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Endogenous SHIP2 does not localize in lipid rafts in 3T3-L1 adipocytes

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Abstract SH2 domain containing inositol polyphosphate 5-phosphatase (SHIP2) dephosphorylates phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) into phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2). SHIP2 knock-out mice demonstrated that SHIP2 acts as a negative regulator of insulin cascade in vivo. Our two-hybrid study showed that SHIP2 interacts with c-Cbl associated protein (CAP) and c-Cbl, implicated in the insulin signaling. As some proteins implicated in insulin signaling, like insulin receptor, CAP, c-Cbl or TC10, were reported to localize in lipid rafts, we addressed the same question for SHIP2. SHIP2 was detected in the non-raft fraction in CHO-IR, C2C12 myotubes and 3T3-L1 adipocytes except when it is overexpressed in CHO-IR, where we detected SHIP2 in the raft fraction.

Keywords: Lipid raft; Caveolin; SHIP2; Insulin receptor

1. Introduction

SH2 domain containing inositol 5-phosphatase (SHIP2), like SHIP1, is a member of the type II polyphosphate 5-phosphatase family. It contains SH2, proline-rich, NPXY and SAM protein–protein interaction domains and is able to dephosphorylate phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) into phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) [1]. Compared to SHIP1 that is restricted to hematopoietic cells, SHIP2 is widely expressed in a wide range of tissues [2]. SHIP2 is phosphorylated in response to growth factors [3] and by insulin in CHO-IR and 3T3-L1 [4,5]. Nevertheless, the role of the phosphorylation of SHIP2 is not well understood. It does not seem to modulate its 5-phosphatase activity but rather its interaction with partners [6]. Data from knock-out mice have identified SHIP2 as a critical and essential negative regulator of insulin signaling [7]. SHIP2 knock-out mice show increased sensitivity to insulin and die soon after birth. Heterozygous adult mice have increased glucose tolerance and insulin sensitivity associated with an increased recruitment of the Glut4 transporter and increased glycogen synthesis in skeletal muscle [7].

Insulin, via phosphatidyl inositol 3-kinase (PI3-K) activation, is known to be important for exerting a variety of metabolic actions (for review, see [8]). By dephosphorylating PtdIns(3,4,5)P_3 into PtdIns(3,4)P_2, SHIP2 regulates negatively PI3-K pathway. Its overexpression in 3T3-L1 adipocytes inhibited insulin-induced activation of Akt/PKB and decreased Glut4 translocation [9]. In the same way, it has been reported that insulin induced Glut4 translocation can also be negatively regulated by expression of SHIP1 [10]. By a two-hybrid study, we have recently shown the interaction between SHIP2, c-Cbl associated protein (CAP) and c-Cbl, providing a potential molecular link between SHIP2 and the insulin cascade [11].

Lipid rafts are described as subdomains of the plasma membrane that contain high concentration of cholesterol and glycosphingolipids. This particular composition can form a liquid-ordered phase which is resistant to some detergent solubilization. Caveolae, that were originally identified as flask-shaped invagination of the plasma membrane in endothelial and epithelial cells, can be viewed as a subset of lipid rafts. They are distinguished from lipid rafts by the presence of the cholesterol binding protein caveolin and are abundant in endothelia, muscles cell types, adipocytes and fibroblasts. A variety of proteins have been shown to partition into caveolae and lipid rafts (for review, see [12]). These microdomains can be isolated on the basis of their resistance to solubilization in non-ionic detergent (Triton X-100) at 4 °C and their low density in sucrose or OptiPrep gradients [13].

Stimulation of the insulin receptor by insulin leads to the activation of several pathways. The major one implicates the IRS proteins, activating the PI3-K. Insulin also stimulates the MAPK pathway via Shc and Gab-1 [14] and the small G-protein TC10 [15]. A possible role for caveolae and caveolin in insulin signaling was suggested by the fact that caveolin-1 becomes phosphorylated upon insulin stimulation in 3T3-L1 [16]. Several laboratories have localized elements of the insulin cascade in caveolae or rafts, including the insulin receptor itself, in 3T3-L1 adipocytes [17]. Furthermore, insulin receptor co-immunoprecipitates with caveolin-1 in freshly isolated rat adipocytes [18] and cholesterol depletion with β-cyclodextrine inhibited insulin-stimulated glucose uptake [19]. CAP, c-Cbl, TC10 and flotillin-1, important proteins to stimulate the insulin-induced Glut4 translocation to the plasma membrane, are described as present in the lipid rafts [20–23]. Here, we report by a detergent-based method of raft purification that endogenous SHIP2 is not observed in the lipid raft in CHO-IR cells, in C2C12 myotubes or 3T3-L1 adipocytes.

