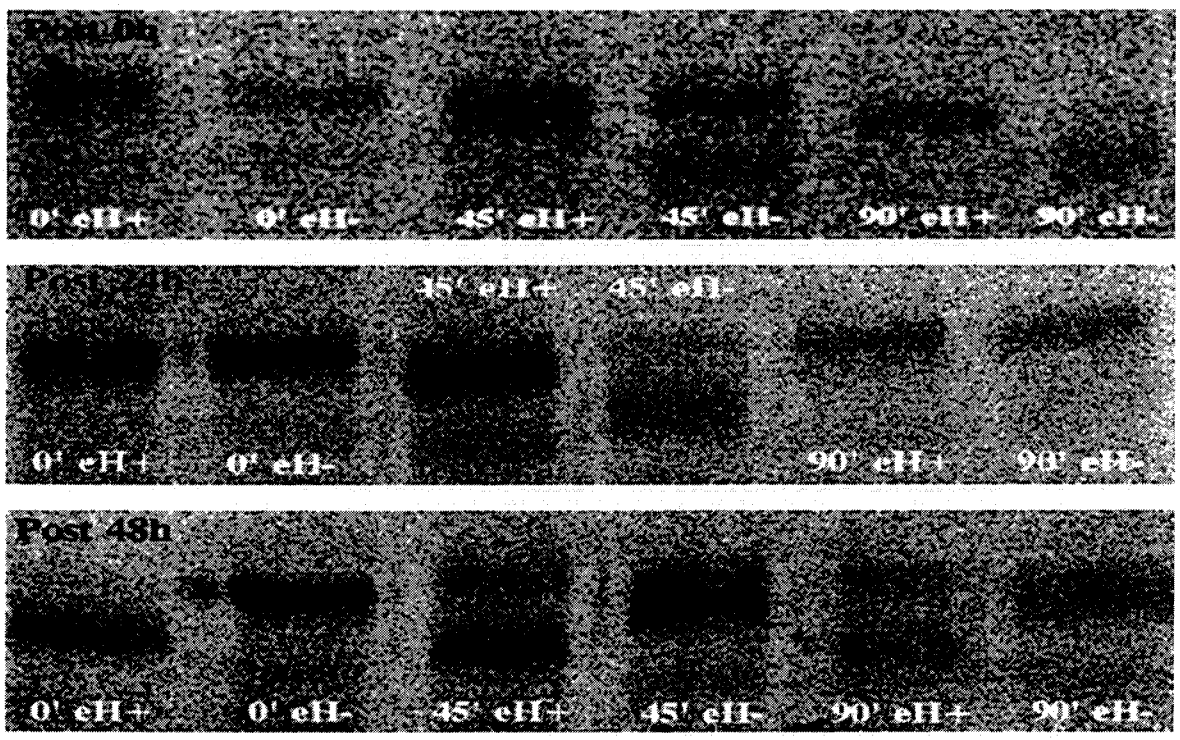


Figure 11: Endoglycosidase H-digestion gels from samples 0, 24 and 48 hours post switching of the medium. Chasing times were 0, 45 and 90 minutes with a negative control for each timepoint.

	0 hours		24 hours		48 hours		3 days	
	endoH+	endoH-	endoH+	endoH-	endoH+	endoH-	endoH+	endoH-
0 min	61	94	88	88	85	75	75	78
45 min	60	67	78	67	**62**	71	**53**	79
90 min	82	NA	93	100	**43**	66	**42**	78

Table 2: Densitometric values of endoglycosidase H-digestions. The numbers indicate percentage of G protein in endo H-resistant form. Timepoints in the first row indicate sampling time counted from the onset of differentiation. EndoH+ indicates endoglycosidase H treatment and endoH- control samples. Chasing times are shown in the first column. Darkened numbers indicate that the division has occurred.

10 Discussion

In vitro studies have been established as one of the best alternatives to study protein trafficking in muscle cells. However, there is a problem with studies of this kind: are these results applicable *in vivo*? - this is a question which can not be ultimately answered without direct studies on a living muscle. Another question constantly met in cell culture studies utilizing viral infection is whether the viral infection affects the results or not? This, too, creates an unphysiological environment and complicates the interpretation of results: one must remember, that viruses do not have access into the muscle *in vivo*. Finally, it is unclear if the ongoing cell's own protein synthesis which has been shown to continue 6-10 hours post-infection [Rahkila et al 1996] affect the results. According to metabolic labeling results in the same study, this would not be the case. Keeping these methodological hindrances in mind, the results of this study can be considered reliable and meaningful.

Timing and sequence

In this study we established timing of the suggested development of the SR in relation with other differentiation-specific events. CK activity and expression of myogenin as well as the fusion index in this study were well in line with the results affirmed before [Woods et al 1997]: CK activity started to rise in one day and achieved its maximum 6 days post-plating; the fusion index reached its peak values one day before that; expression of myogenin was occurring all through the experiment, so there is not much to be derived from this data. It can be concluded that the rearrangements in trafficking pathways, being complete approximately one day post-plating as seen in electroforesis gels, occur far before phenotypic differentiation or fusion of mononucleated cells into a skeletal muscle fiber. Therefore, differentiation-induced changes in VSV G trafficking seem to be among the first steps of differentiation of L6 muscle cells. When present, the division phenomenon can be seen at 45 minutes after the synthesis of G proteins. The molecular mechanism eliciting changes upon transport pathway remains a mystery.

