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REGULATION OF GLUCOSE UPTAKE IN DIFFERENTIATED CELLS

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1. ABSTRACT

Glucose uptake into the cell is mediated by a family of glycosylated membrane proteins, called glucose transporters (GTs) that are able to facilitate passive hexose transfer across the lipid plasma membrane. The tissue-specific transporter isoforms generally differ in their affinity to the natural substrate D-glucose according to the specific functions of the respective organ.

The mechanisms by which external and internal signals regulate glucose uptake into the cells belong to one

of the most extensively studied fields of cell physiology. However, in spite of significant progress in identifying the involved molecular components and signaling pathways, the final cellular mechanism responsible for the short-term regulation of glucose uptake is still a matter of intense debate. The widely accepted translocation hypothesis, which explains transport regulation by exo- and endocytic modulation of the number of GTs in the plasma membrane, insufficiently accounts for the whole insulin-induced transport stimulation and is insufficient to integrate the wide variety of different transport-modulating signals in differentiated tissue cells into a common mechanistic concept.

Some time ago, a novel type of glucose transport regulation has been proposed prevailing in differentiated tissue cells. This mechanism depends on the presence of glucose transporters on microvilli of differentiated cells. The basic framework for this theory was provided by a recently presented novel concept of ion channel regulation via microvillar structures [Lange, K. (1999): Microvillar Ca^{++} signaling: A new view on an old problem. J. Cell. Physiol. 180, 19–35; Lange, K. (2000): Regulation of cell volume via microvillar ion channels**.** J. Cell. Physiol. 185, 21-35; Lange, K. (2000): Microvillar ion channels – Cytoskeletal regulation of ion fluxes. J. Theor. Biol. 206, 561-584], earlier studies on glucose transport regulation and a number actual biochemical findings. Here, a survey on both concepts is given and the ability of the novel mechanism of microvillar transport regulation to integrate a large body of experimental data into the common concept of cellular regulation via microvillar pathways is discussed.

2. INTRODUCTION

The mechanism for glucose uptake into the cell, necessary to provide the cell metabolism with the most important energy-producing substrate, belongs to one of the most ancient biological devices conserved over a long distance of the evolutionary pathway. In the primary form of life, the unicellular organism, a main problem was the transfer of the hydrophilic sugar molecules across the hydrophobic lipid membrane delimitating the internal water space of the cells from the environment. In order to facilitate this process, transport proteins evolved specifically designed to accelerate the normally very low transfer rate of this sugar through lipid bilayers. Thus, glucose transporters are the most ancient players on this scene. At a later state, however, living systems have undergone an enormous complication. Rapid growth of protozoa under favourable environmental conditions is inevitably followed by food depletion at higher cell density. Preservation of live under conditions of starvation was one of the most severe challenges that evolution has to stand. The development of multicellular organisms was an ingenious answer. Most of the recent work in cellular physiology and biochemistry is aimed at understanding the extremely sophisticated mechanisms nature has elaborated in response to nutritional deficiency.

An early organism living on the borderline between uni- and multicellular organisms, the slime mold *Dictyostelium discoideum*, has told us an interesting story about this phase of evolution. The life of this organism starts as a swarm of multiplying cells freely suspended in the nutrient-containing fluid. However, as soon as local nutrient depletion occurs, cell aggregation is initiated. Starving cells secrete cAMP as chemotactic signal that induce other cells to move to the starvation centre where aggregation and differentiation to a multicellular fruiting body occurs. Up to the now-living highly organized animals, the basic processes of cell differentiation remained unchanged; depletion of essential nutritional components results in production of cAMP and cellular differentiation.

The slime mold example suggests that an essential endeavour of differentiation is reduction of cellular food intake. Cell aggregation is only one aspect of this process. A vast amount of further changes in the organization of cell metabolism is induced in parallel. Some of these changes are specifically aimed at reducing the unrestricted uptake of energy-providing substrates across the plasma membrane and submit this process to the superior demands of the organism as a whole. Furthermore, energy metabolism is reorganized for enhanced efficiency.

One of the most important differences between rapidly growing undifferentiated cells and their differentiated counterparts is the type of glucose uptake regulation. Whereas the undifferentiated cell exhibits unrestricted glucose uptake via transporter proteins on the cell surface, the transport process of differentiated cells is precisely tuned to the actual energy demand. A puzzling variety of external and internal transport modulating signals serves this tuning process. In addition, an arsenal of surface receptors on differentiated cells imparts responsibility of various cell functions to hormonal control.

Another complicating event was the biogenetically late introduction of a highly efficient ATP producing pathway, the oxidative phosphorylation of ADP in mitochondria. This ‘modern’ instrument of ATP production works at 19-fold higher efficiency compared to glycolysis alone. However, the oxidative pathway still depends on glycolysis because it starts with pyruvate, the last but one product of glycolysis. The functional integration of this novel pathway into the ancient metabolic framework of glycolytic energy production belongs to one of evolution’s masterpieces. For this purpose, the establishment of a sophisticated system of regulatory mechanisms was necessary.

Up to now, a vast quantity of information about transport regulation by insulin, “insulin-like” effectors and other environmental signals has been accumulated. Yet, the precise mechanism(s) by which all these effects are mediated is still unclear. The only mechanistic concept proposed by Cushman and Wardzala (1) together with Suzuki and Kono (2), the ‘translocation hypothesis’, explains insulin-induced transport stimulation by exocytic insertion of additional glucose transporters from an intracellular vesicular storage pool into the plasma membrane. In spite of a considerable amount of conflicting data and the obvious failure to integrate the great variety of

insulin-independent forms of transport regulation into a common mechanistic framework, up to now, the translocation hypothesis remained the sole mechanistic concept. The way by which various effectors and conditions such as glucose starvation and glucose feeding (glucose-curb), endogenous demand in muscle cells, hydrogen peroxide, SH-reagents, high Ca^{++}, as well as physiochemical conditions (high pH, hyperosmolarity, shear stress) modulate transport rates is still unclear.

More than 10 years ago, a novel mechanism of transport regulation has been proposed able to explain most of the accumulated experimental data (3-5). This idea was induced by the finding that the GluT4 transporters in differentiated 3T3-L1 adipocytes are exclusively located on microvilli. Further, transport of hexoses via these microvillar transporters was found to be low unless shortening of microvilli or complete disorganization of the microvillar actin filament bundle occurs. Thus, the microvillar cytoskeleton was proposed to function as diffusion barrier between the microvillar tip compartment (entrance compartment) and the cytoplasm. However, this novel concept has been widely rejected.

In the meantime, compelling evidence has accumulated supporting the assumption of a regulated microvillar diffusion barrier, which turned out to be a multifunctional device for modulation of substrate and ion fluxes via transporters and ion channels (reviews: 6-8). This concept was shown to imply even one of the most important cellular signal systems, the Ca^{++} signaling pathway (6). In addition, several recent findings complete the general outline of this concept, e.g. the role of phosphatidylinositol 3-kinase in insulin-stimulated transport, some biochemical aspects of microvilli formation, but most importantly, the finding that almost all types of glucose transporters are preferentially localized on microvilli of differentiated tissue cells.

Here, a survey on both concepts is given and the ability of the novel mechanism of microvillar transport regulation to integrate a large body of experimental data into the common concept of cellular regulation via microvillar pathways is discussed. It should be stressed, however, that the latter mechanistic scheme did not invalidate the translocation concept of insulin action since it concerns the immediate rapid component of transport activation preceding exocytosis. Exocytic recruitment of membrane proteins undoubtedly belongs to the well-established consequences of hormonal and even some non-hormonal forms of cell stimulation and clearly contributes to the overall effects on transport.

3. REGULATION OF GLUCOSE METABOLISM IN DIFFERENTIATED AND UNDIFFERENTIATED CELLS

3.1. Metabolic regulation versus membrane limitation

Glucose uptake via the facilitated diffusion system responds to changing cellular energy demands by two different mechanisms (review: 9, 10). The rate of glucose uptake into undifferentiated, rapidly growing cells such as embryonic and tumor cells or preconfluent cells in culture, largely depends on the activity certain rate-limiting glycolytic enzymes, mainly hexokinase and fructose 6-phosphate 1-kinase or other downstream enzymes that are regulated by adenine nucleotides and intermediates of the carbohydrate metabolism itself. A low demand of metabolic energy causes a bank-up of metabolic substrates upstream of the rate-limiting enzyme(s), which finally results in accumulation of intracellular free glucose and decreased net uptake of glucose. This type of glucose uptake regulation, called 'metabolic regulation', is generally characterized by high levels of intracellular free glucose almost reaching the extracellular value, accompanied by considerable lactate production under aerobic conditions.

A completely different way of regulation is observed in muscle cells, adipocytes, and other types of differentiated tissues cells. Here, metabolic activity is regulated by external and internal signals via modulation of the transport activity across the plasma membrane. The 'transport (or membrane)-limited' cell type is characterized by very low concentrations of intracellular free glucose, which are independent of metabolic rates. Membrane limitation is an essential prerequisite for regulation of glucose metabolism by exogenous signals.

Although most of the recent work on transport regulation was done using membrane-regulated cell types, especially adipocytes and muscle cells, very little is known about the nature of the membrane-limited state itself and the specific mechanisms that enable cells to switch from metabolic regulation to the membrane controlled state. Since the days of Otto Warburg, the problem why tumour cells, but not their differentiated counterparts, exhibit aerobic glycolysis has not been adequately resolved. One reason for this failure of classical biochemistry may rest on the original suggestive idea that tumour cells are metabolically disregulated normal cells, which forced research into the wrong direction. In fact, the critical gap in knowledge concerned normal cells, not tumour cells, and one should have better asked for the specific organization of energy metabolism in normal tissues, which enable differentiated cells to coordinate mitochondrial oxidative phosphorylation and glycolysis in a way that avoids excessive lactate production. This problem, although closely related to the phenomenon of membrane limitation and cellular differentiation, remained largely unnoticed and unresolved up to our days.

Another reason for this gap in our knowledge might be the limited access to differentiated cells for experimental work. Thus, in earlier days, work on energy metabolism was done using erythrocytes or ascites tumour cells; both cell types being far away from the differentiated phenotype. Energy metabolism of these cells is clearly regulated on the level of rate-limiting metabolic steps closely resembling that of archaic unicellular organisms. Not until the establishment of cell culture techniques for tissue cells and differentiation procedures for permanent cell lines, could glucose metabolism and transport of higher organisms adequately be studied in systems really reflecting all the 'modern' achievements of higher living organisms.

Table 1. Mammalian facilitated hexose transporters

Glut isoform	Function (k_m for D-glucose)	Tissue specificity	Subcellular organization
Glut 1	5 mM	Nondifferentiated cells,erythrocyte, neuron and glial cells cells, div. blood-tissue barriers	Plasma membrane / microvilli
Glut 2	16-25 mM	Liver, pancreatic ß-cells	Basal microvilli
Glut 3	1-2 mM	Neuron, placenta, testes	Axonal node microvilli
Glut 4	3-5 mM	Insulin-sensitive tissue	Basal microvilli
Glut 5	6 mM (fructose)	Small intestine fructose transporter, testes and sperm	Luminal intestine surface (brush borders)
Glut 6		Nonfunctional pseudogene product	
Glut7		Putative microsomal GT	Part of the G6Pase complex in liver microsomes

3.2. Membane components involved in transport regulation

3.2.1. Glucose transporter proteins

Several different isoforms of transporter proteins are involved in specific tissue functions related to regulatin of hexose metabolism (Table 1)

These various isoforms are clearly designed to serve different functions. Those tissues that exclusively depend on glucose as energy-providing substrate including blood cells, brain, muscle, and fat cells, express the high-affinity transporters Glut1, Glut3, and Glut4. Insulin-regulated tissues such as fat and muscle cells express the Glut4 isoform, whereas all these tissue cells in their undifferentiated (embryonic) states only synthesize Glut1.
In contrast, tissue cells that are involved in homeostasis of blood glucose express the low-affinity isoform Glut2. In these latter cell types, the working point (k_m) of the transporter is shifted to higher concentrations matching the specific tissue function. For instance, pancreatic ß-cells carry Glut2 on their basal microvilli (11) that serve as glucose sensors. Increased glucose uptake in response to hyperglycemia elevates glycolytic ATP within a specific cellular subcompartment and activates insulin secretion via Ca^{++} influx through voltage-sensitive Ca^{++} channels. On the other hand, the homeostatic function of liver cells mainly rests on feeding glucose into the blood stream under hypoglycemic conditions. Stimulated glycogenolysis elevates the intracellular glucose concentration to above 10 mM, sufficient for release of glucose via the low-affinity transporter Glut2.

All mammalian facilitated sugar transporters appear to be glycosylated, presumably at a site located in the large extracellular loop connecting the transmembrane-spanning domains (TMs) TM1 and TM2 (Figure 1). Mutational studies revealed some further structure function relations. The C-terminal cytoplasmic domain is critically involved in glucose binding to the transporter, perhaps by interacting directly with the short intracellular segment connecting TM10 and TM11. Deletion of the last 33 amino acids of Glut1 rendered the protein catalytically inactive, however, truncation of the last 12 amino acids had no effect on the activity. TM10 is assumed to be involved in the conformational change that occurs during the translocation of the sugar through the lipid membrane. The Segments TM7 and TM11 may comprise part of the aqueous channel through which glucose moves.

3.2.2. The insulin receptor

External regulation of cellular energy metabolism affords the presence of surface receptors for regulation of transport and metabolism via extracellular signals. Insulin receptors are expressed in almost all tissues cells. According to the degree of insulin dependence of the respective tissue, the number of insulin receptors on the cell surface varies between 40 in erythrocytes and 200 000 in fat and muscle cells.

Insulin receptors are oligotetramers ($a_2ß_2$). The external portion of the receptor is composed of two a-subunits linked to each other by disulfide bonds. Two ß-subunits form the membrane-spanning and intracellular part of the receptor (Figure 2). The ß-subunits act as tyrosine kinase that is activated by a conformational change of the receptor molecule following insulin binding. This conformation change represents the true signal transduction step through the plasma membrane. Insulin receptor autophosphorylation further generates a cascade of numerous intracellular events.

