Effect of Insulin on Caveolin Enriched Membrane Domains in Rat Liver

Alejandro Balbis, Gerardo Baquiran, Catherine Mounier, and Barry I. Posner

Polypeptide Hormone Laboratory, Faculty of Medicine, McGill University, 3640 University St.,
Suite W315, Montreal, Quebec H3A 2B2, Canada

Running Title: Insulin signaling in liver: role of caveolin-enriched membranes

To whom correspondence should be addressed: Dr Barry I Posner; Tel. (514) 398-4101;
Fax: (514) 398-3923; E-mail: barry.posner@staff.mcgill.ca
SUMMARY

Compartmentalization of signaling molecules may explain, at least in part, how insulin or growth factors achieve specificity. Caveolae/rafts are specialized lipid compartments, which have been implicated in insulin signaling. In the present study we investigated the role of caveolin enriched membrane domains (CMD) in mediating insulin signaling in rat liver. We report the existence of at least two different populations of CMD in rat liver plasma membranes (PM). One population is soluble in Triton-X 100 and appears to be constitutively associated with cytoskeletal elements. The other population of CMD is located in a membrane compartment insoluble in Triton X-100 with light buoyant density and is hence designated CMD/rafts. In response to insulin we found evidence of rapid actin reorganization in rat liver PM along with the association of CMD/rafts and insulin signaling molecules with a cell fraction enriched in cytoskeletal elements. The presence of CMD in liver parenchyma cells was confirmed by the presence of caveolin-1 in primary rat hepatocyte cultures. Cholesterol depletion, effected by incubating hepatocytes with 2mM methyl - β - cyclodextrin (MβCD), did not permeabilize the cells or interfere with clathrin-independent internalization. However at this concentration MβCD perturbed CMD of hepatocyte PM, inhibited insulin-induced Akt activation and glycogen synthesis, but did not affect insulin-induced IRK tyrosine phosphorylation. These events, together with the presence of a functional insulin receptor in CMD of rat liver PM, suggest that insulin signaling is influenced by the interaction of caveolae with cytoskeletal elements in liver.
INTRODUCTION

Although binding of insulin to its receptor is a high affinity specific process, the signaling cascades generated by the activated insulin receptor kinase (IRK)\(^1\) are shared by a number of other growth factors (1-3). Nevertheless the metabolic actions of insulin cannot be reproduced in intensity and quality by other hormones or growth factors. The question of how insulin achieves its specificity of action remains to be satisfactorily resolved. Compartmentalization of signaling molecules in plasma membranes and endosomes may play an important role in determining the specificity of signal transduction (4-7).

Recently, the isolation of detergent-insoluble, low-density membrane fragments from cells suggests that sphingolipid and cholesterol rich domains could exist as a liquid-ordered phase in the membrane (8). These lipid domains, which are known as lipid rafts, can recruit or exclude signaling proteins. Thus, lipid rafts have been implicated in the regulation of hormone and growth factor signaling (8,9). Caveolae, which constitute a subset of lipid rafts, are invaginated cell surface micro-domains, enriched in caveolin oligomers, the major protein constituent of these structures (10,11). Three caveolins (caveolin 1, 2 and 3) have been discovered. Caveolin-1 and caveolin-2 are found most abundantly in adipocyte and endothelial cells, whereas caveolin-3 is found in muscle cells. Caveolae have been implicated in potocytosis, transcytosis, endocytosis independent of clathrin, and signal transduction (11,12). It has been shown that caveolae negatively affect EGF and Src signaling (13). In contrast to these inhibitory effects, a number of studies, mostly in adipocytes, have suggested that intact caveolae are necessary for insulin signaling. In 3T3-L1 adipocytes, IRK was reported to be concentrated in caveolae (14), and to interact with caveolin-1 to modulate insulin signaling (15,16). It has also been reported that insulin induced tyrosine-phosphorylation of caveolin (17). Disrupting the lipid structure of caveolae, by depleting their cholesterol content with \(\beta\)-cyclodextrin, attenuated IRK signaling (14,18). Rafts/caveolae have also been implicated in insulin-stimulated glucose transport in
3T3-L1 adipocytes by a mechanism independent of PI3-kinase (19,20). Recently, caveolin-1 knockout mice have been created, which are viable despite a complete ablation of caveolae (21). These mice show impaired nitric oxide signaling, vascular dysfunction, hyper-proliferation of endothelial cells, abnormalities in lipid homeostasis, insulin resistance and decreased insulin receptor expression in adipose tissue (21-23). These observations suggest that caveolae in adipocytes can contribute to both the strength and specificity of insulin signaling.

There is a paucity of data concerning the role of caveolae on insulin signaling in other insulin-responsive tissues. Although liver contains a lower level of caveolin-1 than that in adipocytes it has nevertheless clearly been shown that caveolin-1 is located in liver parenchymal cells with negligible levels detected in endothelial cells (24,25). Furthermore, caveolae have been demonstrated at the cell surface of hepatocytes using rapid-freeze deep-etching electron microscopy (25). Since liver is an important insulin target tissue we investigated the significance of caveolin enriched membrane domains (CMD) in mediating insulin signaling in rat liver. In the present study we have characterized these entities and have demonstrated a possible function in insulin signaling.
EXPERIMENTAL PROCEDURES

Animals- Female Sprague-Dawley rats, 10 weeks of age, (160-180g bodyweight (BW)) were purchased from Charles River Canada Ltd. (St. Constant, PQ, Canada), housed in an animal facility with 12 h light cycles at 25°C and fed ad libitum on Purina chow. Animals were fasted overnight (16-18 h) before each study.