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Abbreviations: IR, insulin receptor; CAP, c-Cbl associated protein; PI3-K, phosphatidyl inositol 3-kinase; SHIP2, SH2 domain containing inositol 5-phosphatase; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate

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2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM, 4.5 g/l glucose), streptomycin/penicillin, fungizone, fetal bovine serum, horse serum and calf serum were obtained from Gibco (Paisley, UK). Bovine serum albumin, bovine insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Protease inhibitors cocktail was from Roche (Mannheim, Germany). OptiPrep 60% (w/v) in water was obtained from Axis-Shield (Oslo, Norway). Mouse monoclonal antibodies against adaptin-β (clone 74), flotillin-2 (clone 29) and caveolin-1 (clone 2297) and rabbit polyclonal antibodies against caveolin and the insulin receptor β-subunit were purchased from Transduction Lab (Erembodegem, Belgium). Rabbit polyclonal antibody against CAP was from Upstate Biotechnology (Veenendaal, The Netherlands). Mouse monoclonal antibody against c-Cbl (clone A-9) was from Santa Cruz Biotechnology, Inc. The rabbit polyclonal antibody against SHIP2 was provided by C. Erneux and has been previously reported [24]. The mouse monoclonal anti-HA antibody was from Roche (Mannheim, Germany) and the mouse monoclonal anti-His antibody was from Clontech (Palo Alto, CA).

2.2. Cell culture

CHO-IR cells were maintained in Ham's F12 supplemented with fetal bovine serum 10%, 100 U/ml penicillin and 100 mg/ml streptomycin, fungizone 2.5 μg/ml and G-418 0.5% in 5% CO_2/humidified atmosphere at 37 °C. C2C12 cells were maintained in DMEM supplemented with fetal bovine serum 10%, 100 U/ml penicillin and 100 mg/ml streptomycin and fungizone 2.5 μg/ml in 5% CO_2/humidified atmosphere at 37 °C. After confluence, they were differentiated with 2% horse serum for three days. 3T3-L1 fibroblasts, a kind gift of doctor J. Pairault (Paris), were cultured in DMEM supplemented with 10% calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin in 8% CO_2/humidified atmosphere at 37 °C. Differentiation to adipocytes was induced 2 days post-confluence by incubating the cells in DMEM supplemented with fetal bovine serum 10%, 100 U/ml penicillin and 100 mg/ml streptomycin, 160 nM insulin, 0.25 μM dexamethasone and 100 μM IBMX for 2 days. Cells were maintained in the same medium without dexamethasone and IBMX and used between day 7 and 11 following differentiation.

2.3. Preparation of detergent-resistant membranes by OptiPrep gradient centrifugation

Lipid rafts were isolated from CHO-IR, 3T3-L1 adipocytes and C2C12 myotubes using a modification of the protocol of Tansey et al. [25]. CHO-IR were transfected using Superfect (Invitrogen, Merelbeke, Belgium) with the His-tagged SHIP2 and the HA-tagged CAP in pcDNA3. 3T3-L1 and C2C12 cells were untransfected. Before the stimulation with insulin, the cells were serum starved for 16 h. The cells were rinsed twice with ice cold PBS, lysed and scraped in 400 μl TEN buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, protease inhibitors and Triton X-100, 0.5% or 1%). The lysates were homogenized by passage through a 21-gauge needle 10 times followed by centrifugation at $800\times g$, for 10 min after 15 min solubilization. The supernatant was adjusted to 35% (v/v) OptiPrep by adding 585 μl of 60% stock solution of OptiPrep, transferred to the bottom of a centrifuge tube and overlayed successively with 8 ml of 30% OptiPrep (diluted with 0.5× TEN buffer) and 1 ml of TNE buffer. After centrifugation at $197\,000\times g$ for 4 h (SW41, 40 000 rpm), 1 ml fractions were collected from the top of the gradient (designated fractions number 1 (top) to 10 (bottom)). Protein samples of equal volumes taken from each fraction were concentrated with StrataClean (Stratagene), resuspended in Laemmli buffer and boiled for 5 min.

