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Insulin and cellular stress induced glucose uptake in 3T3-L1 adipocytes

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Chapter X

General Discussion.

Over the past years, the field of insulin-induced GLUT4 translocation has witnessed several important breakthroughs, making it at present only a matter of time before the research lines from the insulin receptor downwards and from the GLUT4 membrane-fusion upwards are fully linked [1;2]. Important inroads into the mechanism of GLUT4 vesicle translocation are the identification of the essential contributions of PKB- β and PKC- λ downstream of insulin-induced PI-3'kinase and the identification of the CAP-Cbl-TC10 pathway. Rather than presenting an exhaustive overview of all progress made, this chapter aims to focus on two key aspects in the regulation of glucose uptake in 3T3-L1 adipocytes that appear as a central theme linking the previous chapters. These aspects are 1. Regulation of glucose transporters after membrane-insertion. And 2. The role of PKC- λ in mediating translocation of glucose transporter containing storage vesicles.

Although the involvement of PKB- β in mediating insulin-induced GLUT4 translocation is undisputed [3-6], several lines of evidence (such as arsenite- and osmotic shock induced GLUT4 translocation) illustrate that it is dispensable for initiating GLUT4 vesicle translocation per se (Chapter 3)[7]. Rather, PKB appears to act as a licensing factor, conferring specificity on the insulin signal. Nonetheless, the identification of the downstream effectors involved in mediating this response to PKB and the mechanism through which they are bypassed by cellular stress-induced GLUT4 translocation should provide important insights in insulin-signalling and diabetes.

A hypothetical model on the regulation of GLUT4 mediated glucose uptake after membrane insertion.

Several observations suggest that translocation and GLUT4-mediated glucose uptake can be segregated, suggesting an involvement of different pathways in mediating these events. First, there is a consistent gap in time between the reported $t_{1/2}$ of GLUT4 translocation and the $t_{1/2}$ of glucose uptake, with translocation preceding glucose uptake. Second, the IC_{50} of GLUT4 translocation is roughly ten-fold higher than the IC_{50} of glucose uptake for wortmannin. Importantly, insulin-induced p38 MAPK activation being as sensitive to wortmannin as the latter. Third, there is a consistent discrepancy between the insulin-induced fold increase in plasma-membrane GLUT4 and fold-increase in glucose uptake, with the latter being 1.5-2 fold higher [8-10]. Thus, there appears a p38 MAPK-dependent component involved in regulating glucose uptake after GLUT4 membrane insertion.

Intriguingly, a similar involvement of insulin-induced p38 MAPK signalling has been described for norepinephrine transporters in SK-N-SH cells [11]. In this system the effect was mediated through a p38 MAPK-induced association between the NE transporter and PP2A. Although no association between the GLUT4 transporter and PP2A phosphatase has been described in literature, it is well established that insulin induces dephosphorylation of plasma-membrane localised GLUT4 [12-15], suggesting a similar mechanism may be involved in regulating GLUT4. To describe this phenomenon, the term “intrinsic activation” was coined by Klip and co-workers as a model to account for this phenomenon. In this model, an effect of insulin-induced p38 MAPK on the speed of glucose uptake by the GLUT4 transporter was suggested. A different and alternative model employs data derived from the GLUT1 transporter. Although a facilitative transporter, GLUT1 mediated glucose transport is sensitive to intracellular levels of ATP, through a process termed “intrinsic occlusion” [16-18]. Significant structural and mechanistic similarities between this regulation of GLUT1 and GLUT4 have been described in Chapter 5. According to this hypothesis glucose is transported across the plasma-membrane by an oscillation of the transporter between an outward (sugar accepting from the extracellular environment) and inward (sugar release into the cytosol) facing conformation. Importantly, these two conformations are mutually exclusive. As a consequence, locking the transporter in either conformation using cytochalasin B or by introducing point mutations impedes glucose turnover and consequentially glucose uptake by the cell [19-22]. Intriguingly, ATP-binding to GLUT1 inhibits an anti-GLUT-1 C-terminal antibody binding [17]. Conversely insulin-stimulation unmasks a C-terminal epitope in GLUT4 [23-25].