An essential second step in insulin-induced stimulation of glucose uptake is activation and recruitment of cytoplasmic phosphatidylinositol(PI) 3-kinase to the inner face of the plasma membrane (Figure 3). D-3 phosphorylation of PI lipids yielding PI 3,4-bisphosphate ($PI(3,4)P_2$) and PI 3,4,5-trisphosphate ($PI(3,4,5)P_3$) appears to be an important step downstream of receptor autophosphorylation. A large number of further processes are initiated via this step including stimulation of glucose uptake, of protein kinases, membrane ruffling, and cell motility.

Specific cytoplasmic substrates of the insulin receptor tyrosine kinase, IRS-1 and 2, play an essential role in diversification of the intracellular responses to insulin receptor stimulation. As soon as the ß-subunit is completely phosphorylated, IRS1/2 associates to the cytoplasmic domain of the receptor and is tyrosine-phosphorylated itself. The various, at least twenty phosphorylation sites of IRS-1 allow binding of a large number of different Src homology 2(SH2) motif-containing cytoplasmic proteins that each are starting points of separate signaling pathways to other cellular effector system.

In addition to PI 3-kinase, the actin cytoskeleton has been clearly identified as an essential mechanistic

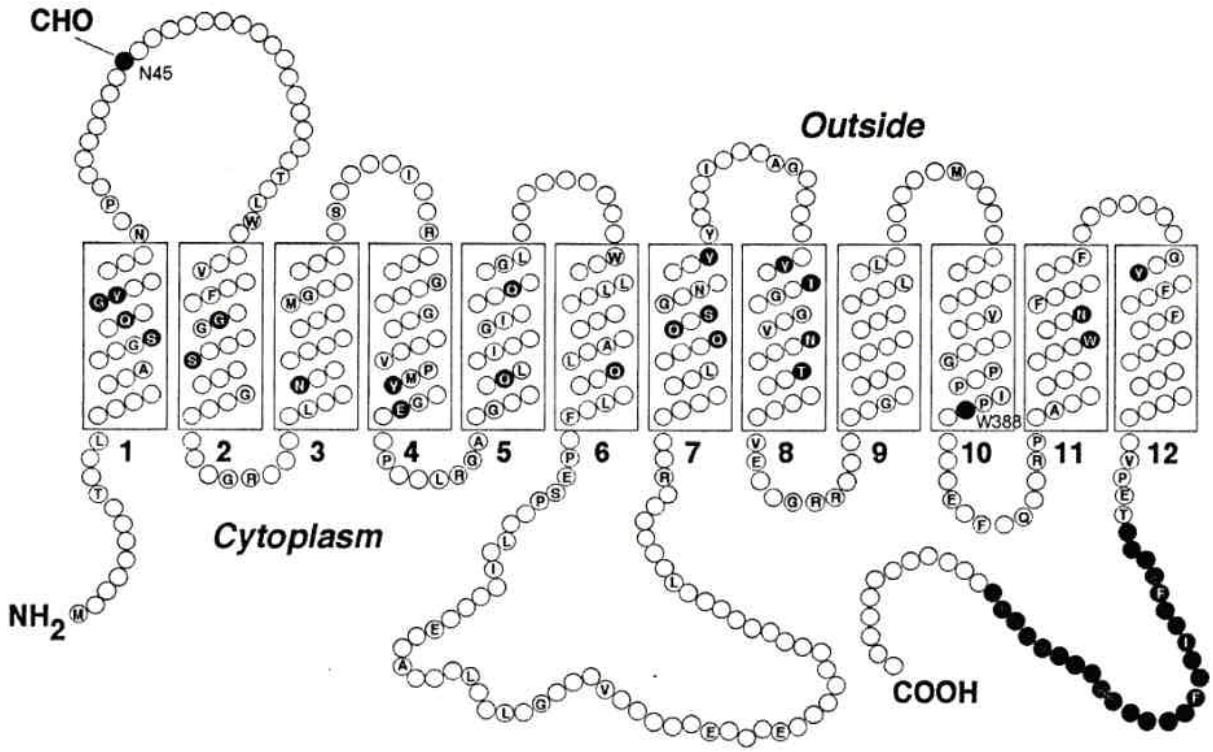

Figure 1. Mammalian facilitated glucose transporter forming twelve TMs. CHO: Potential site for N-glycosylation. Part of the COOH-terminal end (filled circles) is essential for high-affinity binding of D-glucose. TM 10 is immediately involved in the transport process.

component involved in transport stimulation (12-15). As it appears, the PI 3-kinase-induced formation of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ triggers extensive cytoskeletal reorganization, finally resulting in formation of submembrane microfilament networks in the immediate vicinity of activated IRs.

Although it is generally accepted that PI 3-kinase membrane association and activation is essential for insulin-induced stimulation of glucose transport, the further steps of insulin signaling toward glucose uptake stimulation are still unknown. On the other side, Cushman and Wardzala (1) together with Suzuki and Kono (2) (for reviews see: 16, 17) have put forward a distinct idea about the final part of this pathway in their 'translocation theory'. However, although widely accepted, this hypothesis failed to explain all experimental data and, most importantly, yielded no systematic explanation for the events connecting PI 3-kinase activation and stimulation of exocytosis.

4. THE TRANSLOCATION HYPOTHESIS

The experimental basis of the translocation hypothesis strongly depends on subcellular fractionation techniques and especially on the widely held assumption that fractionated sedimentation of cell homogenates yields subcellular (microsomal) fractions that contain only intracellular membrane components and are free from surface components. Although never soundly proved and severely challenged in the past, the results of a large amount of experimental work rest on this assumption.

Early works of Cushman and Wardzala (1) as well as Suzuki and Kono (2) showed that a large portion of transporter proteins originally located in the microsomal fraction of unstimulated cells is found in the plasma membrane fraction of insulin-treated cells. Thus, exocytic transfer of transporters normally residing in an intracellular storage pool was assumed as mechanism of insulin action on transport in adipocytes and other cells (Figure 4).

Further analysis of the kinetics of transporter appearance and disappearance in response to insulin stimulation led to the conclusion that only a 'constantly cycling' transporter pool is compatible with the experimental results. Jhun *et al.* (18) reported that labelled Glut4 is rapidly reduced at the surface of both basal and insulin-stimulated rat adipocytes in steady-state at 37^0C and stoichiometric amounts of Glut4 appear in the microsomal fraction. The equilibrium exchange of Glut4 is describable by two first order rate constants, one for internalisation and one for externalisation. Insulin affects both rate constants, reducing k_{in} by 2.8-fold and increasing k_{out} by 3.3-fold. In contrast, Yang and Holman (19), using 3T3-L1 adipocytes, found the main effect of insulin on exocytosis. The apparent rate constants for endocytosis of Glut4 and Glut1 are only 30% lower in the insulin-stimulated state (k_{in} = 0.08 and 0.093 /min) than in the basal state (0.116 and 0,121 /min) explaining the rapid equilibration of labelled

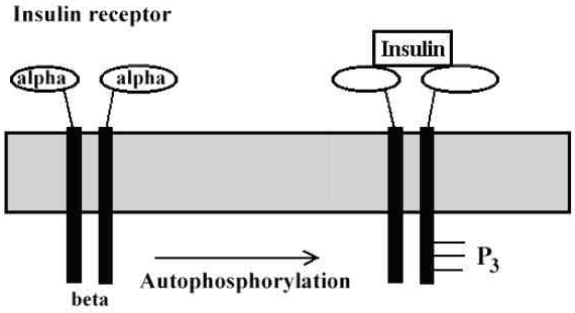

Figure 2. Insulin receptor-mediated signal transfer through the plasma membrane. Ligand binding to the external a-subunits, via conformational changes within the ß-subunit, results in autophosphorylation of the cytoplasmic domain on tyrosine residues.

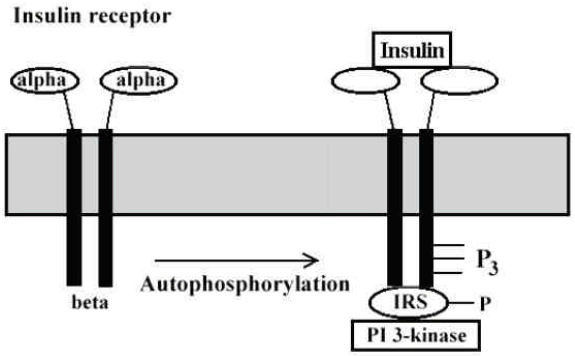

Figure 3. Insulin receptor substrate(IRS)-dependent PI 3-kinase activation and recruitment to the plasma membrane. IRS binding to the tyrosine phosphorylated ß-subunit of the insulin receptor results in tyrosine phosphorylation of IRS and subsequent binding of SH-2 domain-containing proteins such as PI 3-kinase.

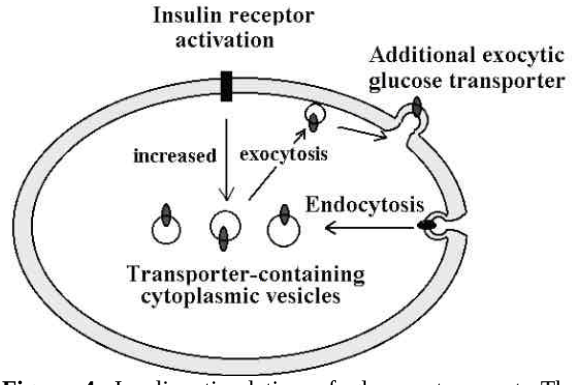

Figure 4. Insulin stimulation of glucose transport. The cellular transporter pool is constantly cycling between the internal vesicle pool and the plasma membrane. Insulin stimulates exocytosis or/and inhibits endocytosis of GT-containing intracellular vesicles to recruit additional glucose transporters to the cell surface.

surface transporters even in unstimulated cells. In contrast, the rate constants for GluT4 and GluT1 exocytosis in the insulin-stimulated state (k_{out} = 086 and 0.096 /min) differ significantly from those in the basal state (0.01 and 0.035 /min). Thus, studies using different experimental approaches yielded quite different results.

Applying the same experimental techniques as for detection of insulin-mediated translocation effects, some forms of insulin-independent transport modulation appear to follow the same type of transporter redistribution from a cytoplasmic pool to the plasma membrane. Thus, glucose deprivation (20), virus transformation (21) high pH and hyperosmolarity (22) action of tumour promoters (23), as well as cold treatment (24) apparently cause an insulin-like exocytic response.

On the other side, a number of other effectors and conditions do not induce transporter redistribution although their action on transport is not additive to that of insulin. Two examples are stimulation of glucose transport by hyperosmotic exposure (25) or by metabolic stress (26) in clone 9 cells. In the latter case, the authors clearly stated that activation of GluT1 transporters pre-existing in the plasma membrane is the predominating mechanism mediating the early response to inhibition of oxidative phosphorylation.

Even the development of various techniques for cell surface labelling of glucose transporters did not solve the main problem of the translocation hypothesis that the appearance of new transporters on the cell surface is unable to fully account for the observed activation of glucose transport (27-29). This obvious shortcoming is reflected by the use of operative or descriptive terms such as “intrinsic activity” or “masking and demasking effects”. Sometimes the conclusions convey a touch of despair: *Insulin-stimulated transport is mediated by a facilitative transport process that is … not labelled strongly by* (the surface label) *ATP-BMPA* (28) or *…the photolabel (ATP-BMPA) can combine with transporters that are at the cell surface but do not fully participate in transport* (27). In another study (29), application of the same labelling technique led to the opposite conclusions: *Insulin-stimulated GluT4 transporters can exist in two distinct states within the adipocyte plasma membrane, one of which is functional and accessible to extracellular substrate, and one, which is non-functional and unable to bind extracellular substrate*.

Dissociation of translocation and transport effects is observed in many experimental systems. Even the total lack of transporter appearance or disappearance on the surface on transport modulation has been reported (30-32). For instance, inhibition of oxidative phosphorylation in clone 9 cells was shown to activate GluT1 pre-existing in the plasma membrane (33-37, 26). Similarly, Fisher and Frost (32) reported that glucose deprivation increased the rate of glucose transport in 3T3-L1 adipocytes 8-10-fold without changing the distribution of GluT1 and GluT4 in the plasma membrane and microsomal fractions. They stated that *the increase in transport activity associated with glucose deprivation does not result from the translocation of either of the glucose transporters known to exist in 3T3-L1 adipocytes.*

In a recent study, Zierler (38) analysed the relation between the insulin-induced translocation and the transport effect reported in five relevant papers. Using a

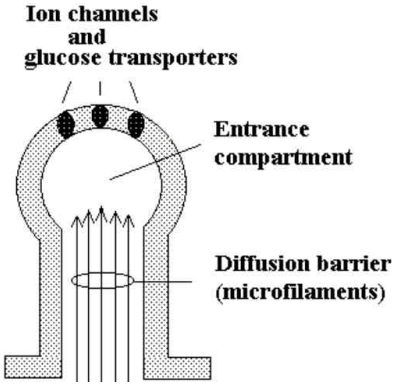

Figure 5. Structural organization of microvilli: The tip membrane contains tissue-specific integral membrane proteins including transporters and ion channels through which ions and substrates enter the tip (entrance) compartment. Entrance compartment and cytoplasm are separated by the microfilament bundle of the shaft region.

model-free mathematical analysis, he came to the conclusion that the concentration of GluT4 in the plasma membrane in response to insulin would have to have been 2- to 7-fold greater than actually observed if translocation alone were to account for the observed increase of glucose uptake. With other words, insulin-induced increase in GT intrinsic activity accounts for more than half the observed increase in glucose uptake.