2.4. Western blotting experiments

Protein samples from OptiPrep gradient fractions were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), which were incubated in 5% non-fat dry milk dissolved in TBSN buffer (20 mM Tris–HCl, pH 7.6, 140 mM NaCl, 0.05% Nonidet P-40), for 1 h at room temperature, to block the non-specific sites. Incubations with primary antibodies were carried out for 2 h at room temperature. After several washings, the membranes were incubated for 1 h with the respective peroxidase-conjugated secondary antibody (Amersham, Bucks, UK) diluted in TBSN. The membranes then underwent another extensive washing in TBSN and the bound antibodies were detected using a chemiluminescence method (Western Lighting, NEN, Boston, USA).

3. Results

3.1. Insulin receptor is observed in lipid rafts in CHO-IR, C2C12 myotubes and 3T3-L1 adipocytes

CHO-IR cells, differentiated C2C12 and 3T3-L1 cells were solubilized with Triton X-100 at 4 °C for 15 min. As the detergent concentration seems to be of crucial importance to detect the insulin receptor in the lipid rafts [26], we tested two concentrations (0.5% or 1%) of Triton X-100. The lysate was loaded on an OptiPrep gradient and centrifuged. One milliliter fractions from the top of the tube were collected and analyzed for rafts and non-rafts markers (Fig. 1). Caveolin or flotillin, known to be associated with lipid rafts [22] and β-adaptin, a

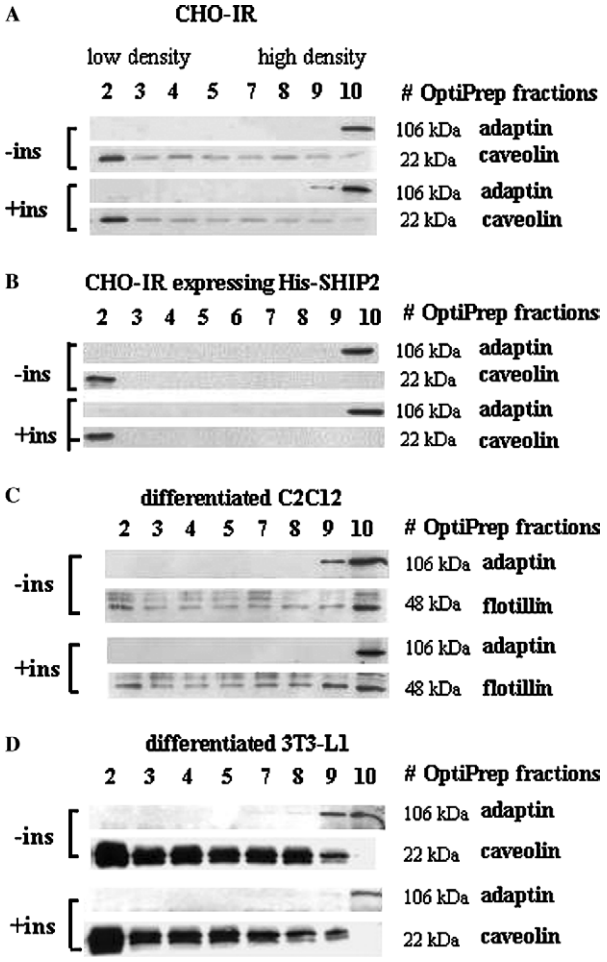

Fig. 1. Western blot analysis of caveolin (caveolae marker) and adaptin-β (clathrin-associated membranes marker) distribution. OptiPrep density gradient fractions were prepared from SHIP2 transfected CHO-IR in 1% Triton X-100 (A), or from wt CHO-IR (B), C2C12 (C) or 3T3-L1 (D) in 0.5% Triton X-100 cells with (+ins) or without (−ins) insulin stimulation 100 nM for 5 min. Equal volumes of OptiPrep density gradient fraction were concentrated, subjected to 13% SDS–PAGE and blotted. Blots were first incubated with the mouse monoclonal adaptin-β antibody and men reprobed with the rabbit polyclonal caveolin antibody.