Materials- Porcine insulin was a gift from Eli Lilly and Co., (Indianapolis, IN, USA). Phenylmethanesulphonic fluoride (PMSF), sodium orthovanadate, methyl-ß-cyclodextrin and most other chemicals were purchased from Sigma (St Louis, MO, USA). Reagents for electrophoresis were from Bio-Rad (Richmond, CA, USA). Kodak X-OMAT AR film was from Picker International (Montreal, PQ, Canada). PVDF Immobilon-P transfer membranes were from Millipore Ltd. (Mississauga, ON, Canada). [U-14C]glucose (300 mCi / mmol) was purchased from NEN Life Sciences Products-DuPont (Wilmington, DE). Magnetic beads (Dynabeads M-280) were from Dynal (Lake Success, NY). An antibody raised against a peptide corresponding to residues 942-969 of the juxtamembrane region of the IRK ß-subunit (anti-960) was prepared and purified on a PAS column as previously described (26 ) and used for Western blotting. For immunoprecipitation of IRK, an antibody directed against the ß-subunit was obtained from the serum of a patient with acanthosis nigricans (27). Polyclonal anti-p85, a polyclonal anti-IRS1 antibody and a specific antibody against Akt2 were purchased from Upstate Biotechnology Incorporated (Lake Placid, NY, USA). Antibodies against Akt1 and phospho-Akt1 (Ser473) were purchased from New England Biolabs, Inc. (Mississauga, Canada). An antibody against actin and iron saturated transferrin were purchased from Sigma (St Louis, MO, USA). A monoclonal anti-phosphotyrosine antibody (anti-PY) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-caveolin-1 was purchased from Transduction Laboratories (Lexington, KY).

Preparation of Sub-cellular Fractions- Rats were anaesthetized and sacrificed by decapitation at the indicated times following intra-jugular injections as described in the
appropriate Figure legends. Livers were exsanguinated, rapidly excised, and minced at scissor point in ice-cold buffer (5 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 1 mM benzamidine, 1 mM PMSF, 1 mM MgCl$_2$, 2 mM NaF, and 2 mM Na$_3$VO$_4$). Plasma membranes (PM), endosomes (ENs), and Microsomes were prepared as previously described (27). A purified Golgi fraction prepared as described (28) was kindly provided by Dr. Bergeron. The protein content of these fractions was measured using a modification of Bradford’s method with BSA as standard (29).

**Isolation of Caveolin Enriched Membrane Domains (CMD) – 1)** Isolation of CMD with Triton X-100: CMD were isolated by a modification of the method of Liu et al (30). Briefly, plasma membranes were pelleted and mixed with 3 ml of ice-cold 1% Triton-X100 in buffer A (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF and 2 mM Na$_3$VO$_4$). The samples were homogenized (10 strokes in glass homogenizer), incubated on ice for 1 hr, adjusted to the same amount of protein, and diluted 1:1 with 80% sucrose in buffer B (50 mM Tris-HCl [pH 7.5] - 150 mM NaCl). The extract (4 ml, between 2 and 5 mg of protein) was loaded on the bottom of a 12 ml ultracentrifuge tube and overlaid with 4 ml each of 30% and 10% sucrose in buffer B. The gradient was centrifuged for 21 h at 29,000 x g in a SW40 Ti rotor (Beckman Instruments) and 1 ml fractions were collected from the top of the tube. Fractions 1-8 (10-30% sucrose gradient), fraction 9 (soluble proteins in the residual 40% sucrose layer), and the pellet, re-suspended in 1ml of ice cold phosphate buffered saline (PBS) were subsequently analyzed by SDS-PAGE and Western Blotting. The amount of protein recovered in the DRM/lipid raft fraction (fractions 4 and 5 of sucrose gradient) and in the Triton insoluble pellet was between 20-100 µg and 400-700 µg respectively. 2) Isolation of CMD with a detergent free method: Briefly, PM was mixed with 3 ml of ice-cold Na$_2$CO$_3$ (200 mM, pH 11) in buffer A. Samples were homogenized (10 strokes in glass homogenizer), sonicated (3 times, 10 seconds each) and incubated on ice for 1 hr. Subsequently, samples were mixed with 80% sucrose, centrifuged overnight and collected as indicated above. 3) In some experiments PM
were homogenized in 1% Triton X-100 and Na$_2$CO$_3$ (pH 11; 200 mM final concentration) in buffer A. After incubating on ice for 1 hr the samples were mixed with an equal volume of 80% sucrose (1:1) and subjected to sucrose gradient centrifugation as described above.

**Immunoblotting** - Fractions 1-8 (100 µl each) from the sucrose gradients were mixed with 50 µl of 3x Laemmli sample buffer subjected to SDS-PAGE (6-12% gel) and then transferred to Immobilon-P membranes for immunoblotting. In some experiments, proteins from these fractions were concentrated with trichloroacetic acid (30) and dissolved in 100 µl of 1x Laemmli buffer. The Triton-insoluble pellet was re-suspended in 1 ml of PBS buffer, mixed with 500 µl of 3 x Laemmli sample buffer and aliquots of 100 µl were used for SDS-PAGE. Fifty µg of protein from fraction 9 and total PM respectively were used for SDS-PAGE. Immunoblotting with anti caveolin-1 antibody showed a non-specific band at 29 kDa when total membranes or fraction 9 were analyzed. This band was absent in the Triton-insoluble pellet and in lipid rafts. Either $^{125}$I$^2$GAR or $^{125}$I$^2$GAM were used as secondary antibody and, following autoradiography at -80°C, appropriate bands were quantified using a BioRad GS-700 Imaging Densitometer.