In this study, the expression of myogenin can also be seen in myoblasts, which should not be the case as myoblasts should begin to express myogenin at the onset of determination. It could be argued that the expression of myogenin occurs on a very low base-level even in

myoblast, but this seems highly impropable. A more obvious reason for this phenomenon would be that part of the myoblasts had begun to differentiate spontaneously upon reaching sufficient confluence; this problem is constantly met in studies with L6 cell culture [Paavo Rahkila, personal communication].

Reason for digression

The results of this study clearly agree with the findings from previous studies [Kellokumpu et al 1995, Rahkila et al 1996, 1997, 1998], showing undisbutably that the trafficking of vesicular stomatitis glycoprotein (VSV G) changes during differentiation. One theory [Rahkila et al 1998] suggests that the G protein somehow gains allowance to deviate from the myoblastic trafficking route while the sarcoplasmic reticulum develops; this could result from the formation/opening of a novel pathway. Alternatively, the G protein could be forced into this new route by some unknown targeting signal.

VSV G protein, which has been shown to target basolaterally in polarized epithelial cells, has been found in the SR in mature muscle fiber [Rahkila et al 1996]. Based on this result, together with the results of this study, as well as later studies by Rahkila and colleagues, it can be suggested that the reason for digression would be the development of the SR. If such is the case, the molecular signals leading to development of the SR, as well as the signals for targeting of VSV G to the SR, remain to be elucidated. Another possible reason for digression of VSV G is that the declining capacity of the Golgi to handle the amount of VSV G forces the unprocessed form onto a new route, as has been suggested to be the case with AchE [Rotundo et al 1989]. Therefore, a recent finding that the influenza virus glycoprotein (WSN HA) does not exhibit digression [Rahkila et al 1998] may be because of the different trafficking mechanims for the two viruses; or because the WSN HA selectively does not gain the allowance to stray from the path. We conclude that the reason for digression would be a development of a novel intracellular compartment - whether or not it will be identified as the SR remains to be investigated.

The location of digression

The results of the endoglycosidase H-digestion suggest that the unprocessed VSV G does not enter the medial Golgi or, alternatively, that the lack of processing derives from the inactivity of the Golgi stacks. The latter case would imply that the differentiation-induced changes in the Golgi apparatus were degenerative, which seems implausible in the light of

continuous functioning of the Golgi in mature muscle cells. It can therefore be concluded that deviation of different forms of G protein occurs either in the CGN or outside the Golgi apparatus - possibly during transit through the IC/VTC-region

Half-digression

The significance of digression of only half of the G protein seems to be at key position in unraveling the mechanism of this type of transport. It is easy to postulate that the portion of VSV G changing in their endo H-sensitivity has remained in pre-differentiation pathway. Other possibility is that all protein circulates through the SR, or whatever the destination, and finally only half of that amount ends up going through the medial Golgi. In this case, a whole new transportation pathway would have been formed into which the digression could occur as speculated; it could therefore be that the endo H-resistant form was to be directed away from this newly formed/opened route. In conclusion, the transport mechanism of endo H-resistant form of VSV G protein awaits to be elucidated.

It is possible that the fraction of the G protein remaining endo H-sensitive was transported to Golgi and then back to the SR/ER endomembranes in the skeletal myofibers, as suggested by Kellokumpu et al (1995). In this case, the glycosylation machinery of the Golgi would have been rendered defective during the redistribution of Golgi, which is not supported by the recent findings of Rahkila et al (1998). We thus conclude that the endo H-sensitive form of VSV G protein did not enter the medial Golgi but was transferred to the newly formed endomembranous destination, which we suggest to be the SR, via other route. The mechanisms of this transport remain a mystery.

Rotundo et al (1989), showed that all the newly synthesized AchE polypeptide chains were transported from the RER to the Golgi apparatus. However, only a subset of these molecules actually transit the Golgi, acquire endo H resistance, and are exported by the cells. It was concluded that muscle cells synthesize many more AchE polypeptides than will become assembled into functional exportable oligomers, and that much of this excess enzyme is sorted after entering the Golgi to another organelle(s) for degradation. Whether similar saturation-based model for deviation can be applied to the case of VSV G is unclear.

Trafficking route and targeting

The present study did not shed much light on the trafficking route for VSV G protein from the compartment of synthesis along the post-differentiation pathway to the SR: is it vesicle- or tubulus mediated, or even direct? Are there any coat proteins involved in this trafficking - and if there are, which are they? Would the transport occur by a direct pathway, through IC, through IC-CGN or along a to-and-fro route?

Further problems

Additional questions in the field of VSV G transport and targeting are many: Are there signals for different destinations or is the half-digression a stochastic phenomenon? In the case of there being a signaling system, what are the signals sorting the G protein into fractions? Moreover, these results do not reveal why the relative amount of unprocessed protein stays constant, and what the reason is for a proportion of 50 % - as opposed to, say, 30/70 % or 80/20 %, as similarly demonstrated in other studies. And finally, the age-old question, are the ER and the SR of the same endomembrane origin?

In conclusion, this study provides us with evidence that the differentiation of mononuclear myoblast cells into a multinucleated muscle fiber induces changes on the transport pathway of VSV G protein, and further that only half of the protein deviates from the original pathway. Spatially, the digression occurs somewhere between the ER and medial Golgi and temporally, before the phenonotypic differentiation of muscle cells. We postulate that the reason for the deviation phenomenon may be the development of a novel intracellular compartment, possibly the SR.

Acknowledgments

We are grateful to Paavo Rahkila for invaluable practical as well as scientific assistance and for critical appraisal of the manuscript. We also like to thank Taru Ikäheimonen and Mikko Tanska for helping with the pictures as well as Veera Kettunen for correcting the language. We thank Timo Takala for providing the means to complete the work.

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