As pointed out by Olefsky (39), tissue-specific expression of the so called insulin-sensitive glucose transporter Glut4 alone is not the only factor conferring insulin-stimulated glucose transport on these tissues because heterologous expression of GluT4 in other tissues is not sufficient to generate insulin-stimulated GluT4 translocation in non-muscle or non-adipose cells. Consequently, additional factors in muscle and adipose cells, related to insulin signaling must exist. As it appears, exocytosis of transporter proteins to the plasma membrane does not completely describe the mechanism by which insulin stimulate glucose transport. Presumably, a yet unrecognised mechanistic feature is involved in transport regulation.

An early concept of membrane limitation (3-5, 40) is able to solve this problem and to close the mechanistic gap between insulin-induced PI 3-kinase activation and transport stimulation.

5. MEMBRANE-LIMITED HEXOSE UTILIZATION

5.1. Structural organization of membrane limitation

Starting point for the studies on membrane-limited systems was the observation of a special type of endogenous regulation of hexose uptake occurring under specific experimental conditions in C6 glioma cells, a permanent cell line, exhibiting membrane-limited glucose metabolism at confluence (41, 42).

In this cell line, inhibition of glycolytic ATP production, either by pretreatment with iodoacetate or by use of 2-deoxyglucose as transport substrate, resulted in a conspicuous time-dependent periodic fluctuation of hexose uptake rates (40, 42). Although uptake rates changed up to 6-fold, the intracellular concentration of free glucose remained at a very low and completely constant level (1-2 orders of magnitude lower than the extracellular concentration) (42). Constant levels of intracellular free glucose in spite of rapidly changing uptake rates pointed to an effective coupling between transport and phosphorylation of glucose. Tight coupling between membrane transport and hexokinase reaction strongly indicates a specific organization of these processes entirely different from that of metabolic regulation.

Further studies revealed that the observed periodic fluctuation of 2-deoxyglucose uptake is triggered by changes in the ATP content of a special cellular compartment, called ‘entrance compartment’ for hexose transport. This compartment, the site of hexose entry into the cell, appeared to be localized within microvilli and other lamellar surface protrusions (40). Moreover, experimental evidence based on 2-deoxyglucose and 3-O-methylglucose uptake kinetics as well as electron microscopic studies suggested that this entrance compartment is separated from the cytoplasmic main compartment by a cytoskeletal diffusion barrier formed by the central core of actin filaments in microvilli (5, 40). The effectiveness of this diffusion barrier is regulated via the cytoplasmic ATP/ADP system, allowing substrate uptake in response to the actual energy demand.

According to these and other studies (3, 4, 43-46) the following outline of the membrane-limited (or -regulated) state has emerged:

- The establishment of membrane-limited hexose metabolism is based on the differentiation-dependent sequestration of glucose transporters (and other integral membrane proteins such ion channels) to special plasma membrane domains localized on the tips of microvilli (3) (Figure 5).

- The entrance compartment is separated from the cell body by a diffusion barrier attenuating the flow of low molecular weight solutes into the cytoplasm. This barrier is formed by the microfilament bundle, a dense central structure tightly connected to the covering lipid membrane of the microvillar shaft.

- The diffusion resistance between the entrance compartment and the cell body is regulated by structural changes of the cytoskeletal core. Elongation or shortening of the microfilaments is triggered by changes in the ATP and ADP concentrations of both the entrance compartment and the cytoplasm. Exogenous signals such as insulin or other growth factors also induce shortening of microvilli or complete integration of the microvillar membrane portion into the plasma membrane by depolymerization and reorganization of the microfilament bundle (3, 5).

The biochemical equipment of the entrance compartment, which allows the autonomous production of

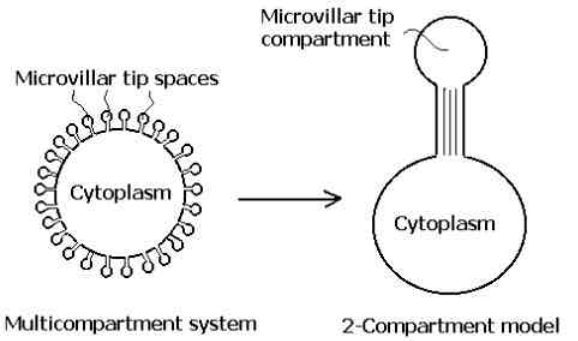

Figure 6. Transformation of the microvillar multi-compartment system into the topologically and functionally equivalent 2-compartment model.

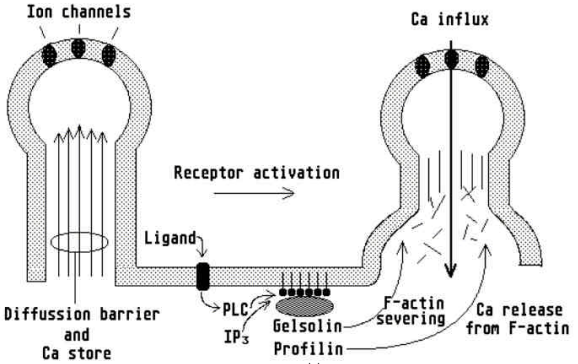

Figure 7. Activation of Ca^{++} signaling by receptor-mediated depolymerization of the microvillar F-actin diffusion barrier allowing coordinated Ca^{++} release from the F-actin Ca^{++} store and Ca^{++} entry into the cytoplasm.

glycolytic ATP as well as the assembly and disassembly of microfilament bundles, imparts to this system self-organizing and self-regulating properties responding to environmental hexose supply. High external glucose concentrations cause reversible elongation of the microfilament bundle, thereby enhancing the internal diffusion resistance of the microvillar pathway for glucose and glucose phosphates (5, 47-49).

5.1.1. The microvillar tip compartment as sensor for changes of environmental conditions

In order to simplify the discussion of this system, the numerous spaces of the microvillar tips are combined giving the topologically equivalent model with one large tip compartment (entrance compartment) (Figure 6). However, the unique arrangement of the finely dispersed peripheral tip spaces in the multicompartment system imparts specific properties on the entrance compartment, which must be kept in mind. For instance, the very small internal water spaces rapidly follow the environmental conditions by equilibration via cation/anion channels and transporters located within the tip membranes. Because of this unique property, the microvillar tip compartment rather reflects the ionic composition of the external medium than that of cytoplasm. Thus, the microvillar tip compartment is qualified as cellular sensor for rapid detection of relevant environmental changes (see 7.1.). On the other hand, microvilli represent a considerable portion of the total membrane area of the cell. Under certain conditions, the microvillar membrane reserve can be used for cell enlargement. In the case of a hypotonic challenge, cells are able to expand their cytoplasm reaching the several-fold of their original volume without membrane damage.

5.1.2. Establishment of the microvillar diffusion barrier is an essential precondition for calcium signaling in differentiated cells

An important differentiation-dependent cytoplasmic parameter is the extremely low cytosolic Ca^{++} concentration that is an absolute prerequisite for Ca^{++} signaling. Maintenance of the steep Ca^{++} gradient between the extra- and intracellular space is essential for regulation of cellular functions by cytosolic Ca^{++}. Numerous important cellular functions such as growth, contraction, secretion, and modulation of the membrane potential are regulated by receptor-mediated Ca^{++} signaling.

As recently proposed, the microvillar diffusion barrier system provides for both the effective blockade of the in- and efflux pathways for cations and the high-affinity Ca^{++} storage system to adjust the low basal [Ca^{++}] levels (7, 8, 50) (Figure 7). According to this concept, cellular Ca^{++} influx can be modulated by the microvillar diffusion barrier (50-54). The phospholipase C(PLC)-coupled Ca^{++} signal pathway triggers Ca^{++} release from microvillar actin filaments by disassembly / reorganization of actin filaments via activation of the PIP/PIP_2-regulated actin-binding and severing proteins profilin, gelsolin/villin, and cofilin (55-57). Due to the biochemical identity of the internal Ca^{++} store and the diffusion barrier system, Ca^{++} release is functionally coupled with the activation of Ca^{++} influx (store-operated Ca^{++} pathway). This mechanism is schematically depicted in Figure 7 (review 6). The morphological aspect of this process was visualized by scanning electron microscopy of vasopressin-stimulated rat hepatocytes (54) and bombesin- and thapsigargin-treated HIT cells (51).

Thus, receptor-stimulated Ca^{++} influx via the microvillar influx pathway is closely related to the proposed mechanism of glucose transport regulation. In the basal state of the cell, transduction of the divalent cations Ca^{++} and Mg^{++} is prevented by the polyanionic nature of the microfilament bundle of microvilli. F-actin bundles behave like cation exchangers representing a hydrated matrix with a high density of fixed charge centres (58, 59 surveyed in 7), to which monovalent cations are loosely associated as clouds of counterions. In the presence of divalent cations such as Mg^{++} and Ca^{++}, the counterion clouds become much smaller and tighter localized to the fixed charge centres, which corresponds to a much tighter binding of the cation (60). Consequently, transduction of Mg^{++} and Ca^{++} through this matrix is highly inhibited and cytoskeletal reorganization is needed to activate the microvillar influx pathway for divalent cations (Figure 7).

5.1.3. Energy metabolism in the membrane-limited state

Since Warburg's observation that tumour cells differ from their normal counterpart by lactate production

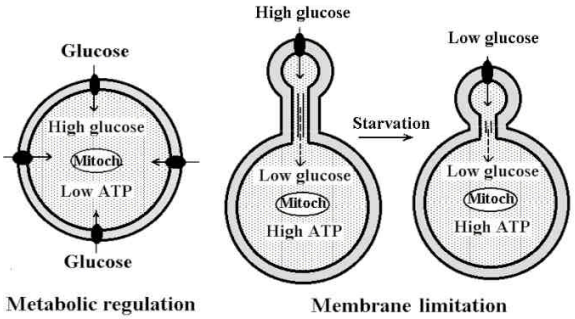

Figure 8. Cellular characteristics of metabolic regulation and membrane limitation. The membrane-limited state allows adaptation of glucose uptake rates to varying levels of external glucose.

under aerobic conditions, the biochemical basis of this and two other metabolic features of malignant cells, inhibition of glycolysis by oxygen (Pasteur effect) and inhibition of respiration by glucose (Crabtree effect) remained largely unexplained. Pasteur and Crabtree effects are generally explained by competition of the two main metabolic pathways, respiration and glycolysis, for their common phosphorylation substrate ADP. However, the question why normal differentiated cells are lacking this competition remained unanswered.

With respect to the type of hexose utilization, transition of non-dividing differentiated cells to the rapidly proliferating state, regardless whether induced by oncogenic transformation or mitogenic stimulation, can be characterized as change from membrane-limitation to metabolic regulation. The membrane-limited state differs from that of metabolic regulation by the special type of glucose uptake regulation at the membrane level that excludes competition of the glycolysis and respiration for adenine nucleotides because the ATP/ADP system acquires a signal status for regulation of glucose entry. Both oncogenic transformation (61, 62) and treatment of quiescent cells by growth factors cause an immediate stimulation of hexose transport activity to maximum values. Abolition of membrane-limitation can be induced as well by metabolic stress, e.g. by 2,4-dinitrophenol-induced reduction of cellular ATP (40), receptor-mediated stimulation (63-66). The reverse process is observed in cell cultures reaching confluence or by cultivation under differentiating conditions (5). Changes between these two states can be verified by experiments.

Loss of membrane-limitation is characterized by two experimental criteria: First, the steady state content of free intracellular glucose increased up to the extracellular level (41, 42) and second, the aerobic lactate production is elevated (49, 67). Increased aerobic lactate production, most obvious after oncogenic transformation, also occurs under other experimental conditions such as inhibition of respiration (ATP depletion) (68) and stimulation by growth factors (64, 65). Both effects indicate that the loss of membrane limitation is accompanied by the re-establishment of competition between respiration and glycolysis for adenine nucleotides. with the concept of microvillar regulation of glucose uptake, abolition of membrane limitation by ATP depletion is accompanied by shortening or even complete integration of microvilli into the plasma membrane (5, 67, 69-73). Thus, in membrane-limited systems, the length of microvilli is critically determined by the cytoplasmic ATP/ADP system, which in turn depends on the availability of C_3 substrates for mitochondrial ATP production. This way, a complete regulatory circuit precisely adapts glucose uptake to cellular ATP demands (Figure 8). Due to this regulatory principle, pyruvate/lactate, produced via the Embden-Meyerhof pathway, is almost exclusively used for oxidative phosphorylation yielding 18 times more ATP per mol glucose than glycolysis alone. Only small amounts of lactate can leave the cell under these conditions. characteristics of metabolic regulation and membrane limitation. The membrane-limited state allows adaptation of glucose uptake rates to varying levels of external glucose.

5.1.4. Surface morphology of the membrane-limited state

During adipose conversion of 3T3-L1 fibroblasts, adipocytes acquire the membrane-limited state as indicated by the appearance of the endogenous regulation of 2-deoxyglucose uptake (5). The morphological aspect of this transition is shown in Figure 9. Adipose conversion is accompanied by the outgrowth of numerous microvilli on the cell surface (Figure 9b) (5). Stimulation of hexose uptake either by endogenous signals (Figure 10a) or by insulin treatment (Figure 10b) is paralleled by significant changes in the shape of microvilli assuming a voluminous sack-like appearance (5). Apart from electron microscopic evidence, enlargement of the internal microvillar compartment was also verified by demonstrating that insulin induces a significant (2-fold) increase of the cellular distribution space for 3-O-methylglucose (5). Furthermore, removal and isolation of microvilli from the cell surface of intact 3T3-L1 adipocytes revealed that microvilli contain nearly 90% of the surface-exposed glucose transporters comprising the total insulin-sensitive transporter pool of these cells (3).

5.1.5. Localization of glucose transporters on microvilli

An essential prerequisite of the outlined concept of membrane limitation is the preferred localization of glucose transporters on microvilli. In the past years, this type of surface distribution has been confirmed in a number of differentiated cell types using immuno-electron microscopy:

- GluT1 is localized on microvilli of the rat oviduct (74).
- GluT1 is localized primarily to basolateral membranes of fully differentiated Caco cells, whereas the differentiation-specific transporter GluT3 was found predominantly on apical microvilli (75).
- GluT1 is localized on the microvillar surfaces of endothelila or epithelial cells of the blood-tissue barriers (76).
- GLUT1 is concentrated at both the microvillous apical plasma membrane and the infolded basal plasma membrane of the syncytiotrophoblast. (77).