**Immuno-isolation of CMDs** - PM was homogenized in Na$_2$CO$_3$ (pH 11) (10 strokes Dounce Homogenizer) and incubated on ice for 1 h followed by centrifugation at 200,000 x g for 1 h. The resultant pellet was again homogenized and now sonicated (3 times, 10 seconds each) in Tris buffered saline (TBS, buffer B) containing proteases inhibitors and 1% albumin as noted above. The treatment with Na$_2$CO$_3$ was carried out in order to remove PM filaments, which could interfere with the immuno-isolation process. Magnetic beads (Dynabeads M-280) were coated with a specific antibody against caveolin-1 or with IgG (negative control) as specified by the manufacturer. Coated beads were incubated with PM for 2 h at 4°C, resuspend and washed four times with TBS (pH 7.5) prior to boiling for 5 minutes in Laemmli sample buffer, and subjecting to SDS-PAGE.

**Cell Culture** - Primary hepatocytes, isolated from 120-140-g male Sprague Dawley rats (Charles River Laboratories, Inc., St. Constant, Canada) by *in situ* liver perfusion with collagenase were
plated on a collagen matrix (Vitrogen-100). Sub-confluent cultures were prepared by seeding 1 x 10^6 cells, onto 9.6 cm^2 six-well plates (Corning, Costar, Cambridge, MA) or 5 x 10^6 cells, onto 78 cm^2 culture dishes (Starstedt Canada, St. Laurent, Canada). Cells were bathed for 24 h in seeding medium (DMEM/Ham’s F-12 containing 10% FBS, 10 mM HEPES, 20 mM NaHCO_3, 500 IU/ml penicillin, and 500 µg/ml streptomycin) and then for 48 h in serum-free medium (SFM) that differed from the seeding medium in that it lacked FBS and contained 1.25 µg/ml fungizone, 0.4 mM ornithine, 2.25 µg/ml L-lactic acid, 2.5 x 10^{-8} M selenium, and 1 x 10^{-8} M ethanolamine. Fresh serum free media was added just before incubation of hepatocytes with MβCD.

**Biotinylation of Cell Surface Proteins** - Detection of IRK located at the cell surface of the hepatocytes was performed as described previously (31). Briefly, hepatocytes were incubated in the presence or absence of MβCD and insulin as noted in Figure Legends. Thereafter, hepatocytes were washed three times with ice-cold PBS-Ca-Mg pH: 7.4 (0.1mM CaCl_2 and 1 mM MgCl_2) and cell surface proteins were biotinylated by incubation with 0.5 mg/ml of Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) in PBS-Ca-Mg for 30 min at 4°C. The reaction was stopped by washing the dishes three times with PBS-Ca-Mg containing 15 mM of glycine. After biotinylation, cell lysates were prepared as described above and IRK was immunoprecipitated with an antibody directed against the α-subunit of the IRK. Immunoprecipitates were boiled in the presence of Laemmli buffer and subjected to SDS-PAGE. Proteins were transferred to Immobilon-P membranes and immunoblotted with α960 or Streptavidin-HRP (Amersham Pharmacia Biotech, Piscataway, USA). Streptavidin binds to biotinylated proteins allowing only the detection of IRK associated with the PM.

**Transferrin Uptake in Primary Hepatocytes** - Transferrin (Tf) internalization in primary hepatocytes was performed as described previously (32). Briefly, hepatocytes were pre-incubated in the presence or absence of 2 mM MβCD for 1 hr at 37°C, and then 1µg of 125I-Tf (around 2 µCi) was added. Iron-saturated Tf was labeled with 125I with the Chloramine-T method as previously described (33). After
incubation at the indicated times at 37°C, cells were washed three times in ice-cold PBS, incubated for 30 seconds with 250 mM of acetic acid containing 500 mM of NaCl followed by neutralization with 1 M of NaAc and washed again three times in ice-cold PBS. Cells were solubilized in 1% Triton X-100 and intracellular radioactivity was determined in a gamma counter and normalized to the protein content. Specific incorporation of Tf into the hepatocytes was the difference between the binding $^{125}$I-Tf minus the binding $^{125}$I-Tf plus 400 µg of non-labeled iron saturated transferrin.

**Glycogen Synthesis**- Glycogen synthesis was determined by incorporation of [U-$^{14}$C]glucose into glycogen as previously described (34). Briefly, hepatocytes (1×10$^6$ cells) were serum-deprived for 4 h, pre-incubated in absence or presence of 2 mM of MβCD for 1 hour at 37 °C and subsequently incubated for 2 hours in serum free media containing 15 mM [U-$^{14}$C]glucose and insulin (100 nM), in absence of MβCD. Incubations were stopped by three rapid washes with ice cold PBS and cells were solubilized in 1 ml of 0.1 M NaOH. The samples were then boiled in the presence of 2 mg of carrier glycogen and precipitated over-night in 70% ethanol at -20°C. After centrifugation, the precipitated glycogen was resuspended in 500 µl of water, incubated for 5 min at 70°C, and incorporated radioactivity was determined by scintillation counting.
RESULTS

Caveolin-1 Distribution in Rat Liver Subcellular Fractions

Caveolin-1 has been observed to be largely localized to the plasma membrane of selected cell types, in 50- to 100-nm omega-shaped invaginations termed caveolae. (10,11). These caveolin-enriched micro-domains (CMD) are characterized by their light buoyant density and resistance to solubilization by Triton X-100 at 4°C (10,11). In this study we examined the distribution of caveolin-1 in rat liver subcellular fractions by immunoblotting the various fractions using a specific antibody against this protein. As shown in Figure 1a, caveolin-1 was detected principally in PM and microsomes, and was barely detectable in endosomes or Golgi fractions.