clathrin-associated soluble-membranes marker, was used to detect, respectively, the raft (fraction 2) and non-raft fractions (fraction 10) in our gradients. The results on the three types of cells generally reproduce the literature on other cells: caveolin mostly in rafts, adaptin mostly in non-raft fraction and flotillin in both but mostly in non-raft fraction. In 3T3-L1, caveolin is very abundant, spilling over to other fraction but not to the non-raft fraction. In each cell type, insulin receptor is detected in both fractions (Fig. 2A), as reported by Huo and Kimura who have isolated the lipid rafts by detergent-free methods [27,28]. Although no effect of insulin on the distribution of the IR was previously described in 3T3-L1 adipocytes [17], we observed most of the time that IR level is lower in raft fraction after insulin stimulation. This could be due to the endocytosis of the receptor which follows stimulation. On the other hand, in contrast to the results published by Gustavsson et al. [17] and others [16,29], we do not solubilize completely the IR by using a detergent-based method for lipid rafts preparation; even with 1% Triton X-100, we still observed the IR in the raft fraction in adipocytes (Fig. 2B).

3.2. *Endogenous SHIP2 does not localize in lipid rafts of CHO-IR, differentiated C2C12 cells or 3T3-L1 adipocytes*

The CAP/c-Cbl complex was shown to be present in lipid rafts in adipocytes [20] and SHIP2 was shown to associate to CAP and c-Cbl in CHO-IR [11]. We therefore addressed the question whether SHIP2 localizes in these caveolin enriched detergent resistant fractions. CHO-IR cells were co-transfected with HA-tagged CAP and His-tagged SHIP2, and solubilized with 1% Triton X-100. Non-transfected CHO-IR, C2C12 or 3T3-L1 cells were solubilized with 0.5% Triton X-100. OptiPrep gradients were performed and each gradient was analyzed for rafts and non-rafts markers (as in Fig. 1). In SHIP2-transfected CHO-IR, SHIP2 was detected in both raft and non-raft fractions, independently of insulin stimulation of the cells (Fig. 3A). On the other hand, endogenous SHIP2 was only observed in the non-raft fraction in non-transfected CHO-IR cells, in C2C12 myotubes and in 3T3-L1 adipocytes (Fig. 3B). We have also tested the presence of SHIP2 in the raft fraction after 1 and 15 min insulin stimulation and we cannot detect SHIP2 in the raft fraction (Fig. 3C).

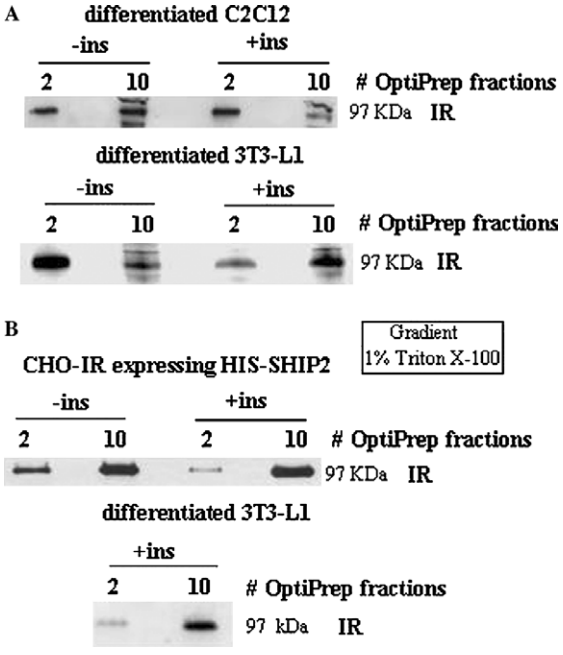

Fig. 2. Western blot analysis of insulin receptor distribution in 0.5% Triton X-100/OptiPrep density gradient. (A) Fraction 2 (raft fraction) and 10 (non-raft fraction) were prepared from C2C12 or 3T3-L1 cells stimulated (+ins) or not (−ins) by insulin 100 nM for 5 min. (B) One percent Triton X-100/OptiPrep density gradient fraction 2 (raft fraction) and 1.0 (non-raft fraction) were prepared from SHIP2 transfected CHO-IR or 3T3-L1 cells stimulated (+ins) or not (−ins) by insulin 100 nM for 5 min.