Involvement of a defect in turnover after inhibition of p38 MAPK in insulin-stimulated glucose uptake can be observed employing a mannose-derived photoaffinity labelling compound (ATB-BMPA) [28;29] on SB203580 treated adipocytes. As can be seen in Fig. 1A pre-treatment of 3T3-L1 adipocytes with SB203580 does not affect insulin-induced translocation of the GLUT4 transporter from the LDM fraction towards the PM fraction of the transporters. Application of the photoaffinity labelling compound shows a reduction in the amount of accessible exofacial glucose binding sites in the GLUT4 transporter after treatment with SB203580 (see Fig. 1A). A further consideration with respect to these data is that during its initial stages of membrane fusion, the GLUT4 transporter is known to transit through a stage wherein the transporter (although already present in the PM fraction) is not yet fully accessible to the extracellular environment [26;27]. This phenomenon (termed “occlusion”) could equally well be responsible for the effects observed.

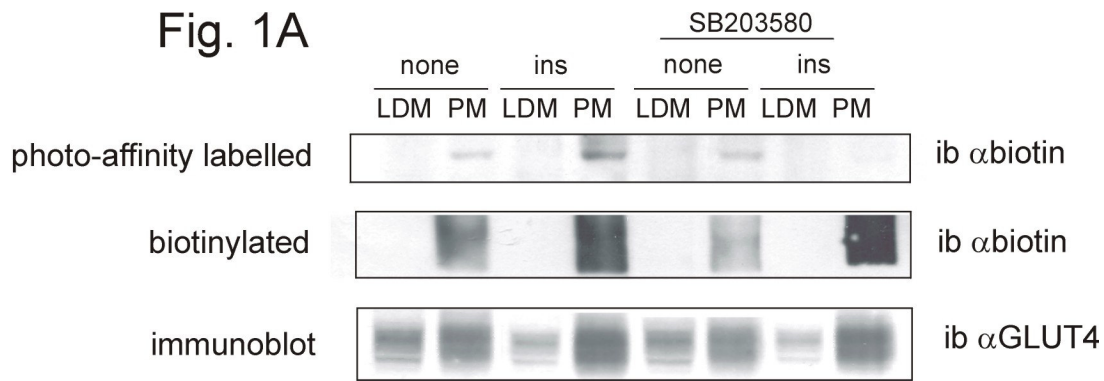


Fig. 1B

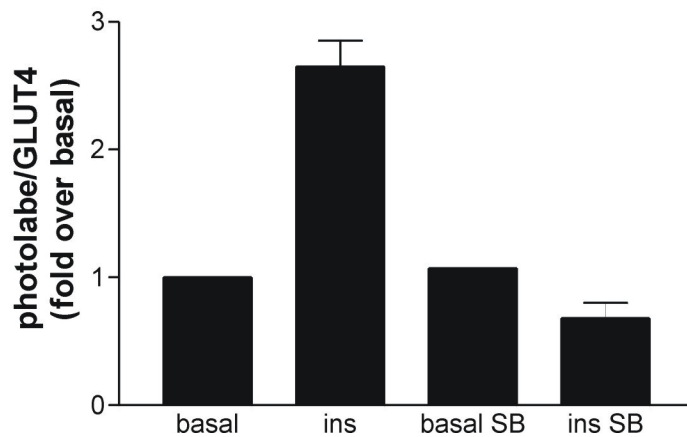


Fig. 1 effects of SB203580 on insulin-induced GLUT4 translocation.