Isolation of CMD from Rat Liver PM with Triton X-100

We subsequently analyzed the distribution of caveolin-1 in rat liver PM following incubation of PM preparations with 1% Triton X-100 and subsequent sucrose gradient flotation analysis as described in Experimental Procedures. Caveolin-1 was found in the Triton X-100 insoluble pellet and at the 10-30% sucrose interface (Fractions 4 & 5 in Fig.2a, left panel), which constituted detergent resistant membranes (DRM) with a buoyant density characteristic of lipid rafts (DRM/rafts). When corrected for protein content in each fraction, around 60% of caveolin-1 was found in the pellet, 10% in DRM/rafts at the 10-30% sucrose interface, and the remainder was solubilized as indicated by its presence in the load zone (fraction 9) beneath the sucrose gradient (Fig. 3d, white bars). Caveolin-1 was enriched in the pellet and the DRM/rafts (10-30% sucrose interface) to the extent of 11 and 7 fold respectively relative to the original PM lysate.

The PM fractions we prepared have been characterized in detail and appear to be quite pure although there is some contamination with endoplasmic reticulum (ER) (35). Microsomes contain both caveolin-1 (Figs. 1a and 2a, right panel) and abundant ER elements. Therefore it is possible that a substantial portion of PM caveolin-1 is due to contamination with microsomes.
We therefore subjected microsomes treated with Triton X-100 to sucrose gradient fractionation and compared caveolin-1 distribution from this compartment (Fig. 2a, right panel) to that from PM. Caveolin-1 from microsomes was found in DRM/rafts and in the soluble fraction (Fraction 9). In contrast to PM, no caveolin-1 was found in the Triton-insoluble pellet from microsomes (Fig. 2a, right panel), indicating a different distribution of caveolin-1 in these two compartments.

To compare further the DRM/rafts from PM with those from microsomes we respectively subjected them to SDS-PAGE and subsequent silver staining. As seen in Figure 2c, the DRM/rafts from PM displayed a protein pattern distinctly different from that observed in those isolated from microsomes, indicating their distinction from one another.

**Caveolin-1 and the PM Cytoskeleton**

It has been previously noted that the PM preparation used in our work contains abundant filaments (35,36). Furthermore, after treatment of the PM with Triton X-100 and subsequent centrifugation, the filamentous structures in the resultant pellet were found to be enriched in actin (36). In our study we found that actin was enriched only in the PM fraction, and not microsomes, Golgi or endosomes, relative to its concentration in whole liver homogenate (Fig. 1b, compare lanes H and PM). When the distribution of actin was analyzed in PM sub-fractions, resolved by sucrose density centrifugation, we found that, as with caveolin-1 (Fig. 2b, left panel), actin was enriched in the pellet and the DRM/rafts (10-30% sucrose interface) to the extent of 5 and 2 - fold respectively relative to the original PM lysate. We also found that in microsomes actin, in parallel with caveolin-1 distribution, was found in DRM/rafts and the soluble proteins of fraction 9; whereas none was found in the pellet (Fig. 2b, right panel). These results suggest that the PM micro-domains containing caveolin-1 also contain insoluble actin-rich structures perhaps in association with one another.
Characterization of CMD in Rat Liver PM

CMD have also been prepared by treating membranes with Na$_2$CO$_3$ (pH 11) (37). It was previously shown that treatment of rat liver PM preparations with Na$_2$CO$_3$ (pH 11) solubilized the filaments associated with the membrane while leaving the latter intact (36). Since a high proportion of caveolin-1 in PM is associated with the Triton X-100 insoluble pellet (Fig 2a, left panel), we carried out sucrose gradient flotation analysis of Na$_2$CO$_3$-treated PM in order to dismantle the cytoskeleton and thus determine the characteristics of this pool of caveolin-1. PM lysates were incubated with Na$_2$CO$_3$ (pH 11) and/or Triton X-100, and subsequently fractionated by sucrose density centrifugation (Fig.3). When PM lysates were treated only with Na$_2$CO$_3$ (pH 11) a substantial proportion was found at the interface between 10 and 30% sucrose (Fig. 3b, top panel & 3d, black bars) with some observed in fractions 6 to 8. As previously observed (36) following Na$_2$CO$_3$ treatment actin was also removed from the pellet (Fig.3b, lower panel) and now largely appeared as soluble components (Fraction 9) but also in association with entities of a buoyant density comparable to CMDs (compare Figs. 3a & b, lower panels, see also 3e, black bars).

In order to evaluate further the characteristics of the CMD associated with the Triton-insoluble pellet, PM were treated with a combination of Triton X-100 and Na$_2$CO$_3$ (pH 11) prior to sucrose gradient fractionation. This treatment released most of the actin and caveolin-1 from the pellet (Fig.3c, 3d & 3e, gray bars). Thus, we found that the bulk of caveolin-1 and actin were now present as soluble entities in Fraction 9 (Fig.3c, 3d & 3e black bars). In addition, around 10 % of caveolin-1 was present in the sucrose gradient but in less dense fractions compared to those derived from treating PM with Triton X-100 alone (compare Fig 3a and 3c, top panels). Since prior treatment of PM with both, Triton X-100 and Na$_2$CO$_3$ (pH 11) removed actin (Fig. 3c, lower panel & 3e) and other peripheral proteins (e.g. IRS1 and p85, data not shown) from the DRM/rafts fraction, we suggest that the CMD contained in this fraction have reduced density
due to the removal of associated peripheral proteins. The total protein content in each of these fractions is shown in Figure 3f.