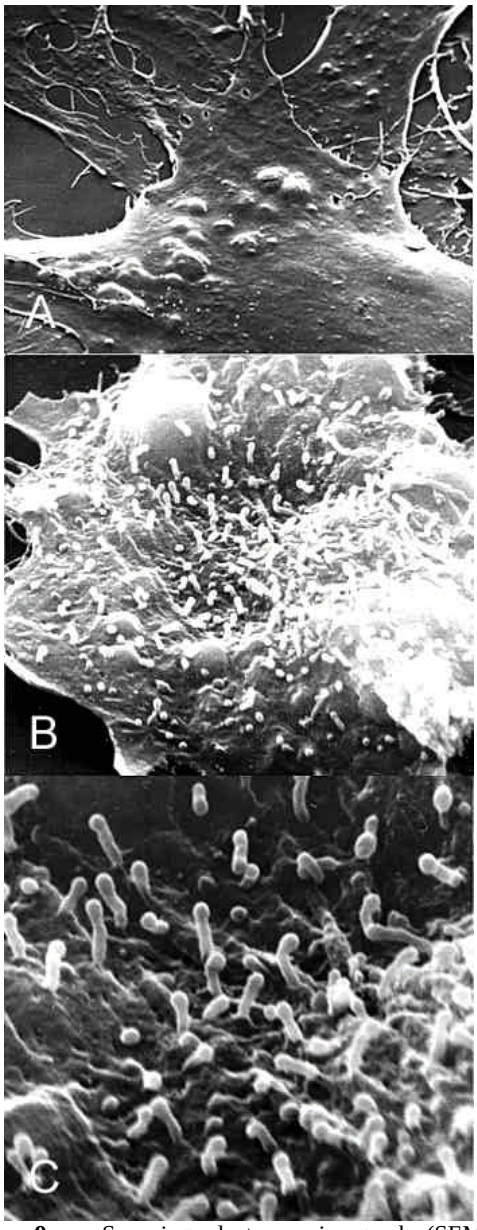

Figure 9. a. Scanning electron micrograph (SEM) of surface of undifferentiated 3T3-L1 cells. **b and c. S**urface of differentiated 3T3-L1 adipocytes.

- GluT2 is specifically localized to microvilli of pancreatic ß-cells (11).
- Reduced GluT2 on microvilli of ß-cells is correlated with diabetic loss of glucose-stimulated insulin secretion (78, 79).
- GluT2 is localized in sinusoidal microvilli of hepatocytes (80).
- GluT5 (fructose transporter) is found on apical microvilli in S3 proximal tubules of the rat kidney (81).

Further evidence has been acquired by studying isolated microvillar membrane fractions with immunoblot analysis and cytochalasin B binding techniques:

- GluT1 is found in MV of rod outer segments of the photoreceptor cells (corresponding to microvillar surfaces) colocalized with hexokinase, aldolase, GAPDH, phosphoglycerat kinase, and LDH (82).
- GluT4 is almost exclusively present in isolated microvilli of differentiated 3T3-L1 adipocytes (3, 4).

Insulin stimulates hexose uptake in 3T3-L1 cells up to 10-fold, consequently, about 90% of the total surface pool of GTs must be localized on microvilli. Using the cytochalasin B-binding technique, exactly this portion was found in microvilli-derived vesicle fractions from 3T3-L1 adipocytes (3, 4). The explanation for this clustering of transporter proteins in microvillar membranes turned out be surprisingly simple and revealed a new, hitherto unknown aspect of cellular differentiation.

5.1.6. Formation of microvilli on the surface of differentiated cells

In rapidly dividing cells, translocation of newly synthesized proteins to the plasma membrane continuously occurs by exocytosis of trans-Golgi vesicles. This process leads to insertion of additional lipids and integral membrane proteins into the cell surface (Figure 11). Subsequently, inserted integral membrane proteins migrate out of their original lipid microenvironment and rapidly scatter over the whole cell surface by lateral diffusion.

This type of surface processing of integral membrane proteins is significantly changed in resting ($G_{1/0}$-arrested) or differentiated cells. Soon after induction of adipose conversion by cultivation (48 h) of 3T3-L1 cells with dexamethasone, isobutylmethylxanthine, and insulin, SEM revealed a large number of small spherical surface protrusions (Figure 9b). At later stages of cultivation, these exocytic blebs grow out from the plasma membrane forming the tips of new microvilli (Figure 9c).

In non-dividing or differentiated cells, exocytosis results in the formation of stable spherical membrane blebs that are not integrated into the plasma membrane. Stabilization of these surface protrusions is due to a proteoglycane coat that inhibits lateral diffusion of the integral membrane proteins located in this membrane domain. Following exocytosis, a submembrane bundle of actin filaments forms the central core structures of the growing microvillus that bears the original exocytic membrane domain at the tip (Figure 12). Achler *et al.* (83) described the cell surface events accompanying microvilli formation on intestinal epithelium: *Formation of the basolateral microvilli required polymerisation of actin and proceeded at glycocalyx-studded plaques that resembled the dense plaques located at the tips of the apical microvilli.*

The original protein composition of exocytic vesicles is preserved in microvillar tip domains, which contain all those tissue-specific integral membrane proteins that are expressed in the differentiated cell. This type of surface distribution of integral membrane proteins on differentiated cells has been confirmed by biochemical techniques in the following way (4).

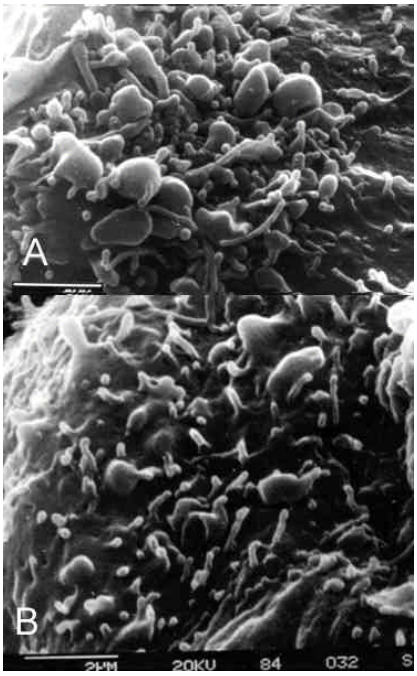

Figure 10. a. SEM of the surface of 3T3-L1 adipocyte after local ATP depletion during a short incubation with 2-deoxyglucose (1mM; 40s). **b.** SEM of the surface of an insulin-treated 3T3-L1 adipocyte (10mU/ml; 10min).

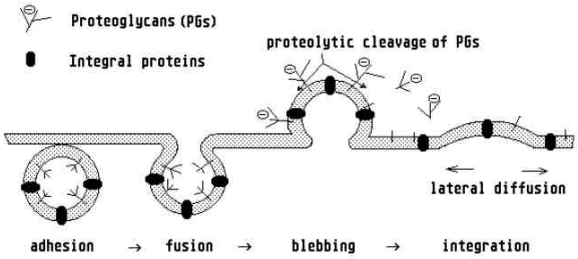

Figure 11. Membrane insertion into the plasma membrane of rapidly growing cells.

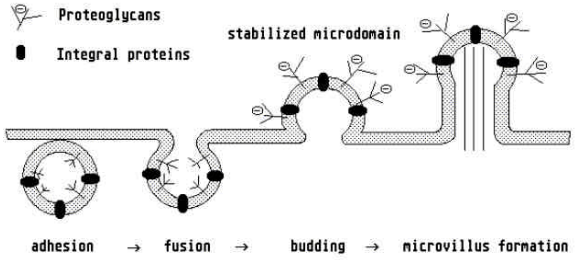

Figure 12. Exocytosis and microvilli formation in differentiated cells. Surface coat molecules (proteoglycans) stabilize the exocytic membrane domain and prevent lateral diffusion of integral membrane proteins.

5.1.7. Restricted mobility of integral membrane proteins in exocytic blebs of differentiated cells.

Some time ago, the existence of a novel type of glucose transporter in adipocytes and muscle cells has been discovered (for review see 84). This transporter species, named Glut4, is only expressed in insulin-sensitive tissues. In cultured 3T3-L1 adipocytes, both Glut4 and the erythrocyte/brain transporter type Glut1 are expressed at the same time. However, in contrast to Glut1 that is synthesized in both 3T3-L1 fibroblasts and adipocytes, Glut4 does not appear before day 4 after induction of adipose conversion (85, 86). Since at this time the formation of microvilli on the adipocyte surface has started, the distribution of the Glut4 protein between plasma membrane and microvilli was used to test the above postulated inhibition of lateral mobility in the microvilli-forming surface blebs. If it is true, that part of the Glut1 protein is expressed prior to adipose conversion, this transporter isoform should occur within both the plasma membrane fraction and microvillar vesicles; in contrast, Glut4 should be found in the microvillar fraction only. This type of transporter distribution was indeed observed. Whereas Glut1 occurred in equal amounts in both the plasma membrane and the microvilli fraction, Glut4 was detected almost exclusively in the microvillar fraction (4). The result of this study strongly supports the idea of restricted lateral mobility in newly inserted membrane areas of differentiated adipocytes. Moreover, the exclusive localization of transporter proteins on microvilli of differentiated cells is explained in a rather compelling way. Even those integral membrane proteins that have reached the plasma membrane vanish with time by proteolytic degradation.

Restricted lateral mobility of integral membrane proteins depends on the presence of proteoglycans on the cell surface (87, 88). Calvo *et al.* (89) reported the increased expression of surface coat components in 3T3 L-1 fibroblasts during their differentiation to adipocytes. The transmembrane connection between proteoglycans of the surface coat and the microfilaments of microvilli has been demonstrated by Carothers Carraway *et al.* (90). Thus, proteoglycans are intra- and extracellularly connected with each other forming domain-stabilizing cage structures (Figure 13).

The combined effects of the external and internal connections decrease the lateral mobility of integral membrane proteins within the plane of the lipid bilayer. Restricted mobility of integral proteins in glycocalyx-covered plasma membrane regions has been described more than 20 years ago. Studying the cell surface distribution of insulin receptors on human placental syncytiotrophoblasts, Nelson *et al.* (87) noted: *Insulin receptors are specifically associated with the glycocalyx region of microvilli on the surface membranes of microvilli. No insulin receptors were detectable in association with the intermicrovillous plasma membrane even though its glycocalyx is in direct continuity with the glycocalyx of microvilli…which suggests that there is not complete freedom of lateral mobility of the insulin receptors in the surface membrane of this tissue.* The exclusive localization of insulin receptors on microvilli of lymphocytes (91), hepatocytes and hepatoma cells (92), pancreatic ß-cells (93), and 3T3 L-1 adipocytes (94, 95) has been confirmed later on. The EGF receptor is another example for the microvillar localization of cell surface receptors (96, 97).

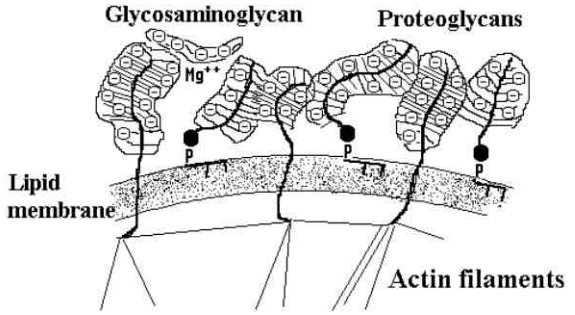

Figure 13. Stabilized exocytic membrane domain. The surface coat of proteoglycans (and glycosaminoglycans) is connected via transmembrane core proteins with intracellular actin filaments. The resulting cage structure prevents integral proteins to leave the membrane domain.

In proliferating cells, the surface coat is removed by serum proteases such as plasmin and thrombin, which are activated by tissue-specific ectoproteases of the plasmin activator type. These ectoenzymes are tightly bound to the cell surface via proteoglycans. During adipose differentiation in the presence of dexamethasone, the expression of plasmin activator is diminished and that of a specific protease inhibitor, plasmin activator inhibitor, is induced. This inhibitor is also bound to the cell surface coat as complex with its substrate the plasmin activator (98, 99). The involvement of serum proteases for cell differentiation also follows from the fact that cultivation of 3T3-L1 fibroblasts with heparinized medium immediately induces adipose conversion and microvilli formation (100-101); also, omission of serum is a widely used condition to differentiate cells in culture.

Microvilli formation is generally observed in cultured cells during growth arrest in G_0/G_1 phases of the cell cycle or in the differentiated state (100, 102-104). Under these conditions, newly synthesized functional membrane proteins are exclusively located on microvilli tips and are thus no longer able to support maximal rates of cellular metabolism because the cytoskeletal diffusion barrier of the microvillar shaft attenuates the flux of hexoses and ions into the cytoplasm. The specific metabolic state of membrane- or transport-limitation is established.

5.2. Biochemistry of microvilli formation

5.2.1. Roles of PI kinases and actin binding proteins

The specific organization of the microvillar cell surface is essentially determined by the presence of phosphatidylinositol (PI) kinases, especially the PI 4- and 5-kinases, which are involved in PIP and PIP_2 formation. The interaction between PIP/PIP_2 and several actin-binding proteins, most importantly of the gelsolin/cofilin/villin family is the basis for the translocation of these capping proteins and associated actin monomers to PI 4(5)-kinase-containing membrane domains. Membrane microdomains containing these enzymes are, thus, the destination points of actin monomer transfer from the cytoplasm to the plasma membrane (105). In the case of gelsolin and villin, the cytoplasmic 1:2 complex consisting of one gelsolin and two actin monomers liberates one actin monomer after binding of the complex to phospholipids. The membrane bound 1:1 complex acts as nucleation points for the formation of microfilaments at the cytoplasmic side of the plasma membrane (106). On this way, actin monomers are shuttled from the cytoplasm to the inner face of the plasma membrane and used for the localized assembly of new microfilaments. Gelsolin and villin are both identified as components of microvilli in epithelial cells (83, 104, 107, 108). Increased expression (103) or microinjection of villin into fibroblasts results in the loss of stress fibers and the formation of large surface microvilli (109). Thus, the occurrence and activation of PI 4(5)-kinase on the inner face of the plasma membrane initiates a rapid and spatially directed reorganization of cytoplasmic actin to the cortical locations of PI 4(5)-kinases (105). Most interestingly, PI 4-kinase has been identified as a component of the GluT4-containing vesicles from rat adipocytes (110) and of HeLa and CHO cell microsomes most likely containing microvilli-derived membrane components (111, 112).