Adipocytes were pre-treated with 10 μ M SB203580 for 15 minutes and subsequently stimulated with 100 nM insulin for 15 minutes as indicated. Cells were subjected to subcellular fractionation and equal amounts of protein were subjected to immunoblot analysis. Panel A. Representative immunoblots of subcellular fractionation experiments. The top frame shows the results obtained in duplicate when cells were treated with photoaffinity labelling agent (ATB-BMPA) following established procedures. The presence of the photolabel is probed by immunoblotting using an antibody against the biotin moiety present in this compound. The lower frame shows an experiment performed in triplicate of subcellular fractionation samples obtained from 3T3-L1 adipocytes subjected to the biotinylation assay. These samples were also probed with an anti-biotin antibody. The lowest frame illustrate data of subcellular fractionated cells when probed with an antibody against GLUT4. This analysis was performed routinely in the experiments shown in the frames above. Panel B. Immunoblots were quantified on a Lumi-Imager and expressed as relative amounts of photolabel per amount of GLUT4 resident in the PM fraction. Data shown are means of an experiment performed in duplicate \pm SEM.

However, treatment of the intact adipocytes with periodate after insulin-stimulation causes oxidation of vicinal diol groups in exposed sugar-side chains of the GLUT4 transporter allowing subsequent reaction of these sugars with a biotinylating reagent. As can be observed in Fig. 1A, when performing this procedure no effect of SB203580 on the magnitude of insulin-induced exposure to the extracellular environment was observed, thus ruling out an effect of SB203580 on transition through the occluded state. The dramatic effect of SB203580-treatment on glucose turnover by the insulin-responsive GLUT4 transporter can be particularly well grasped when expressing the relative levels of photolabel over the levels of GLUT4 present in the PM-fraction (i.e. indicating the amount of photolabel per amount of GLUT4). As can be seen in Fig. 1B, when analysed as such the amount of photolabel binding to the GLUT4 transporter is completely reduced. This amount of reduction is even more pronounced than the observed reduction in glucose uptake observed after SB203580 treatment (~30%, see also Chapter 3 and 7) and indeed more than when isolating photoaffinity-labelled samples of whole cells using streptavidin bead pulldown (as shown by us in a recently submitted manuscript). This could indicate the turnover effects observed (and hence, the GLUT4 transporters undergoing post-membrane insertion regulation by insulin) are localised in a specialised plasma-membrane subdomain, although much more data is required to substantiate this intriguing suggestion. Another important realisation in these assays is that, though labelling the exofacial glucose binding site of the GLUT4 transporter, the photoaffinity labelling compound cannot be transported into the cell [28;29]. Consequentially, these observations strongly suggest that treatment with SB203580 does not affect the intrinsic activity of the GLUT4 transporter, for in that case the photoaffinity labelling should not have been reduced.

Thus, it seems that insulin-induced p38 MAPK activity assists in GLUT4 conformational oscillation possibly in conjunction with the aforementioned effects of ATP, either through modification of the GLUT4 transporter itself (i.e. dephosphorylation) or through binding of additional regulatory proteins (such as Daxx [30]). Preliminary experiments employing plasma-membrane derived vesicles after stimulation with insulin (containing GLUT4), isolated in the presence or absence of exogenously supplied ATP provide some further information on this process. In this analysis it was observed that the presence of 10mM ATP (similar to the level of ATP in adipocytes, see Chapter 4) markedly inhibited the amount of glucose taken up in the isolated PM-derived vesicles (unpublished observations). At a higher level of cellular organisation, such a mechanism would ensure the available glucose being shunted towards the cells most deprived in ATP and consequently most in

need of glucose. Furthermore, cellular stress-induced p38 MAPK activation would aid in enhancing the glucose turnover of transporters in the plasma-membrane, providing the cells with the energy required to face the damage sustained by the cellular-stress encountered.

Another prime candidate in regulating such an effect is the chaperone protein-family. Several proteins appear to be regulated by a molecular chaperone complex composed of Cdc37 and the associated chaperone HSP90. Association of this complex may be involved in regulating the proper conformation of these “client”-proteins [31-33]. Protein kinases regulated by this complex are unable to fold properly and consequently undergo degradation after prolonged treatment with geldanamycin and/or radicicol, two structurally unrelated inhibitors that interact with the ATP-binding pocket of HSP90 [34;35]. Intriguingly, short term incubation of 3T3-L1 adipocytes with these inhibitors for only 15 minutes prior to insulin stimulation resulted in a 30% inhibition of glucose uptake without an apparent effect on GLUT4 translocation (Fig. 2A and B). Thus, interference with HSP90-activity reiterates the effects of p38 MAPK inhibition, suggesting a possible link between these two pathways.