3.3. *Detection of CAP and c-Cbl in lipid rafts in 3T3-L1 adipocytes*

As CAP and c-Cbl were reported to be recruited to the lipid rafts following insulin stimulation [20], we wanted to test whether CAP and c-Cbl could be detected in the lipid rafts of 3T3-L1 (Fig. 4). Although the major part of endogenous CAP was detected in the non-lipid raft fraction, we showed that a small part localizes in the raft fraction, independently of insulin stimulation. We cannot detect endogenous c-Cbl in this fraction contrary to Baumann et al. [20] who detected it with a non-detergent-based method.

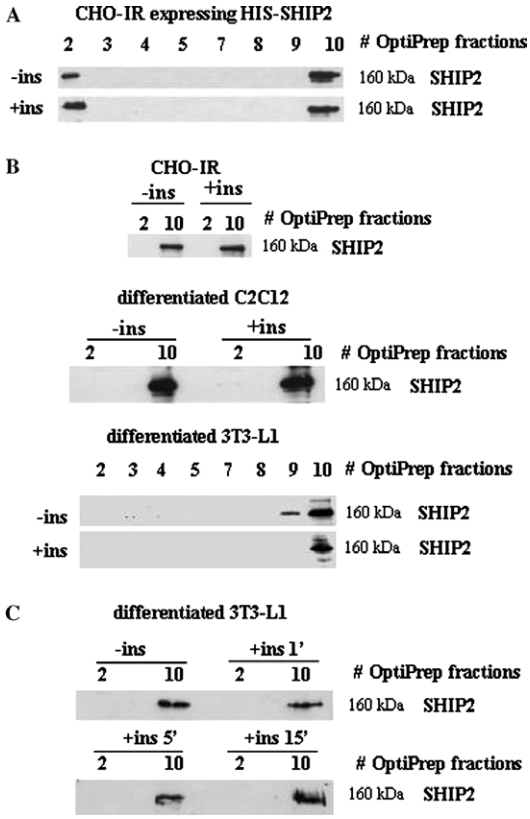

Fig. 3. (A) Western blot analysis of SHIP2 distribution. One percent Triton X-100/OptiPrep density gradient fraction 2 (raft fraction) to 10 (non-raft fraction) were prepared from pCDNA3.his-SHIP2 transfected CHO-IR cells and stimulated (+ins) or not (−ins) by insulin 100 nM for 5 min. (B) About 0.5% Triton X-100/OptiPrep density gradient fraction 2 (raft fraction) and/to 10 (non-raft fraction) prepared from CHO-IR, C2C12 or 3T3-L1 cells stimulated (+ins) or not (−ins) by insulin 100 nM for 5 min. (C) Western blot analysis of SHIP2 in 0.5% Triton X-100/OptiPrep density gradient fraction 2 (raft fraction) and 10 (non-raft fraction) prepared from 3T3-L1 cells and stimulated for 1, 5 and 15 min by 100 nM insulin.

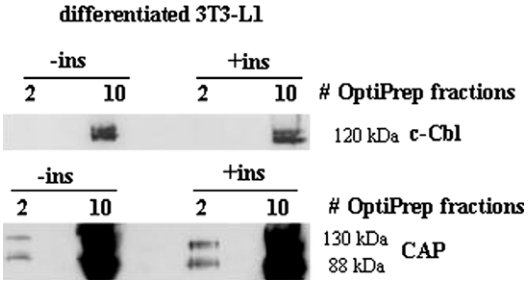

Fig. 4. Western blot analysis of c-Cbl or CAP in 0.5% Triton X-100/OptiPrep density gradient fraction 2 (raft fraction) and 10 (non-raft fraction) prepared from differentiated 3T3-L1 cells stimulated (+ins) or not (−ins) by insulin 100 nM for 5 min.