Subsequent to the formation of microfilament bundles, the plasma membrane tightly covers the sides of the bundle, in a zipper-like fashion, due to the formation of specific noncovalent interactions between certain phospholipids and F-actin domains. These interactions have been demonstrated and studied by St. Onge and Gicquaud (113, 114), Grimard et al., (115) and by Taylor and Taylor (116). Using electron microscopy, they observed the formation of 2-dimensional paracrystalline sheets of parallel fibres in registers or in a net-like organization on phospholipids lipid layers. Binding between phospholipids and F-actin depends on the presence of divalent bridging ions such as Mg++ or Ca++ or positively charged lipids. Gicquaud (117) proposed a model in which a limited number of phospholipid molecules interact with specific sites on the actin molecule.

5.2.2. Specific linker proteins are required for stable residence of integral membrane proteins in microvilli

The preferred or exclusive localization of various types of transporters and ion channels including nonselective cation, Na+, K+ and anion channels on microvilli is documented for a multitude of different cell types. Most likely, stable residence of membrane proteins in microvilli affords direct or indirect binding of their cytoplasmic domains to microvillar actin filaments. Otherwise, unfixed membrane proteins would leave the microvillar membrane domain during transient stimulation.

Recently, several authors (107, 118-124) have demonstrated the central role of specific linker proteins such as the ezrin/radixin/moetin (ERM) proteins for the formation and maintenance of microvilli (review see 125). According to these studies, the presence of activated (phosphorylated) ERM linker proteins is an essential precondition for the development of microvillar cell surfaces (126). Moreover, Matsui et al. (121) pointed out that activation of moesin requires its phosphorylation and the formation of PIP2. Thus, both the localized action of PI (4)P 5-kinase and the activation of linker proteins are essential for microvilli formation.

A rapidly increasing number of integral membrane proteins, localized on microvilli, including

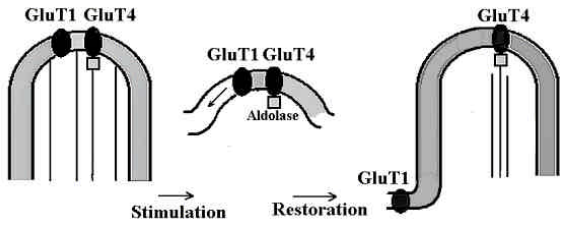

Figure 14. Functional difference between the F-actin-linked glucose transporter GluT4 and the non-linked GluT1. Following activation of the microvillar influx pathway, GluT4, but not GluT1, is again starting point for microvillus formation.

glucose transporters and ion channels, have been shown to interact with the actin cytoskeleton via specific linker proteins. Presently known examples are the K^+ channel (127), epithelial Na^+ channel (128, 129), erythrocyte anion channel (130), CFTR (131), Na^+/K^+-ATPase (132), the adhesion receptors CD44, CD43, and I-CAM (119), and Na^+/H^+ exchanger (133). As it appears, a specific group of functional proteins is selected to reside exclusively within microvillar membranes because of their stable binding to cortical actin filaments, whereas other proteins lacking binding sequences for linker proteins, may have limited residence times within microvilli.

Recently, Kao *et al.* (134) demonstrated that the insulin-sensitive glucose transporter GluT4 (but not Glut1) is connected to the cytoskeleton via a specific linker protein, the glycolytic enzyme fructose 1,6-bisphosphate (FDP) aldolase. This interaction is modulated by insulin as well as by glycolytic intermediates. The aldolase substrate FDP and to a much lesser extend glyceraldehyde 3-phosphate (G3P) inhibit binding of the enzyme to GluT4. In intact 3T3-L1 adipocytes, insulin action decreases the interaction between GluT4 and aldolase. Moreover, treatment of permeabilized cells with FDP or 2-deoxyglucose (2DG) and microinjection of aldolase-specific antibodies inhibits insulin-induced GluT4 translocation.

Whereas the interaction of aldolase with actin filaments is textbook knowledge (135, 136), the cytoskeletal association of Glut4 via aldolase is a novel aspect with far-reaching consequences. Metabolic regulation of the association between cytoskeleton and aldolase is suggested by the finding that the interaction of F-actin with aldolase is inhibited by FDP, G3P, fructose 1-phosphate, and dihydroxyacetone phosphate (136). Within the range of physiological metabolite concentrations, the binding between aldolase and GluT4 appears to be affected by FDP alone (134). As it appears, low concentrations of intermediates from the upper part of the glycolytic pathway favors cytoskeletal fixation of glucose transporters within microvillar surface structures. Since low intermediates are characteristic for starvation conditions, it is an intriguing assumption that one mechanistic aspect of deprivation-induced cell differentiation may be the formation of cytoskeleton-transporter interactions in order to generate or stabilize microvillar structures and to establish or stabilize the differentiated status of membrane limitation.

On the other hand, activation of the microvillar influx pathway via insulin receptor activation usually leads to a period of increased glucose uptake and high metabolite levels. Under theses conditions, the cytoskeletal connection to transporters is transiently lost as shown by Kao *et al.* (134). Transient release of the cytoskeletal fixation allows down regulation of the transporter activity via endocytosis of transporters.

Together, these data suggest that formation of GluT4-containing microvilli is essentially determined by several mechanistic aspects:

- Shuttling of actin monomers by actin-binding and capping proteins to the sites of PIP and PIP_2 formation, the location of PI 4-kinase that is component of GluT4-containing exocytic vesicles (110).
- Association of the transporter protein, GluT4, with actin filaments via FDP aldolase facilitates the formation of microvilli and allows the restoration of the original microvillar organization subsequent to receptor-mediated or starvation-induced activation (Figure 14).
- The cytoskeletal association of GluT4 is subject to metabolic regulation by the aldolase substrate FDP reflecting the state of cellular glucose supply.

6. INSULIN ACTION ON GLUCOSE UPTAKE

6.1. Cell surface morphology of insulin action

SEM was used to study changes in the cell surface morphology occurring on 3T3-L1 adipocytes after short (5-10 min) periods of insulin treatment. As shown in Figure 10 b, microvilli lose their original shapes assuming sack-like appearances. In some cases, the original small upper part of the microvillus is still visible on the doming membrane giving the whole structure a bagpipe appearance. At later stages of insulin action, microvillar membranes are largely integrated into the adjacent plasma membrane.

Abolition of the microvillar diffusion barrier results in a conspicuous enlargement of the cell volume, which could be confirmed by 3-O-methylglucose (3-OMG) equilibration experiments. In 3T3-L1 adipocytes, the time course of the insulin-induced increase in the distribution space of 3-OMG follows the same time- and dose-dependency as that of glucose uptake stimulation by insulin (5). Using the same experimental protocol, even the rapid initial uptake of 3-OMG into the microvillar entrance compartment of unstimulated adipocytes could be demonstrated. Equilibration of the microvillar tip compartment precedes the long-lasting basal influx kinetics. The experiments further showed that the rate of the initial component of basal cells is identical with the uptake rate of insulin-stimulated cells (5). These findings clearly show that all transporters localized on microvilli tips are present in a completely active state, whereas glucose uptake into the cell is impeded by the microvillar diffusion barrier. The same biphasic uptake kinetics of 3-

OMG have been observed by Whitesell *et al.* (137) for 3T3-L1 cells, with a rapid initial half time of equilibration of 1.7 s and a second slower component with a half time of 23 s.

Thus, using clearly arranged experimental approaches as well as correlated morphological and functional data confirm the diffusion barrier concept for glucose uptake regulation.

6.2. Glut4-containing vesicles can be sheared from the cell surface

The mechanism of insulin action on hexose transport was further studied by isolation of a cell surface-derived membrane fraction consisting of microvilli or tips of the microvilli. This fraction was prepared from intact 3T3-L1 adipocytes by use of a hydrodynamic shearing technique originally developed by Carothers Carraway *et al.* (138) for isolation of microvilli from tumour cells and used later on for microvilli isolation from cultured cells (122). The technique is based on gentle shearing forces exerted on the cell surface by pressing a cell suspension through hypodermic needles. Although devoid of microvilli, most of the cells (80-90%) remained intact after this procedure. The cells were then subjected to homogenization and subcellular fractionation according to the protocol of McKeel and Jaret, modified by Gibbs *et al.* (139).

As shown by cytochalasin B binding, the microvillar fraction isolated from 3T3-L1 adipocytes contains 90% of the surface exposed glucose transporters including the total insulin-sensitive transporter pool of these cells (3, 4). Only 5% of the total cellular transporter protein was found in the plasma membrane, nearly 60% in the microvilli fraction, and 37% in the microsomal fraction (3, 4). Pretreatment of the cells with insulin (10 min), shifted about 40% of the transporters from the microvillar fraction to the plasma membrane fraction.

6.3. Microvillar vesicles are part of microsomal fractions

Applying the same sedimentation protocol without the shearing procedure prior to cell homogenization, the total insulin-sensitive transporter pool was found in the microsomal fraction. Obviously, the cell surface-derived microvillar vesicle fraction, generated by the low-force shearing technique, is also formed during normal homogenization using the Teflon-glass homogenizer. In either case, hydrodynamic shearing forces were used. However, the two methods differ considerably in the strength of the generated shear forces, which mainly depend on the dimension of the aperture through which the cell suspension is pressed. In contrast to the Teflon-glass homogenizer usually having a clearance of 90 µm, the needles used for microvilli preparation have an internal diameter of 600 and 500 µm. Consequently, the needle technique exerts much less shear stress on the cells than Teflon-glass homogenization. These experiments clearly demonstrate that even much smaller shearing forces than those exerted by the Teflon-glass homogenizer, are able to pull off microvilli and related structures from the cell surface and to generate a distinct vesicle population with sedimentation properties quite similar to those of the internal membrane systems.

This finding implies far-reaching consequences. Since the proteins characteristic for microsomal fractions, so-called marker proteins, are present in both, intracellular membranes (endoplasmic reticulum, transgolgi vesicles) and microvillar membranes, a distinction between these vesicle fractions by 'marker enzymes' is not possible. Both membrane fractions contain the same components because the internal membranes are the immediate precursors of the microvillar membranes and, as pointed out above, the protein composition of the original exocytic membrane domain is completely preserved in the microvillar tip membrane. As a consequence of this finding, the general agreement about the intracellular location and function of several important functional proteins, especially those attributed to the endoplasmic reticulum, has seriously to be questioned.

Already two important 'microsomal' systems with widely accepted intracellular localization, the receptor-regulated Ca^{++} store/Ca channel (51, 53) and the insulin-sensitive glucose transporter pool (3) have been identified as microvillar components. However, a number of further systems with putative intracellular localization remain to be re-evaluated. For instance, one of the most important components of microsomal fractions, the drug metabolising cytochrome P450 system, has been shown to occur and function on microvilli of different cell types (141, 142). A cell surface function of this multifunctional oxidative system appears to be a convincing alternative to the currently accepted view that detoxication occurs deep within the cell when toxic compounds already have flooded all sensible cellular targets. A possible model for the epithelial handling of xenobiotics including the peripheral function of the P450 system is just discussed (143).

The widely used techniques of cell fractionation for the isolation of intracellular membranes should have been re-evaluated almost two decades ago when Carothers Carraway *et al.* (138) for the first time used a hydrodynamic shearing technique for the isolation of microvilli from tumour cells. Later on, Pakkanen and Vaheri (122) as well as Lange and Brandt (3, 4) applied the same technique for microvilli isolation from cultured cells. In the latter publication, a decisive experiment was described, clearly demonstrating the microvillar origin of a large portion of the microsomal fractions. The use of this gentle variant of normal homogenization by Teflon-glass homogenizers for isolation of an inside-out fraction of microvilli-derived vesicles should have caused all experimenters to seriously suspect the generally accepted, but never soundly proved, assumption that microsomal fractions contain intracellular membranes only.

6.4. Differentiation between vesicles of microvillar and intracellular origin

Much experimental work has been employed for the purification and characterization of glucose transporter-containing vesicles from the 'light microsomal' fraction of

adipocytes. Biber and Lienhard (140) introduced an immuno-adsorption technique for the isolation of vesicles containing glucose transporters from homogenates of 3T3-L1 adipocytes. They used an antiserum raised against a COOH-terminal peptide of the transporter. This part of the transporter molecule is located at the cytoplasmic surface of the membrane. If one expects that the transporter-containing vesicles are part of the endoplasmatic reticulum or transgolgi system, it is right to assume that these vesicles carry the cytoplasmic domains on their external surface. Consequently, vesicle fractions isolated by this technique only comprises vesicles of intracellular origin but are necessarily devoid of microvilli-derived components known to maintain their original out-side out orientation (144).

6.5. Role of exocytosis in insulin action

The translocation theory is supported by a large body of data obtained by various experimental protocols. Nevertheless, there are disagreements as to the quantitative contribution of recruitment as discussed by Stephens and Pilch (16). In many cases, the rate of insulin-enhanced glucose uptake is greater than the degree of translocation as assessed by Western blot and cytochalasin B binding.