In summary, the treatment of PM with a combination of Triton X-100 and Na$_2$CO$_3$ (pH 11) demonstrates that only 10% of the PM pool of caveolin-1 is found in DRM/rafts while the remainder is in CMD found in fraction 9 and presumably solubilized (Fig. 3d, gray columns). Around 60% of this latter Triton-soluble pool of caveolin-1 is tightly associated with the cytoskeleton (Fig. 3d, white bars), and is released from the cytoskeleton as low buoyant density CMD in the presence of Na$_2$CO$_3$ alone (Fig 3d, black bars). The remaining 35% which appears in fraction 9 following treatment with either Triton (Fig.3d, white bar) or Na$_2$CO$_3$ (Fig.3d, black bar) appears to represent a distinct population of CMD which have not yet been characterized.

CMD in Cultured Primary Rat Hepatocytes
To confirm the presence of CMD in parenchymal cells, we then examined primary rat hepatocyte cultures. Rat liver hepatocytes were homogenized in 1% Triton X-100 followed by sucrose gradient flotation analysis as described in Experimental Procedures. As shown in Figure 5a (black circles and control Western blot) the distribution of caveolin-1 in rat liver hepatocytes was similar to that observed in liver PM (compare with Figure 2a, left panel), suggesting that the main source of caveolin-1 in liver are the parenchymal cells. CMD and rafts are rich in cholesterol, and partial extraction of the latter from cells is a commonly used method to disrupt the morphology and function of caveolar structures (38). We sought to use methyl-$\beta$-cyclodextrin (M$eta$CD) to selectively extract cholesterol from the hepatocyte cell surface (39), and thus characterize CMDs therein. However, M$eta$CD can also remove cholesterol from PM domains other than CMD/rafts (38). For example it has been observed that 10 mM
MβCD strongly reduced both transferrin and EGF internalization, indicating a perturbation of clathrin-dependent internalization (40). We therefore initially sought to determine the concentration of MβCD, which selectively affects CMD at the cell surface without disturbing the rest of the PM.

To do this we employed a method, which evaluates the integrity of the PM based on the accessibility of sulfo-biotin, used to label cell surface proteins (31), to the cellular interior. Therefore we determined the maximal concentration of MβCD, which minimally effected permeability by incubating primary hepatocytes with different concentrations of MβCD (0.5 to 10 mM), followed by labeling the cells with sulfo-biotin. Cell lysates were then prepared and immunoprecipitated with an antibody against the intracellular protein p85 (the regulatory subunit of PI3-kinase). Immuno-isolated p85 was subjected to SDS-PAGE, transferred to Immobilon-P membranes and analyzed by blotting with streptavidin, which binds with high affinity to biotin. As seen in Figure 4a, in control hepatocytes and cells treated with 0.5, 1.0 and 2 mM MβCD, little or no p85 was labeled with biotin. However at higher concentrations of MβCD the reaction of sulfo-biotin with p85 was readily observed and increased progressively. These results indicate that a concentration of MβCD higher than 2 mM disrupts the integrity of PM.

To confirm the lack of any effect of 2 mM of MβCD on non-CMD domains, we investigated the effect of 2 mM MβCD on the internalization of transferrin and IRK. Transferrin uptake into hepatocytes, pre-treated with 2 mM MβCD, was measured after incubation with transferrin for 15 or 60 minutes. As seen in Figure 4b, 2 mM of MβCD had no effect on the internalization of transferrin. We used cell surface biotinylation to assess the effect of MβCD on insulin-induced IRK internalization. Hepatocytes were pre-incubated in presence or absence of 2 mM of MβCD followed by stimulation with insulin for the indicated times. Cell surface proteins were
subsequently labeled with Sulfo-biotin, lysates were prepared and IRKs immunoprecipitated. The levels of both total and PM-associated IRK were detected by immunoblotting with α960 and streptavidin respectively. It can be seen in Figure 4c, that 2 mM of MβCD did not inhibit insulin-induced IRK internalization.

Having demonstrated that 2 mM MβCD has a minimal effect on both, the integrity of the PM, and clathrin-dependent internalization, we then determined the effect of 2 mM MβCD on CMDs in hepatocytes. As shown in Figure 5 (a & b) MβCD induced the redistribution of caveolin-1 from the Triton X-100 insoluble pellet to the fraction 9 (Fig. 5a & b). In Triton-treated hepatocytes, the distribution of caveolin-1 at the 10%/30% sucrose gradient interface (fractions 4 & 5) reflects CMDs coming from both PM and microsomes. However, as shown in Figure 2a, all the caveolin-1 found in the Triton X-100 insoluble pellet originates from the PM. Therefore treatment of hepatocytes with 2 mM MβCD produces a dissociation of caveolin-1 from PM-associated cytoskeletal elements to the soluble fraction. Although 2 mM of MβCD could also affect caveolin-1 in DRM/rafts derived from PM this effect would be masked by the presence of caveolin-1 DRM/rafts from microsomes, which are not accessed by 2 mM MβCD. In summary, these observations confirm the presence of caveolin-1 in hepatocytes and also demonstrate that MβCD can selectively affect CMD at the PM of the hepatocytes.