A hypothetical model on the role of PKC- λ signalling and adipocyte-polarity.

Both in arsenite-induced GLUT4 translocation (Chapter 3) and in PMA-induced GLUT1 translocation (Chapter 8 and 9), PKC- λ appears as a central mediator regulating the subcellular localisation of GLUT transporter containing vesicles. However, curiously, in neither instance there is evidence for the activation of PKC- λ . Importantly, in case of PKC- λ , the second required phosphorylation site (equivalent to S⁴⁷³ of PKB) is replaced by an glutamic acid [36-38]. Consequentially, basal levels of PKC- λ activity are already quite high, with only a 1.5-2 fold induction after stimulation with insulin [39;40] (Chapter 3.). Thus, rather than activation, the precise subcellular localisation of PKC- λ could be the important factor in regulating vesicle transport. A possible mechanism is provided by some key observations made in other cell-biological mechanisms. Both in the establishment of cellular polarity in *C. elegans* as well as in defining the zona occludens and the site of tight junction formation in epithelial cells PKC- λ plays an essential role [41-46].

Fig 2A

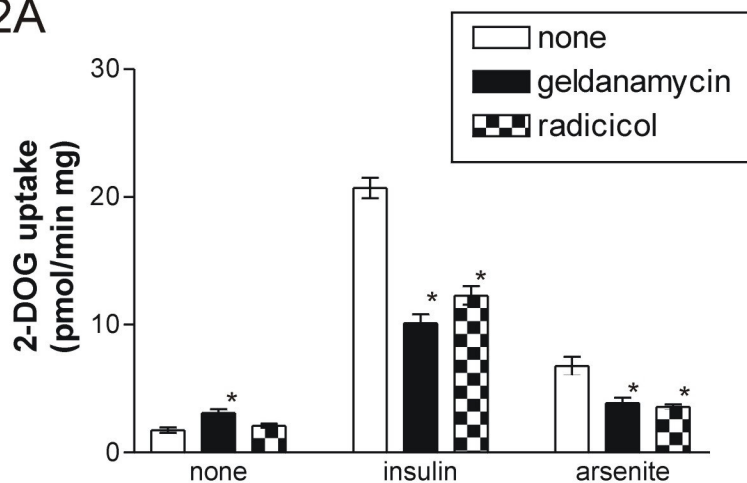


Fig 2B

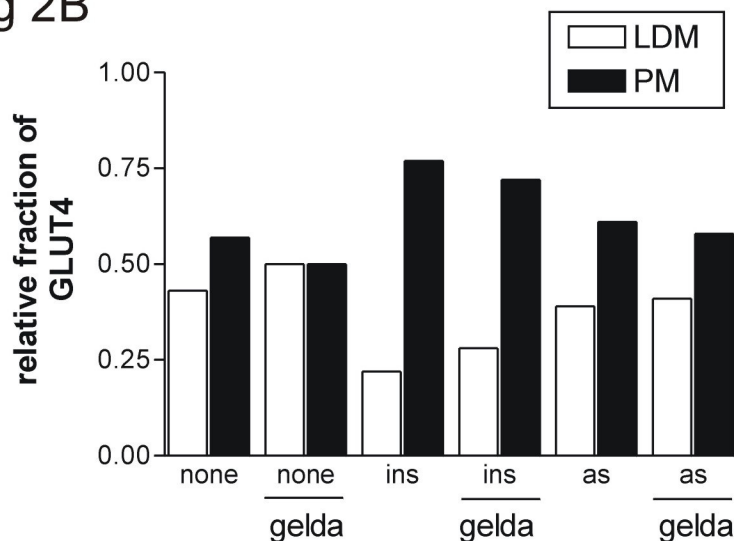


Fig. 2 effects of inhibition of HSP90 on insulin- and arsenite-induced glucose uptake.