The assumption of two different transport-enhancing processes proceeding at different time scales offers an explanation for the dissociation of insulin-induced transport stimulation and transporter translocation in 3T3-L1 adipocytes (145-147, 140) and rat adipocytes (148, 149). Part of the observed insulin-induced transport stimulation may indeed be due to translocation of glucose transporters from an intracellular compartment to the cell surface by exocytic events. Intracellular vesicles are inserted into the plasma membrane during insulin-induced exocytosis. However, in contrast to the fast initial effect of insulin on the microvillar pathway occurring with a half time of about 2 min, exocytic events usually proceed at a much larger time scales with half times of 10-20 min. Most likely, the exocytic portion accounts for 35-40% of the total insulin action (38). The exocytic portion accounts for a 1.5- to 2-fold increase in transporter content of the plasma membrane after 15-30 minutes of insulin action. Transport stimulation within 10 minutes of insulin action, however, is largely due to the activation of the microvillar transport pathway via transporters already present on the cell surface prior to insulin action.

Own studies using 3T3-L1 adipocytes have yielded clear evidence for the contribution of exocytosis to enhanced transport and transporter appearance on the cell surface. Addition of insulin to the culture medium 18 hours before the transport assay yielded an increment of 50-100% to the maximal insulin effect on transport. The additional surface population of transporters induced by the first addition of insulin causes glucose uptake stimulation immediately after their appearance on the cell surface because the exocytic blebs are still lacking the cytoskeletal diffusion barrier. Later on, outgrowth of these exocytic domains on microvillar tips increasingly reduces the uptake rates. The new microvilli population then contributes to the overall insulin-induced transport activity by an additional increase of 50 -100% compared with controls that are not treated with insulin the day before (data not published). These data closely correspond to those of Zierler (38).

6.6. Phosphatidylinositol 3-kinase (PI 3-kinase)-dependent activation of the microvillar influx pathway

Although morphological studies on 3T3-L1 adipocytes (5) have clearly shown that insulin critically alters the structural organization of microvilli, the biochemical mechanism by which insulin receptor activation modulates the cytoskeletal diffusion barrier remained unclear. In contrast to receptor-mediated Ca^{++} signaling, which is initiated by F-actin depolymerization via the PLC pathway, insulin signaling critically depends on activation and translocation of cytoplasmic PI 3-kinase to the plasma membrane. Activation of membrane PI 3-kinase is accompanied by extensive surface membrane dynamics, called membrane ruffling, which is an energy-dependent cytoskeleton-driven process essentially involved in cell motility. The cytoplasmic surface of the ruffling membrane is a site of extensive actin polymerisation generating a 3-dimensional F-actin network that provides the driving force for the observed membrane deformations. However, membrane ruffling is only one aspect of the cytoskeletal response on PI 3-kinase activation. Ruffling can be readily observed by light microscopy. The other cytoskeletal aspect of PI 3-kinase activation, which is only perceptible on the electron microscopic level, is the loss of the internal structural organization of microvilli giving rise to their shape changes.

Using isolated rat hepatocytes, the dependence of the insulin-induced microvillar shape change on PI 3-kinase activation has been clearly demonstrated. Wortmannin completely inhibited insulin-induced shape changes of microvilli on these cells (150). In contrast, the PLC-mediated vasopressin action on microvilli morphology, which closely resembles the effect of insulin on microvilli, is wortmannin-insensitive. The involvement of PI 3-kinase in microvillar shape changes is further strengthened by the earlier finding that phenyl arsine oxide (PAO) inhibits insulin-stimulated glucose transport in fat and muscle cells. Recently, phenylarsine oxide (PAO) has been identified as potent inhibitor of PI 3-kinase (151). As a consequence, PAO should be able inhibit insulin-induced shape changes of microvilli and surface ruffling as well. Own SEM studies have confirmed this assumption (data unpublished).

Recent biochemical findings have contributed to a better understanding of the role of PI 3-kinase products in cytoskeletal reorganisation in differentiated cells with microvillar surfaces.

6.6.1. Complete barbed end capping of cytoplasmic actin filaments is a precondition for the stable existence of microvillar cell surfaces

Capping proteins play a specific role in the maintenance of the microvillar surface. In the basal (unstimulated) state, microvilli only expose the slowly growing pointed ends of their microfilaments to the cytoplasm. Microfilaments with shielded barbed ends but

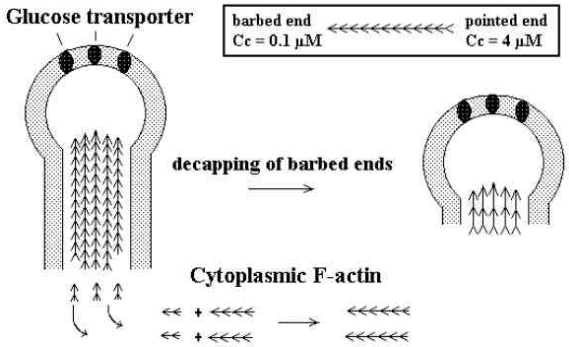

Figure 15. Decapping-triggered disassembly of the microvillar diffusion barrier. Decapped cytoplasmic actin filaments grow at their (high-affinity) barbed ends using free cytoplasmic actin monomers as well as monomers dissociated from the (low-affinity) pointed ends of microvillar F-actin.

exposing their pointed ends are stable only at or above the high critical monomer concentration (C_c) at this end of 4 µM. At monomer concentrations below this value, monomers dissociate from these ends. In contrast, the rapidly growing barbed ends of microfilaments exhibit a 40-fold lower C_c of 0.1 µM. Thus, the exposed pointed ends of microvilli are stable only when all cytoplasmic microfilaments are barbed end-blocked by capping proteins. In this case, the cytoplasmic monomer concentration equals the high C_c of the pointed ends. Otherwise, the length of microvillar microfilaments would be reduced by pointed-end disassembly in favour of barbed-end elongation of cytoplasmic filaments.

The metastabile state of the actin cytoskeleton in cells with microvillar surfaces can be used to trigger rapid cytoskeletal changes in response to specific signals acting on the capping state of microfilaments. The system closely resembles a strained spring, able to release large forces via a trigger mechanism operated by a small trigger force. Receptor-mediated decapping of F-actin has been reported for several cellular models including FMLP-activated neutrophils (152-154), thrombin-stimulated platelets (155), and EGF receptor-mediated activation of adenocarcinoma cells (156). Recent findings point to a novel mechanism of PI 3-kinase-dependent decapping of cytoplasmic F-actin, which is able to trigger rapid changes of the microvillar diffusion barrier.

6.6.2. Role of PI 3-kinase in cytoskeletal disorganization of microvilli

The specific role of actin binding and severing proteins such as profilin and gelsolin/cofilin in Ca^{++} signaling has been shortly depicted in a previous paragraph (see "Activation of ionic pathways"). These proteins are released from the membrane via receptor-mediated activation of PLC to cleave PIP/PIP_2 even when these phospholipids are shielded by the gelsolin/cofilin proteins (56, 57). Release of actin-binding/capping proteins from their original binding sites at the plasma membrane cause microfilament depolymerization and reorganization as visualized by shortening of microvilli and ballooning of the adjacent plasma membrane. Although insulin signaling is independent of PLC, the morphology of insulin action is quite similar.

A recent study of Lu *et al.* (157) of the downstream targets of PI 3-kinase closed the mechanistic gap between PI 3-kinase activation and reorganization of microvillar F-actin. These authors found that the PI 3-kinase products, phosphatidylinositol 3,4- bisphosphate ($PI(3,4)P_2$) and 3,4,5-trisphosphate ($PI(3,4,5)P_3$), bind with up to 10-fold ($PI(3,4)P_2$; K_d = 1.1 µM) higher affinity to profilin (157) than $PI(4,5)P_2$. A similar affinity change may be assumed for gelsolin (158) and other phospholipid-regulated capping proteins. This interpretation is supported by Hartwig *et al.* (155) who demonstrated that D-3 and even D-4 polyphosphoinositides uncap cytoplasmic F-actin in permeabilized resting platelets and by Barkalow *et al.* (159) who showed that thrombin stimulation of platelets results in release of gelsolin and capping protein from barbed ends of cytoplasmic F-actin. Obviously, the high affinity of 3-phosphorylated membrane lipids give rise to rapid redistribution of PI-regulated actin-binding and capping proteins from cytoplasmic F-actin to the sites of PI 3-kinase action at the inner face of the plasma membrane, most likely at the foot points of microvilli where this enzyme develops its highest activity (see paragraph 6.6.5.). Thus, PI 3-kinase-induced redistribution of capping proteins may be the mechanistic basis for both, localized membrane ruffling and microvilli shortening.

Decapping of barbed ends on cytoplasmic microfilaments results in extensive monomer addition to these sites reducing the actin monomer concentration in the cytoplasm to levels far below the C_c of the pointed ends. As schematically shown in Figure 15, the lowered cytoplasmic monomer concentration causes disassembly of microvillar filaments at their exposed pointed ends, shortening of microvilli, and reduction of the diffusion barrier function.

6.6.3. Decapping of F-actin initiates membrane ruffling

The second important aspect of insulin-induced actin reorganization is translocation of actin to the plasma membrane accompanied by membrane ruffling. Since capping proteins carry actin monomers from the cytoplasm to the plasma membrane, localized formation of D-3 phospholipids also cause submembrane microfilament assembly. In addition, actin monomers liberated from the pointed ends of microvillar F-actin may contribute to the formation of the 'ruffling' network below microvilli.

Thus, membrane ruffling in response to PI 3-kinase activation is induced by directed transfer and assembly of actin monomers to the immediate vicinity of activated insulin receptors (Figure 13). The physiological relevance of this vectorial activation of cytoskeletal growth may rest in the oriented initiation of cell motility by gradients of activating ligands (chemotaxis).

On the other hand, the formation of a localized submembrane microfilament network gives rise to mechanical membrane stress at the ruffling sites, which may further contribute to the unravelling of microvilli and

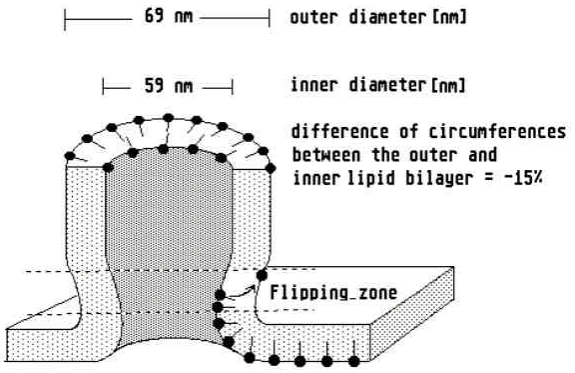

Figure 16. High membrane curvature occuring at the transition region from the plasma membrane to the microvillar shaft. Due to spatial distortion of the bilayer, phospholipids within this membrane domain can translocate between the two lipid bilayers (flipping). Dimensions are taken from microvilli of rat hepatocytes (Figure 18 a).

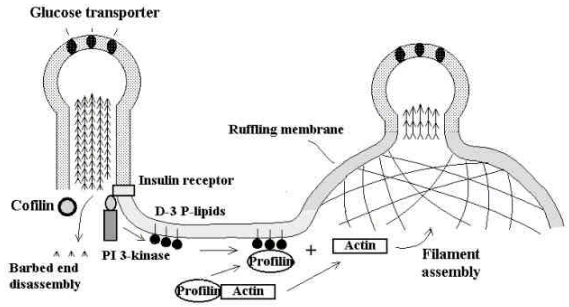

Figure 17. Insulin signaling events leading to microvillar shape change and formation of submembraneous microfilament networks (membrane ruffling). High-affinity binding of actin-binding proteins to D-3 phosphorylated phospholipids directs a flux of actin monomers to the sites of activated insulin receptors/PI 3-kinase. Using these monomers and those from the pointed ends of microvillar filaments, a 3-dimensional microfilament network is formed beneath the plasma membrane, which exerts an isotropic outwardly directed force (black arrows) blowing up this membrane area like a gas-filled balloon (see SEM in Fig. 6b, 14b). The high membrane curvature at the transitional region between the plasma membrane and the microvillar shaft additionally activates the PI 3-kinase by 2 orders of magnitude.

integration of their membrane portions into the plasma membrane. PI 3-kinase-induced membrane ruffling may be seen as an auxiliary mechanism necessary for shortening of the microvillar diffusion barrier.

6.6.4. Insulin-induced activation of the ADF/cofilin system accelerates pointed-end dissociation.

Recent findings point to the involvement of a further mechanistic component involved in insulin-induced cytoskeletal reorganization downstream of PI 3-kinase. The actin-binding and depolymerizing factor (ADH)/ cofilin belongs to a family of 15 - 20 kDa proteins that are regulated by phosphorylation on serine (160). In the basal state, these proteins were found as inactive phosphoprotein (P-cofilin) in the cytoplasm. Following insulin or EGF receptor activation, P-cofilin is dephosphorylated and translocated to the plasma membrane. The mechanism of dephosphorylation is still unclear, however, this reaction appears to depend directly or indirectly on activation of PI 3-kinase since inhibition of this enzyme by wortmannin completely inhibits dephosphorylation of cofilin (161).

As shown by Carlier *et al.* (162) the main functionally relevant effect of ADF/cofilin on F-actin is a 25-fold increase in the rate of monomer dissociation from the pointed ends, while the rate of dissociation from the barbed ends remains unchanged. Accelerated pointed-end dissociation excellently fits into the picture of cytoskeletal reorganization by insulin-induced activation of PI 3-kinase. Otherwise, unravelling of the microvillar microfilaments from their slowly reacting pointed ends via simple monomer dissociation would be to slow to explain the rapid early effects of insulin on transport and microvillar shapes proceeding with a half time of about 2 minutes.

On the other side, inactivation of cofilin by phosphorylation was shown to depend on Rho-mediated activation of Rho/LIM kinases (163), a pathway common to that of ERM protein activation, which is essential for microvilli formation. ERM phosphorylation also depends on PI lipids such as PIP_2 and PIP and, thus, on the location of PI 4- and 5-kinase activity on lipid membranes (161, 164). Obviously, the Rho-initiated kinase pathway provides for ERM activation and cofilin deactivation, two essential steps involved in microvilli regeneration following receptor-mediated activation.