4. Discussion

SHIP2 was demonstrated to be involved in insulin signaling by knock-out mice data [7] and to be able to interact with CAP and c-Cbl by our previous study [11]. Considering Saltiel, Pessin and co-workers work about the CAP/c-Cbl pathway and the fact that SHIP1 was detected in lipid rafts [30,31], we addressed the question whether SHIP2 localizes in caveolae. We used a detergent-based method to isolate raft subdomains of CHO-IR, C2C12 myotubes and 3T3-L1 adipocytes and analyzed the presence of SHIP2, IR, CAP and c-Cbl by Western blotting. A small part of IR and CAP was detected in the raft fraction, but we cannot detect either SHIP2 or c-Cbl in the caveolin enriched fraction. It seems unlikely that SHIP2 could be solubilized from the rafts fraction by Triton X-100 as described for IR as we can detect the transfected SHIP2 in the lipid rafts of CHO-IR. The presence of SHIP2 in raft structure of transfected CHO-IR cells could be due to overexpression of the protein which could escape from its correct localization. We thus showed here that SHIP2, although implicated in insulin signaling, is not found in the lipid rafts.

The arguments for lipid rafts existence and function rely on indirect methods such as effect of cholesterol depletion and use of non-ionic detergents extraction and a number of recent studies have revealed that these methods could create rafts by themselves [32,33]. These studies have produced conflicting results [32,33]. Lipid rafts, enriched in cholesterol and sphingolipids, are difficult to visualize in living cells. However, rafts were suggested to be implicated in many cellular processes such as signaling, apoptosis, ion channel function, and it is now widely accepted that membrane proteins and lipids can be organized into microdomains in eukaryotic cell membranes (for recent reviews, see [12,34]). Many studies have focused on classifying protein as raft or non-rafts proteins and determining the size of these microdomains [32,35]. On the other hand, data about the presence of certain proteins in the lipid rafts are controversial. For example, depending on the method used, IR localizes in the lipid rafts or not [16,17,29]. Although several studies showed that the components of CAP/c-Cbl and TC10 cascade localize in the caveolae lipid rafts, a recent study analyzed the lipid rafts content by immunoisolation of rat adipocytes caveolae and found no evidence for association of insulin receptor with caveolin by co-immunoprecipitation or by immunoisolation [36]. Thus, it is not clear if the proteins involved in insulin signaling are in the lipid rafts. Pessin, Saltiel and colleagues proposed a new pathway for insulin signaling: stimulation of the IR, by insulin, would enhance phosphorylation of c-Cbl through the adapter protein APS. The c-Cbl/CAP complex would then dissociate from the receptor and migrate to lipid rafts to recruit CrkII and activate TC10 via the GTPase activating protein C3G. Although this model is appealing, nothing is known on how c-Cbl moves to the lipid rafts after phosphorylation. If insulin receptor itself is in the lipid rafts, the phosphorylation of c-Cbl would happen there; however, c-Cbl was not observed in rafts in the absence of insulin [20]. CAP itself complexes with c-Cbl even in the absence of insulin [37] and yet CAP localizes in the lipid raft independently of insulin stimulation [20]. These facts are not compatible with the model and our understanding of how the insulin receptor activates the CAP/c-Cbl cascade has still to be enlarged. Development of methods like immunoisolation [36] will perhaps help us in resolving these questions.

Several studies demonstrated that SHIP2 is implicated in insulin signaling [7,9]. Insulin-stimulated glucose disposal in skeletal muscles, one of the major sites of insulin action [38], account for more than 80% of postprandial glucose uptake [39], localizing GLUT4 to the sarcolemma. It was demonstrated that in these cells, SHIP2 co-localizes at Z-lines and the sarcolemma with filamin, an actin binding protein implicated in cell migration [40]. SHIP2 could be implicated in several processes in the cell, and only an undetectable part could interact with CAP, in the lipid rafts. By its interaction with filamin, SHIP2 could localize near PtdIns(3,4,5)P_3 and regulate Glut4 translocation. As the main role of SHIP2 could be to decrease PtdIns(3,4,5)P_3 produced by the PI3-K, SHIP2 has to localize near the PI3-K which does not reside in the lipid rafts in 3T3-L1 adipocytes [28]. The fact that CAP and c-Cbl were found, for the major part at least, outside of cavolae could explain that they can interact with SHIP2. Whether lipid rafts are important for insulin signaling in muscles and adipocytes is not yet clearly demonstrated and the different studies are contradictory, but the data we present here demonstrate that with presently available methodology about rafts purification, SHIP2 does not appear to be recruited there.

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