**Insulin-induced Redistribution of Caveolin-1 and Actin in PM Sub-Cellular Fractions**

As shown in Figure 6, the distribution of caveolin-1 was profoundly affected following treatment of the rats with insulin (1.5 µg /100g body weight). The caveolin-1 content in the Triton-insoluble pellet and in the total PM increased by 30 seconds and substantially subsided by 5 mins following insulin treatment. In contrast, the amount of caveolin-1 in DRM/rafts dramatically decreased in the same interval of time (Fig.6a). The insulin-stimulated increase of caveolin-1 content in total PM may reflect the translocation of caveolin-1 to PM from other cellular
compartments. In the same time interval (zero to 5 mins) after insulin the amount of actin in total PM was not altered (Fig.6b, black symbols). However the actin content of the Triton-insoluble pellet increased significantly at 30 seconds and 5 mins after insulin by 122 ± 8% and 146 ± 13% respectively (Fig. 6b, white symbols); and decreased in the DRM/rafts isolated at the 10-30% sucrose interface (Fig.6b, black triangles). We suggest that this reflects the promotion by insulin of actin reorganization in hepatocytes.

**Impact of Insulin on the Distribution of Caveolin-1 in PM Sub-Cellular fractions**

We considered several possibilities to explain the disappearance of caveolin-1 from PM DRM/rafts after insulin stimulation. Previous reports have described an interaction between the insulin receptor and caveolin-1 (15,16). Thus, a complex formed by IRK and caveolin-1 could be internalized into endosomes. We therefore evaluated the content of caveolin-1 in endosomes after insulin stimulation. As previously observed (41), insulin induced a rapid increase of IRK in endosomes at 2 minutes with a return to near basal levels by 15 minutes (Fig. 7a). In the same time-interval no caveolin-1 was detected in endosomes (Fig. 7a). Thus, internalization of caveolin-1 to endosomes is unlikely to account for the insulin-dependent decrease of caveolin-1 associated with DRM/rafts as noted in Fig.6a.

To determine if CMD contained in DRM/rafts associate with the Triton-insoluble pellet after insulin stimulation, we examined the combined effect of Na$_2$CO$_3$ (pH 11) and Triton X-100 on the distribution of caveolin-1 as seen in Figure 3c. Thus, the PM preparation from insulin-stimulated (5 minutes) rats was divided into two aliquots, one half was homogenized in the presence of 1% Triton X-100 and the other half was homogenized with 1% Triton X-100 and Na$_2$CO$_3$ (pH 11) prior to sub-fractionation by sucrose gradient centrifugation. When PM was isolated from rats receiving insulin and treated with Triton X-100 alone we observed low levels of caveolin-1 in buoyant elements compared to PM from control rats (Fig.6a and 7b, top panel). However, when
PM from insulin treated rats was solubilized in Triton X-100 and Na₂CO₃ (pH 11), we observed the appearance of caveolin-1 in structures with a range of buoyant density (fractions 1 to 5, Fig. 7b, lower panel). We suggest that this indicates insulin-induced association of the CMD contained in the DRM/rafts fraction with the Triton-insoluble actin-cytoskeleton of PM, and that treatment with Na₂CO₃ (pH 11) dissociates these CMD from their association with cytoskeletal elements. We suggest that their greater buoyancy reflects the removal by Na₂CO₃ of associated peripheral proteins as discussed in respect to Figure 3c.

**Distribution of IRK, IRS1 and p85 in Triton-insoluble Compartments of PM**

In view of a number of studies indicating that insulin signaling in adipocytes involves caveolin-containing structures (12,23) and our observations above we investigated the distribution of IRK and downstream signaling molecules in PM sub-compartments following insulin treatment (Fig. 8). More than 90% of the insulin receptor content of PM derived from control rats was solubilized with 1% Triton X-100. Only a small amount (around 2%) was found in DRM/rafts and almost nothing was detectable in the pellet (Fig. 8a, 0 minutes). Following insulin treatment IRK increased transiently in DRM/rafts and progressively in the pellet. In both fractions the IRK was tyrosine phosphorylated (Fig. 8a, 0.5 to 5 minutes). Previous work showed that Triton X-100 can solubilize IRK located in CMD (14). Since we found low levels of IRK in the Triton-insoluble compartments derived from PM, we determined the content of IRK in CMD isolated in the absence of Triton X-100 using magnetic beads coated with anti caveolin-1 antibodies to selectively prepare CMD structures. With this technique, the amount of IRK detected in CMD was more abundant than that seen in DRM/rafts (Fig. 8b). Thus, it appears that a substantial proportion of IRK is localized to CMD in liver, and is solubilized by treatment with 1% Triton X-100, as previously observed (14).
Following sucrose density fractionation of PM homogenized in 1% Triton X-100 from control rats around 5-8% of p85 and IRS1 were found in DRM/rafts (fractions 4 and 5) and 20-30% in the pellet (Fig. 8c). As is evident on comparing Fig. 8c with Figs. 2a & b, IRS1 and p85 show substantial co-localization with caveolin-1 and actin. In previous study we showed that following insulin treatment p85 was recruited to liver PM (6). Here we have found that the bulk of p85 translocated to PM following insulin was directed to the Triton-insoluble pellet (Fig. 8c).