6.6.5. Activation of PI 3-kinase by high membrane curvature

A further remarkable property of the PI 3-kinase reaction is the strong dependence of its activity on membrane curvature (165). In 50-nm vesicles, the PI 3-kinase activity is 100 times greater than on 300-nm vesicles. On the surface of differentiated cells, the 50-nm curvature is almost exclusively found on microvilli. An especially complicated form of high membrane curvature occurs within the transition region between the plasma membrane and the microvillar shaft, presumably a region of facilitated transbilayer flipping of membrane lipids during microvilli elongation (143) (Figure 16).

Phospholipids within this region are much more mobile than those located within the highly ordered even plasma membrane, allowing rapid steric reorientation of these lipids for interaction with the PI 3- kinase. According to this picture, sites of high membrane curvature should be locations of high PI 3-kinase activity, increased formation of 3-phosphorylated phosphatidylinositols, and thus, sites of high ruffling activity.

Summing up, the action of insulin on glucose transport is the direct consequence of PI 3-kinase activation and translocation to the plasma membrane. Downstream of receptor-mediated PI 3-kinase translocation/activation, four mechanistic components are involved in the activation of the microvillar influx pathway (Figure 17):

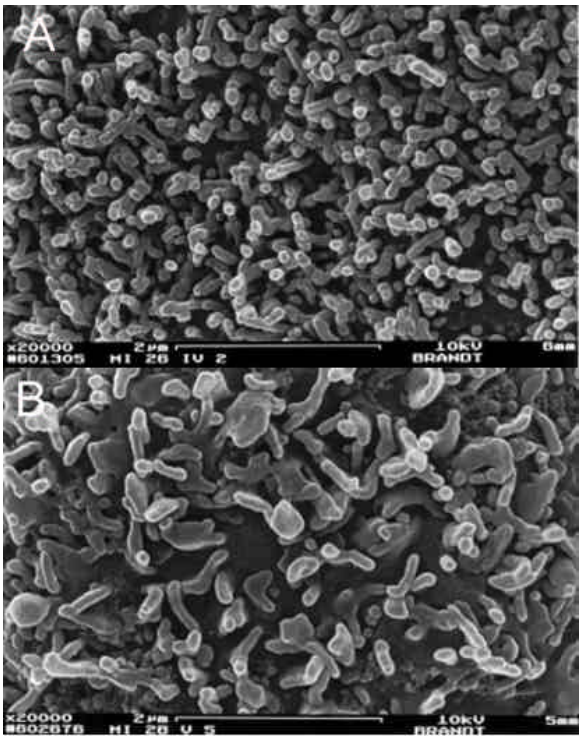

Figure 18. Insulin action on the surface morphology of isolated rat hepatocytes: **a**. unstimulated cell surface; **b**. insulin-stimulated (5 min) cell surface.

- PI 3-kinase activity is strongly potentiated by high membrane curvature at the foot points of microvilli.
- Capping proteins blocking barbed ends of cytoplasmic F-actin are transferred to D3-phosphorylated PIP and PIP_2 moieties in the immediate vicinity of activated insulin receptors/PI 3-kinase. Monomer addition at decapped barbed ends reduce the cytoplasmic actin monomer concentration to levels below the C_c of the pointed ends of F–actin, thereby shortening microvilli through monomer dissociation from the pointed ends of microvillar F-actin.
- Pointed end dissociation of actin monomers is specifically accelerated by PI 3-kinase-dependent activation of the ADH/cofilin system.
- Both, pointed end dissociation of microvillar F-actin and monomer shuttling to the submembrane binding sites of capping proteins result in extensive formation of a 3-dimensional actin networks that imparts mechanical strain on the stimulated membrane regions (membrane ruffling) supporting the retraction of microvilli.

This interpretation is strongly supported by SEM of insulin-induced changes of the surface morphology of isolated rat hepatocytes (150) and 3T3-L1 adipocytes (5). Within a few minutes of insulin action, large ballooned plasma membrane areas have formed on which the original microvillar tips are still visible (Figure 18 b). The surface morphology of insulin action of hepatocytes is almost identical to that of 3T3-L1 adipocytes (Figure 10 b). The dome- or sack-like curvatures of the plasma membrane around the microvilli are due to rapid actin network formation following PI 3-kinase-induced cytoskeletal reorganization (membrane ruffling), assumed to facilitate the shortening process of microvilli by exerting a tangential strain on the plasma membrane. Later on, complete integration of the microvillar membrane domains into the ballooning areas occurs.

6.6.6. Insulin receptors are localized on microvilli

The events following insulin binding to its receptor on the cell surface were subject to extensive investigations. In various cell types, unoccupied insulin receptors were detected on microvilli. Immediately following insulin binding, these receptors appear to leave the microvillar membrane domains moving to the intervillous part of the cell surface.

In a recent publication, Carpentier and McClain (166) summarized the state of knowledge in the following way:

- In its unoccupied and unstimulated state, insulin receptors associate with microvilli on the cell surface. This preferential association is dependent on the integrity of the cytoplasmic tail of the receptor.
- Insulin binding releases the constraint maintaining the receptor on microvilli and does so via receptor kinase activation and autophosphorylation of three tyrosine residues present in the regulatory domain.
- The insulin receptor complex, freely mobile on the cell surface, next associates with the internalisation gates, the clathrin-coated pits, via signal sequences in the juxtamembrane domain of the cytoplasmic tail of the receptor.

Part of this knowledge comes from studies using deletion mutants of the insulin receptor expressed in CHO cells (91), in which the structural requirements for the ligand-specific redistribution of the receptor have been determined. Electron microscopic analysis revealed a preferential initial association of ^{125}J-insulin with microvilli at low temperatures. Depending on the incubation time at 37^0C, this association shifted from microvillous to 'nonvillous' domains in all cell lines in which insulin receptors were normally autophosphorylated. In contrast, in four autophosphorylation-deficient cell lines, the receptors were unable to leave microvilli after insulin binding even during incubation at 37^0C. The authors concluded that the unstimulated insulin receptor underlies constraints maintaining it on microvilli. Following stimulation of receptor kinase and autophosphorylation, the receptor is released from this constraint. The presented electron microscopic pictures, however clearly demonstrate that all mutants with intact receptor autophosphorylation, most obviously the wild type cells, respond to incubation with ^{125}J-insulin at 37^0C with conspicuous shape changes of microvilli into large sack-like surface protrusions carrying the labelled receptors at their bases (Carpentier et al., 1992). This picture closely corresponds to the insulin-induced shape changes of microvilli on 3T3-L1 adipocytes that we had documented by REM techniques (Lange et al., 1990) and clearly demonstrates that insulin activation of cells with intact receptor autophosphorylation results in shape changes of microvilli. Unfortunately, transmission

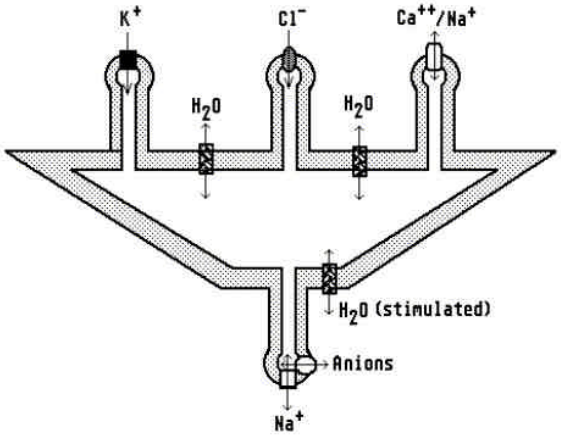

Figure 19. Distribution of ion and water channels on the surface of polarized epithelial cells. The upper part of the scheme represents the basal cell surface bearing water channels on the plasma membrane and K^+ and Cl^- (anion) and nonselective cation channels on microvilli. Lower part: Apical microvilli are equipped with Na^+ channels and various types of large anion channels. Transient appearance of water channels on the apical plasma membrane is due to receptor-stimulated exocytosis.

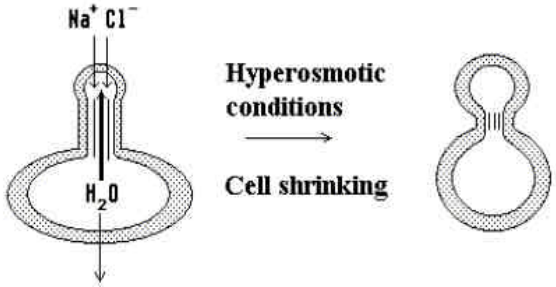

Figure 20. Activation of microvillar ion channels by hypertonic exposure. The microvillar tip (entrance) compartment rapidly equilibrates with the hyperosmotic external medium. Water flow through the microvillar diffusion dilates the entrance compartment and activates NaCl influx by shortening the diffusion barrier.

electron microscopy, in contrast to REM, is an inadequate technique to observe changes in the microvillar organization of the cell surface.

The results of these studies are in accord with the presented concept of microvillar transport regulation confirming some of its most important mechanistic aspects:

- First, insulin receptor activation is necessary to initiate microvillar shape changes.
- Second, insulin-induced shape changes of microvilli are due to intracellular processes downstream of receptor phosphorylation.
- Glucose transporters, insulin receptors, and the diffusion barrier are colocalized within microvilli, which may be taken as a functional unit involved in transport regulation.

The postulated 'constraint maintaining the receptor on microvilli' can be explained by the rigid structural organization of microvilli and not necessarily by direct or indirect linking to the cytoskeleton as proposed by Carpentier *et al.* (91).

7. Insulin-independent forms of glucose transport modulation

7.1. Transport activation by hyperosmotic conditions

In a recent survey (8), the implications of the diffusion barrier concept on the mechanism of cell volume regulation were discussed. The ability for rapid volume regulation following an anisotonic challenge is a systematic property of cells with microvillar surfaces emerging from the specific distribution of ion and water channels on the surface of differentiated cell. Here, the consequences of hypertonic treatment for cells with microvillar surfaces are shortly described and their relevance for transport modulation is pointed out.

7.1.1. Distribution of ion and water channels on the cell surface

Following the proposed concept of regulated microvillar pathways, functional relevant integral membrane proteins of differentiated cells are primarily located on the tips of microvilli, whereas the surface of the cell body is largely depleted from these components. Depending on their specific anchorage to the actin cytoskeleton, various membrane proteins appear to be resident within the microvillar structure, whereas other proteins that are not linked to microfilaments can leave the exocytic membrane domain. Thus, a specific set of ion channels, characteristic for the respective tissue type, is located beyond the diffusion barrier on microvillar tips.

According to the present state of knowledge, ion and water channels of epithelial cells are distributed in the following way (Figure 19):

- Water channels are localized on the basal plasma membrane (167-170) but are largely absent from the apical plasma membrane except after vasopressin stimulation (171, 172). Basal microvilli are devoid of water channels (167, 173-175).
- K^+ channels (176-178), Cl^- channels (179, 180), ATP-permeable anion channels (51, 52), and Ca^{++}/Na^+ permeable non-selective cation channels (180-183) are present on basal microvilli.
- Epithelial Na^+ channel (128, 184-186), anion channels with Cl^-, ATP, and osmolyte permeability such as MDR (187-189), CFTR (190), MRP/ MOAT (191), and monocarboxylate transporter MTC (192), and, occasionally, water channels (167, 193) are detected on apical microvilli of polarized epithelial cells.

7.1.2. Hyperosmotic treatment co-activates glucose transport and ion fluxes

Due to the specific arrangement of ion channels on microvilli of differentiated cells, the increase of external osmolarity primarily results in a corresponding increase of osmotic activity within the entrance compartment by NaCl or osmolyte uptake (Figure 20). Since microvillar membranes are largely devoid of water channels, the hypertonic entrance compartment draws water from the

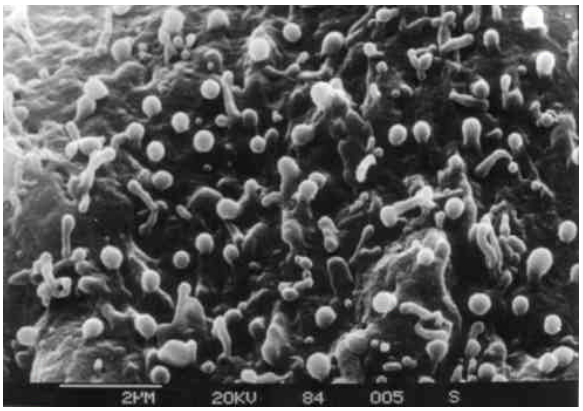

Figure 21. SEM of the surface of a 3T3-L1 adipocyte after 12h of glucose starvation.

cytoplasm through the diffusion barrier. The finely dispersed surface spaces of the entrance compartment responds much more rapidly to osmotic changes than the cytoplasm does by water exit through the plasma membrane. Consequently, the entrance compartment rapidly swells accompanied by shortening of the microvillar shafts. The resulting decrease of the diffusion barrier function allows entry of not only Na^+ and Cl^- but also of glucose from the entrance compartment into the cytoplasm. This activation mechanism for microvillar pathways also applies to cells lacking water channels.

The hypertonic enlargement of the microvillar tip compartment is accelerated by microfilament disassembly within this compartment due to reduction of G-actin and ATP concentrations. Dilution of both components critically lowers the stability of actin filaments at their fast reacting, barbed ends exposed to the entrance compartment. Terminal ATP-actin subunits confer stability to the barbed ends of the filament and allow rapid elongation by addition of further ATP-actin monomers. In contrast, barbed end subunits containing ADP instead of ATP (or ADP-P_i), initiate, rapid dissociation of monomers, which results in the sudden decomposition of large regions at the barbed ends of microfilaments (dynamic instability). Filament shortening proceeds unless addition of ATP-G-actin again stabilizes the filament ends (review see 194). Because actin filaments are always oriented with their barbed ends to the top of microvilli, the filament ends at the tip compartment sensitively respond to swelling-induced changes of ATP and actin monomer concentrations with dynamic instability.

The proposed mechanism of hypertonic volume response is strongly supported by the observations of Winterhager and Stieve (195) and Korten and Böttcher (196) who observed that microvilli do not shrink under hypertonic conditions but, instead, dilate. Hypertonic treatment increases microvillar diameters 2- to 3-fold and decreases the total number of microvilli most probably due to integration into the plasma membrane.