Panel A. 3T3-L1 adipocytes were pre-treated with 10 μ M of either geldanamycin or radicicol for 30 minutes and subsequently stimulated with 100 nM insulin for 15 minutes or 0.5 mM arsenite for half an hour and assayed for glucose uptake as indicated. Data shown are means \pm sem of three independent experiments each performed in triplicate. An * indicates $p < 0.05$. Panel B. 3T3-L1 adipocytes were pre-treated with 10 μ M of geldanamycin (gelda) for 30 minutes and subsequently stimulated with insulin (ins) or arsenite (as) as described above. The cells were subjected to subcellular fractionation and the relative amounts of GLUT4 residing in either the intracellular microsomes (LDM) or the plasma-membrane (PM) are indicated.

In these processes a complex consisting of activated Cdc42, PAR3 and PAR6 serve to tether PKC- λ to a particular site at the plasma-membrane thus turning this rather constitutively active PKC in a well defined, localised signal mediating cellular polarity. PKC- λ subsequently phosphorylates the tumour suppressor protein mLGL, inducing this syntaxin-4 binding protein to release syntaxin-4, enabling v-SNARE/ t-SNARE mediated vesicle fusion to occur at a pre-determined site at the plasma-membrane and consequentially establishing a polarised cell [47;48]. Though the regulation of mLGL may be of importance, preliminary evidence on tomosyn, a mLGL-family member, suggests this protein-family acts as a supplementary regulator [49]. Perhaps it is the central ability of this Rho-GTPase, PAR3/6, PKC- λ complex to give direction to cellular events such as the establishment of tight junctions, migration and asymmetric cell-division that provides us with an important clue to its role in insulin signalling. Though the rounded 3T3-L1 adipocyte can hardly be perceived as a polar cell, on a micro-scale polarity may exist. After all, in response to insulin, the GLUT4 vesicles have to be transported in a given, possibly even pre-determined direction in order for the v- and the t-SNARES to link up and to allow membrane fusion. Support for this suggestion is provided by the observed inhibition of insulin-induced GLUT4 translocation by ectopically expressed ASIP/PAR3 [50]. The importance of PI-3'kinase signalling in cell-polarity has also been established in processes such as wound-healing and oncogenic metastasis [51-53].

To conceptualise this hypothesis is to perceive the “rosetta”-structures as jellyfish afloat in a sea of lipids with their actin-based tentacles dangling in the adipocyte-cytoplasm ready to ensnare any passing GLUT-Storage Vesicle. After insulin-stimulation captured GSVs will subsequently be hauled in by localised actin depolymerisation to merge with the jellyfish body (Fig. 3).

To describe this model in its molecular components, the rim of the jellyfish in this model is formed by a clustering of caveolae (microscopically seen as rosettes [54], Fig. 3B). This clustering causes a highly localised concentration of the CAP-Cbl complex and downstream signalling-components when translocated towards the caveolae after insulin-stimulation [55;56] (Fig. 3C). Furthermore, the body, formed by the so-called adipocyte-caves [57], will be circumvented by the specialised adipocyte cav-actin protruding downwards from the plasma-membrane [58] (Fig. 3A).

Kinesin carries the GLUT-1 and -4 Storage Vesicles along the microtubular (MT) and actin cytoskeleton, though this movement has been suggested to occur non-directional [59-62].