**Effect of Cholesterol Extraction on Insulin Signaling in Primary Hepatocytes**

The insulin-dependent translocation of CMD with the characteristics of DRM/rafts, IRK and several important signaling molecules to the PM cytoskeleton suggests that these events are coupled and relevant for insulin signaling. To assess this hypothesis further we examined insulin signaling in rat primary hepatocytes after disrupting CMD using MßCD. We used concentrations of MßCD up to 2 mM, which, as shown in Figure 4 & 5, selectively disrupt PM-CMD. Pre-treatment of hepatocytes with 1 mM or 2 mM of MßCD had no effect on insulin-induced IRK tyrosine-phosphorylation (Fig 9a). However, insulin-induced Akt phosphorylation/activation was decreased when hepatocytes were pre-incubated with 1 mM or 2 mM of MßCD (Fig. 9b & c). Such an inhibition was more pronounced at 15 minutes than at 2 minutes after insulin stimulation (Fig. 9b & c). The antibody used in this study to assess Akt activation detects mainly Akt1 phosphorylated at Ser473. Since recent studies suggest that Akt2, but not Akt1 is involved in the metabolic actions of insulin (42), we have also used a specific antibody against Akt2, which detects both the phosphorylated and non-phosphorylated Akt2. As shown in Figure 9d, MßCD inhibits insulin-induced Akt2 activation. Similar to Akt1, Akt2 inhibition was particularly noticeable at 15 minutes after insulin. We also assessed the impact of cholesterol depletion on glycogen synthesis, which is dependent in hepatocytes on PI3-kinase/Akt activation (34). As seen in Fig. 9e, 2 mM MßCD significantly inhibited the effect of insulin on glycogen synthesis.
Taken together our data suggest that there is a correlation between the association of CMDs with the cytoskeleton and insulin signaling.
DISCUSSION

Liver contains a low level of caveolin-1 in comparison with other tissues especially adipocytes (43). Although it was suggested that the source of caveolin-1 in liver could be endothelial and not parenchymal cells (44) there have been several studies showing clearly the presence of caveolin-1 in hepatocytes with negligible amounts in rat liver endothelial cells (24,25,45). Also, the presence of caveolin has been demonstrated in caveolae at the surface of hepatocytes (25). In our work, we too have demonstrated the presence of caveolin-1 in primary rat hepatocytes. We showed a similar distribution of caveolin-1 in liver and hepatocytes after Triton X-100 solubilization and sucrose flotation analysis (compare Figs. 2a & 5) further supporting the view that parenchymal cells are a significant source of caveolin-1 in liver. Thus, our data are consistent with the previous work of others (24,25).

At the surface of the adipocytes or endothelial cells, caveolin-1 is found in invaginated structures termed caveolae, which are characterized by their light buoyant density and resistance to solubilization in Triton X-100 (10). However, our results show that most caveolin-1 at the PM is found in light buoyant structures, which are soluble in Triton X-100 and are associated with the PM cytoskeleton (Fig.3). Only 10% of caveolin-1 was found in DRMs/rafts. Detergent-soluble caveolin have been identified in intracellular compartments (46,47). Since our data on PM are not due to contamination by internal membranes (see Fig.2), we conclude that at least two populations of CMD exist in PM of the hepatocytes that must differ in their lipid and protein composition (Fig.3). The insolubility of rafts/caveolae in Triton X-100 is determined, at least in part, by the high level of cholesterol and lipid with saturated acyl chains associated with these domains. Our data showing that around 90% of caveolin-1 at the PM, located in lipid domains soluble in cold Triton X-100 (Fig.3), suggest that this pool of caveolin-1 may not be enriched in cholesterol. However, a low concentration of MβCD (2 mM) partially disrupted the pool of Triton-soluble caveolin-1 associated with the cytoskeleton without affecting the integrity
of the PM or clathrin-mediated internalization (Fig.4). Thus, caveolin-1 would appear to be in lipid domains, enriched in cholesterol, but nevertheless solubilized in cold Triton X-100. Consistent with this hypothesis, a previous work has shown that PM derived from hepatocytes possess caveolin-1 organized in caveolae together with structures containing scattered caveolin (25). Also, it has been proposed there is a coexistence in the apical PM of different cholesterol-enriched lipid rafts based on their relative solubility in non-ionic detergents, such as Triton X-100 or Lubrol WX (48).

**Insulin Signaling: a Role for CMD in PM**

In response to insulin we found that CMD associated with the DRM/rafts fraction of the PM rapidly disappeared (Fig.6a). This was not due to internalization of caveolin-IRK complexes into endosomes (Fig.7a). Indeed, in response to insulin, these buoyant density domains become associated with PM-cytoskeletal elements (Fig.7b). Besides CMD of DRM/rafts, insulin also induces rapid recruitment of IRK, p85, IRS1 to a cytoskeleton-enriched fraction derived from PM (Fig.8). All these events were accompanied by rapid actin reorganization as evidenced in Fig.6b, suggesting that the actin cytoskeleton could be the target of CMD and signaling proteins. This hypothesis is supported by previous studies carried out in L6 myotubes, which demonstrated that, in response to insulin, p85 was translocated to newly formed actin containing structures (49). PI3-kinase and subsequent Rac-1 activation may be involved in this effect since both wortmannin (49-52) and a dominant negative Rac-1 (49) inhibited insulin-stimulated actin reorganization and signaling. A close relation between caveolae/raft and the actin cytoskeleton has been previously observed. Thus the F-actin cross-linking protein, filamin, associates with caveolin-1, and both proteins co-localize with stress fibers when analyzed by immunofluorescence (53). Also, it was recently found that 3T3 L1 adipocytes have a unique cortical F-actin structure that is associated with raft/caveolae at the PM (54). In summary, our results suggest that (i) insulin induces the association of CMD with the characteristics of
caveolae and insulin-signaling molecules with the PM actin-cytoskeleton and (ii) CMD appear to be relevant for insulin signaling in hepatocytes. We have also compared the effect of EGF on the distribution of caveolin-1 and signaling molecules in CMD isolated from PM with those of insulin. EGF induced rapid recruitment of p85, actin and caveolin-1 to the raft fraction with no increase of these proteins in the cytoskeleton-enriched fraction. Thus, the insulin effects observed in this study seem to be specific for this hormone.