Moreover, using X-ray microanalysis, Schrärmeyer *et al.* (197) presented direct evidence that the entrance compartment rather reflects environmental than cytoplasmic ionic conditions. Microvilli of photoreceptor cells contain higher Na^+ and lower K^+ concentrations than the cytosol. The authors concluded that the rhabdomeric plasma membrane is permeable to these ions allowing rapid equilibraton of the microvillar compartment with the extracellular medium.

Similar observations were reported by Lumpkin and Hudspeth (180, 198) and by Postma *et al.* (183). Applying fura-2 /confocal laser scanning microscopy they demonstrated high Ca^{++} concentrations in the tip compartments of hair cell microvilli: *Unstimulated hair cells showed a tip blush of enhanced fluorescence at the hair bundles top, which attribute to Ca^{++} permeation through transduction channels open at rest. Upon mechanical stimulation, individual stereocilia displayed increased fluorescence that originated near the tips and spread towards their bases* (180).

Further compelling arguments in favour of the proposed mechanism come from studies, which demonstrated that hyperosmotic stimulation of glucose transport is always accompanied by co-activation of ionic fluxes (22, 199-204). Moreover, hypertonic pretreatment completely prevents any further insulin stimulation of glucose transport (25). Similarly, glucose starvation-stimulated transport cannot be further activated by hypertonic treatment (204) indicating at least partial identity of the involved mechanisms.

Hypertonicity as well as insulin receptor activation affects the diffusion barrier function by disorganization of the microvillar cytoskeleton. They do this, however, on different ways. Whereas insulin, via activation of phosphatidylinositol 3-kinase, induces shortening of the microvillar actin filaments from their cytoplasmic ends (150), hypertonicity induces disassembly of microfilaments at the tips of microvilli by dilution of the internal water space. An almost identical mechanism underlies starvation-induced activation of glucose transport.

7.2. Glucose starvation-induced transport stimulation

Transport stimulation by glucose-starvation induces shortening of the diffusion barrier by disassembly of the microfilament bundles from the top of microvilli. Similar to hypertonic treatment, glucose starvation critically lowers the ATP concentration within the tip compartment, thereby enhancing the dynamic instability at the barbed filament ends.

Starvation-induced and insulin receptor-mediated transport stimulation can be differentiated by SEM (150). In 3T3-L1 adipocytes, insulin initiates transient structural changes at the basis of microvilli giving rise to domed membrane areas on which the upper parts of microvilli remained unaffected (Figure 10b). This picture suggests a mechanism of microvilli shortening that starts from the cytoplasmic side. In contrast, glucose starvation ultimately results in the complete loss of the shaft region accompanied by enlargement of the microvillar tips, which then appears to protrude directly from the cell surface (Figure 21). This picture clearly points to a shortening mechanism that starts

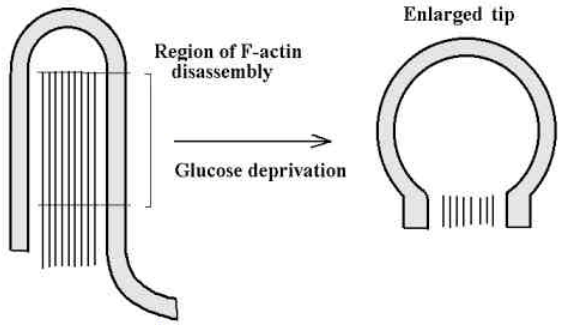

Figure 22. Glucose starvation- (and osmotic shock-) induced disassembly of the diffusion barrier starts from the tips of microvilli. The membrane portion covering the shaft region of disassembled microfilaments is integrated into the enlarged spherical tip of the original microvillus.

from the top of the microvilli as schematically shown in Figure 22. Due to the loss of cytoskeletal support, the membrane of the upper shaft region is integrated into the tip domain, increasing the microvillar tip diameter from 200 to 400 µm (Figure 21).

Interestingly, glucose starvation-induced (8- to 10-fold) transport activation is not accompanied by 'transporter redistribution between plasma membrane and microsomal fractions' as assessed by fractionation techniques. Fisher and Frost (32) stated that *the increase in transport activity associated with glucose deprivation does not result from translocation of either of the glucose transporters known to exist in 3T3-L1 adipocytes*.

The observed changes in the surface morphology of starved 3T3-L1 adipocytes (Figure 21) provide a compelling explanation for this behaviour. In contrast to insulin-treated cells, on which a large portion of microvilli is completely integrated into the ruffling plasma membrane, the surface of glucose-starved cells exhibit intact spherical blebs representing the enlarged tip domains of the original microvilli. Most likely, these surface blebs are not integrated into the plasma membrane because they are still stabilized by the surface coat. Common techniques of cell homogenization transform these surface vesicles into a homogeneous vesicle population exhibiting microsomal rather than plasma membrane properties. The same fractionation behaviour is observed after hyperosmotic stimulation. The difference in the subcellular fractionation behaviour between glucose starvation and insulin action may be due to the removal of surface coat components by insulin receptor-mediated activation of surface proteases.

The effect of glucose-starvation on the cell surface is closely related to that of hyperosmotic activation. Both conditions cause shortening of the diffusion barrier by disassembly of the microfilament bundles from the top of microvilli. During hyperosmotic challenge, dilation of this compartment is caused by osmotic salt (NaCl) and (cytoplasmic) water entry. Similarly, glucose starvation-induced microvilli shortening leads to cell volume increase (5) most likely due to activation of ionic pathways. Thus, glucose starvation as well as hyperosmolarity enhances in- or efflux activities for both glucose and ions via a common mechanism.

Costimulation of transporter and channel activities is a clear consequence of the proposed general role of microvilli in regulation of transmembrane substrate and ion fluxes. The involvement of microvilli in Ca^{++} signaling also explains the observed co-activation of hexose transport and Ca^{++} signaling by bombesin in Swiss 3T3 fibroblasts (205, 206) and by insulin in ß-cells (207), CHO cells (208), hepatocytes (209) and pancreatic ß-cells (210).

On the other hand, the concept of microvillar channel and transporter regulation opens a direct access to the phenomenon of cell swelling induced by receptor activation as shown for the insulin action. Al-Habori *et al.* (211) and Baquet *et al.* (212) have demonstrated that insulin-induced stimulation of ionic influx pathways in hepatocytes can be completely mimicked by hyposmotic cell volume increase, which is another way to shorten the microvillar diffusion barriers and to increase ion and substrate fluxes along this pathway (reviewed in 8). Some authors even considered cell volume changes to be a second messenger for transmission of hormonal and environmental signals (213).

7.3.Transport stimulation by metabolic depletion

The mechanism of transport stimulation by metabolic depletion is another example for the regulatory function of the cytoskeletal diffusion barrier. This type of uptake stimulation closely resembles that of hypertonic treatment and glucose starvation. Again shortening of the cytoskeletal diffusion barrier is caused by ATP depletion, however, due to inhibition of the mitochondrial ATP production, cytoplasmic ATP is involved. Consequently, disassembly of the diffusion barrier is initiated from the cytoplasmic ends of the filament bundle. The morphological consequences of cytoplasmic ATP depletion have been described for various cell types:

- Anoxic hepatocytes display globular microvilli and bleb formation (69).
- Lowered ATP results in thinning of microvillar actin in renal cultured cells (72).
- Proximal tubule cells lose microvilli during hypoxia or metabolic depletion accompanied by changes in actin cytoskeleton (70, 71).
- In hypoxic placental trophoblasts, reduction in number and shortening of microvilli is accompanied by elevated glucose consumption and lactate production (67).
- White *et al.* (214): Microvillar actin bundles are disrupted in anoxic proximal tubule cells due to cytoplasmic ATP depletion. Within individual cells, all microvilli collapsed simultaneously and microvillar actin filaments including villin underwent a parallel translocation to a perinuclear location.

A large body of experimental data on transport stimulation by metabolic depletion has accumulated for the liver cell line clone 9 that responds to ATP depletion by a significantly (up to 12-fold) increased glucose uptake rate.

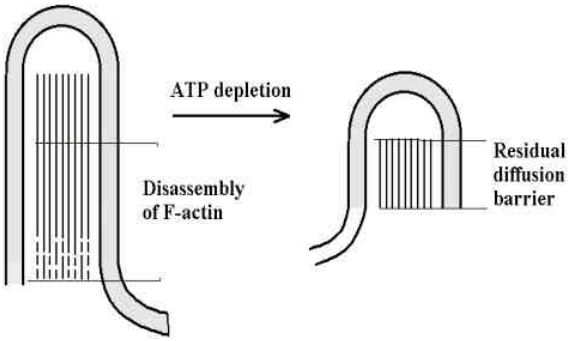

Figure 23. Disassembly of the microvillar diffusion barrier by ATP depletion. In contrast to glucose starvation (Figure 18), microfilaments are shortened from the cytoplasmic side during metabolic depletion.

Again, no change in the transporter distribution between subcellular fractions could be observed (26, 37, 33).

Together, these data suggest that cytoplasmic ATP depletion results in similar changes of the microvillar diffusion barrier, as does hyposmotic treatment or glucose starvation. All three conditions cause disassembly of the microvillar F-actin and, consequently, shortening of microvilli. Because the stabilizing surface coat is not affected, the original microvillar tip domains remain intact appearing as small bleb-like surface protrusions (Figure 23). Following cell homogenization and centrifugation, these surface blebs are recovered in right-side-out orientation within the microsomal fraction.

7.4. Further evidence for the involvement of the actin cytoskeleton in glucose transport modulation

Several recent publications have stressed the role of the actin cytoskeleton in insulin-induced transport stimulation, although the exact function of F-actin in insulin signaling remained unclear. Wang, *et al.* (12) described the inhibitory action of the F-actin-disrupting agents, Cytochalasin D and latrunculin B, on the insulin-induced reorganization of the actin cytoskeleton in 3T3-L1 adipocytes, which is accompanied by a 50% inhibition of insulin stimulated glucose transport. PI 3-kinase activity is not changed by these agents. Cytochalasin D also decreases by about 50% the insulin-dependent redistribution of GluT1 and GluT4 transporters to the plasma membrane fraction. Similarly, Omata *et al.* (15) observed a reduction of insulin-stimulated glucose transport after treatment of isolated rat adipocytes with latrunculin A and the complete inhibition of GluT4 translocation to the plasma membrane. The same results were obtained after disorganization of the actin cytoskeleton via Rab or Rho/Rac inactivation (13, 14).

Since the work of Sampath and Pollard (215) it is known that *in vitro*, cytochalasin D only slows down the rate of actin polymerisation but does not completely stop this process. Consequently, there is no reason to assume that application of cytochalasin D to cells may cause microfilament disruption. As these authors pointed out, the main reduction of the polymerisation rate occurs at the barbed ends of the filaments, which are in the case of microvillar F-actin completely membrane shielded. Thus, an action on the basal transport rate is not to be expected. The reduction of insulin-stimulation of transport by cytochalasin D may indicate that PI 3-kinase-induced F-actin reorganization into submembraneous networks (membrane ruffling; see Figure 11) is a necessary mechanistic component for full activation of glucose transport by insulin.

8. CONCLUSIONS

A novel mechanism of glucose uptake regulation is proposed, which emerges as a special case of a general form of in- and efflux regulation for substrates and ions via microvillar pathways. The establishment of the specific structural organization necessary for this type of regulation, the microvillar surface, is characteristic of resting and differentiated cells. The consequences of this type of surface organization meets the requirements of differentiated tissue cells for regulation of essential cellular functions by external signals such as hormones, growth factors, and transmitters and to respond to various environmental conditions including high or low levels of glucose supply, metabolic stress, and unusual thermal or osmotic conditions.

One of the most important aspects of this surface reorganisation is the differentiation-dependent switch of energy metabolism from metabolic regulation to membrane limitation, which allows the effective integration of oxidative phosphorylation into the ancient framework of glycolytic energy production. Furthermore, the formation of microvillar cell surfaces creates essential preconditions for the function of the Ca^{++} signal pathway, the main signaling system of differentiated tissue cells. Actin filaments of microvilli represent two essential components of this pathway. First, the highly effective diffusion barrier enables the cell to maintain a Ca^{++} gradient of four orders of magnitude by preventing the entry of divalent cations into the cytoplasm. Second, the ability of F-actin for high-affinity Ca^{++} storage allows cytoplasmic Ca^{++} to return quickly from high signaling to low basal levels. The same cytoskeletal structure that regulates receptor-operated Ca^{++} fluxes is also involved in modulation of glucose uptake and in activation of ionic fluxes in response to changes of environmental osmotic conditions (8).

Numerous further adaptations of microvillar functions exist in living systems, the most important of which can be found in various sensory cells mediating sound, light and taste perception via changes of the membrane potential. Another field is electrical signal transduction in the nervous system. Nerve cells do not express microvilli on the cell body but, instead, dendritic spines, the post-synaptic receptors of nerve cells representing the functional analogues of microvilli. Even dendritic spines are able to change their length and shape in response to synaptic activity, thereby, precisely adjusting their sensitivity to the functional load. A further adaptation of high physiological relevance is the polarized microvillar surface of epithelial cells, which appears to be a

sophisticated defense system protecting the cell against external cytotoxic compounds as well as anisosmotic conditions. As it appears, the microvillar structure is an ancient, highly conserved biological device that has been continuously modified to fit the ever-changing demands of life.

The proposed microvillar mechanism of glucose uptake regulation is compatible with most of the established data on this field. It explains by only one common final mechanism so different types of transport modulation as the insulin action, the action of high and low external glucose, of hyperosmotic environment, and metabolic depletion. Many other puzzling features such as costimulation of glucose and ion fluxes turn out to be intrinsic properties of the proposed mechanism. The concept completes the translocation mechanism of transport regulation, solves most of its inconsistencies and shortcomings, and may contribute to a better comprehension of diabetes, obesity, and hypertension.

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