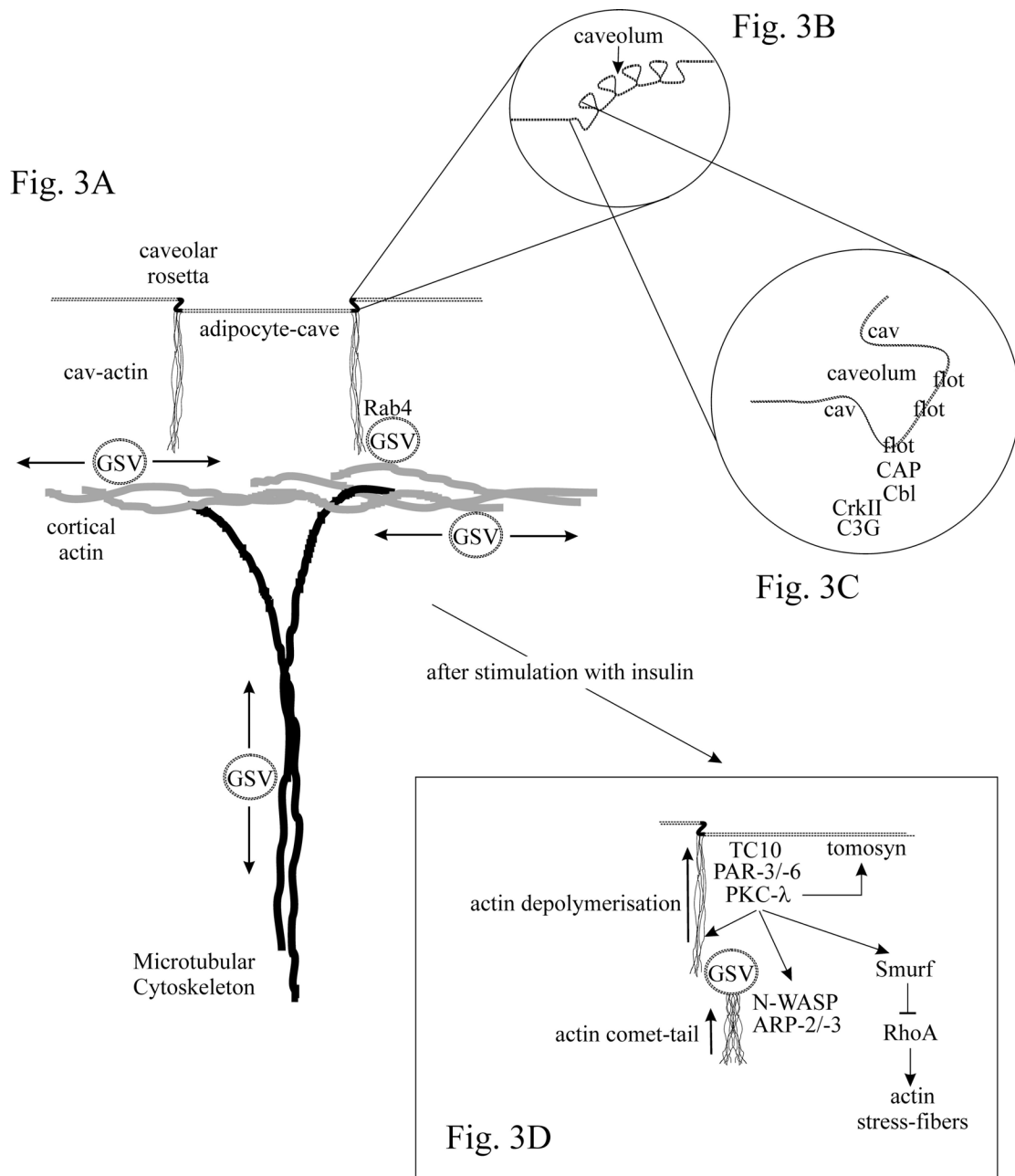


Fig. 3 A hypothetical model on the mechanism of insulin-induced GLUT4 translocation in 3T3-L1 adipocytes.

Panel A. depicted are the adipocyte-caves locked between caveolar-rosetta structures with associated cav-actin structures. The cortical actin runs underneath the plasma-membrane and is linked to the cell interior through the microtubular cytoskeleton.

Panel B. Enlargement of de caveolar-rosetta. Panel C. Enlargement of a single caveolium, illustrating how translocation of CAP-Cbl forms a localised enrichment in insulin-signalling components. Panel D. suggested processes in action after insulin stimulation. The direction of GSV-translocation mediated by the combined force of cav-actin depolymerisation and N-WASP induced actin polymerisation are indicated with arrows. Arrows emanating from the TC10-PAR-3/-6-PKC-λ complex illustrate activation steps. Inhibitory actions are indicated with a barred arrow. See the list of abbreviations for a description of the components depicted.

Nonetheless, as a mere matter of chance, this would result in movement of the GSVs along the microtubular cytoskeleton outwards, away from the tubulo-vesicular structures towards the plasma-membrane. Subsequent transfer of the vesicles to a similar (if not identical) transport system then carries the vesicles along the cortical actin structure running parallel with the plasma-membrane (see Chapter 2). The vesicle is then positioned to link up with the adipocyte cav-actin structure (see Fig 3A), leading to a tethered complex in the juxta-membrane compartment through the activities of proteins such as Rab4 [63-65] and/or LIMK [66-69]. Subsequent stimulation with insulin then induces a highly localised activation of the CAP-Cbl-TC10 signalling route in the near vicinity of the cav-actin structures. The hauling in of tethered vesicles can then occur simply by shortening cav-actin spikes with the attached GSV bringing these vesicles in close proximity to the (then activated) v-SNARE- and exocyst-complexes (see Chapter 1). Aside from cav-actin shortening, the N-WASP/Arp2-3 mediated formation of actin comet-tails under the control of TC10 may contribute to provide an additional driving force mediating this vesicular translocation [70-72](Fig 3D). When employing a model as described here, the puzzling deleterious effects of both actin stabilisation and actin depolymerisation on insulin-induced GLUT4 translocation (as described in Chapter 2) are easily resolved. Meanwhile, the adipocyte-cave (the body of the jellyfish) is formed by “normal” plasma-membrane, if only deeply invaginated into the adipocyte cell-body [57]. Consequentially, the previously-mentioned association of the insulin receptor and cav-1 (Chapter 2) ensures the localised generation of pip_3 in (but not wholly limited to) the adipocyte-caves [73-75]. In this, the caveolar structures circumventing the adipocyte-caves also serve to concentrate pip_3 by presenting a boundary to the randomised diffusion of this important signalling component. Second, the enhancement of available surface for biological processes is a well established cellular mechanism. As a final consideration, mergence of the lipid-base GSV with caveolae would result in a disruption of the caveolum, which is irreconcilable with the events observed after insulin-stimulation. Thus, the composition of the adipocyte-caves presents the adipocyte with the opportunity to translocate all GLUT4 containing vesicles at hand into the adipocyte-cave domain with its own complement of pip_3 (and possibly PKB- β , v-SNAREs and exocyst complexes) without further deleterious consequences for subsequent membrane-fusion events. In fibroblasts, the continues remodelling of the microtubules is thought to enable the microtubules to search and capture special sites in the cortex [76]. In this aspect, the regulation of the microtubular cytoskeleton could also be a key factor in insulin-signalling. Regrettably, little is know about the regulation of the microtubular network by insulin signalling

pathways, its interaction with the adipocyte cortical actin and the role of known important players therein such as APC, CLIP-170 and IQGAP [76]. Furthermore, much remains to be elucidated on the genesis of the adipocyte caves. Though the observation of extracellular-matrix present in the lumen of these structures could provide an important clue for an involvement of outside-in signalling [57]. In tight junction establishment outside-in signalling is of importance and PAR-complex associating proteins such as the transmembrane protein CRB3 and the adapter protein PALS1 play an essential part in this process [42]. However, their involvement in adipocyte morphology, if any, is at present also unknown. Nonetheless the concept of “adipocyte cell-polarity” certainly merits further scientific enquiry. It is expected that the adaptation of FRET-based technologies such as FLAIR [77] and RAICHU [78;79] to the dissection of TC10 signalling pathways in 3T3-L1 adipocytes will provide novel insights into the mechanism of insulin-induced GLUT4 translocation.

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