The presence of IRK in CMD of DRM/rafts could be considered as a strong argument in favor of the significance of caveolae for insulin signaling. However, the available data concerning this issue is not clear. This work, as well as an earlier study, show that IRK is barely detectable in CMD isolated with Triton X-100. In contrast, we observed substantial levels of IRK in PM-CMD prepared by immuno-isolation with an anti caveolin-1 antibody attached to magnetic beads in absence of detergent (Fig. 8b). The presence of IRK in CMD has been confirmed by electron microscopy and immunofluorescence in 3T3 L1 adipocytes. In this work the authors showed that the IRK co-fractionated with CMD when Na₃CO₂ (pH 11) but not Triton X-100 was used to extract CMD. They suggested that this was due to selective solubilization of IRK by this detergent. Our data conform to this observation. In contrast to these results, Souto et al (55) analyzed the presence of IRK in adipocyte caveolae by immunopurification of caveolae and electron microscopy combined with immunogold labeling. Both techniques showed that there is a lack of IRK in caveolae. In summary, we suggest that the presence of a functional IRK in PM-CMD together with an insulin-induced association of CMD of DRM/rafts with the cytoskeleton support the view that insulin signals through PM caveolae in liver.

**Cholesterol Depletion in Primary Hepatocytes: Effect on Insulin Signaling**

The constitutive association of Triton-soluble CMD with PM-cytoskeleton and the subsequent translocation of Triton-insoluble CMD (DRMs/rafts) to this latter compartment in response to
insulin, make almost impossible any further analysis of the role of CMD on insulin signaling in rat liver. Although treatment of the PM with Na₂CO₃ (pH 11) can disrupt the association between the cytoskeleton and CMD, this also interferes with the association of these lipid domains with insulin-signaling proteins (i.e. IRS1, p85, etc). Thus, in order to evaluate further the importance of CMD on insulin signaling in rat liver we continued our studies in primary hepatocyte cultures. We used MβCD to extract cholesterol from the PM of the hepatocytes as a tool to investigate the role of CMD on insulin signaling (38). CMD are enriched in cholesterol, therefore these membrane domains are more sensitive to cholesterol extraction compared to the rest of the PM. However, the concentration of MβCD used to extract cholesterol from CMD must be carefully determined since, as suggested in this and previous work (40), non-CMD PM is also affected by cholesterol depletion. Thus, in this work, we found that 2 mM MβCD partially disrupted hepatocyte cell surface CMD without permeabilizing the cell to sulfo-biotin or affecting clathrin dependent internalization (Fig.4). Under this circumstance extraction of cholesterol from hepatocyte PM did not interfere with the insulin-induced tyrosine-phosphorylation of IRK but inhibited the downstream activation of PKB/Akt. When a low concentration of MβCD was used (1 mM or 2mM), we observed partial inhibition of Akt activation, principally at 15 minutes but not at 2 minutes after insulin. Thus, our results suggest that CMD could be involved in the modulation of insulin signaling at later times after insulin administration. It is also of considerable interest that insulin-induced glycogen synthesis is inhibited by 2 mM of MβCD. This is in accordance with the dependence of insulin-induced glycogen synthesis on PI3-kinase/Akt activation in hepatocytes (34).

We have also demonstrated that clathrin-mediated endocytosis is not affected by 2 mM of MβCD since neither IRK nor transferrin internalization was inhibited when hepatocytes were pre-incubated with this concentration of MβCD (Fig.4). Thus, CMD could be essential for insulin signaling after IRK is internalized and may modulate the translocation of insulin-signaling
proteins downstream of IRK to a specific sub-cellular compartment. In accordance with this hypothesis, a previous work carried out in adipocytes has shown that β-cyclodextrin inhibits the insulin-induced association of IRS1 with IRK without affecting the activation of the latter (18). Consequently, IRS1 tyrosine phosphorylation and Akt activation were inhibited (18). Although liver contains a low level of caveolin-1 compared to adipose tissue, cholesterol depletion in hepatocytes induces effects on insulin signaling similar to those observed in adipocytes. In agreement with a role of CMD in insulin signaling, it has been recently demonstrated that caveolin-1 null mice are insulin resistant and also show a drastic reduction in the level of IRK in adipocytes (23). The author of this work hypothesized that caveolin–1 could be necessary for proper stabilization of IRK in adipocytes since IRK mRNA levels were not affected in caveolin-1 null mice (23). Our data are consistent with a role for CMD in insulin signaling in hepatocytes. Since this conclusion relies on the effect of MβCD which, as discussed above, is not absolutely specific for CMD, further work is required to define this role conclusively.

In summary we report the existence of at least two different populations of CMD in rat liver PM. One population of CMD is soluble in Triton-X 100 and appears to be constitutively associated with cytoskeletal elements. A second population of CMD is located in a membrane compartment insoluble in Triton X-100 with light buoyant density (DRM/rafts). These micro-domains behave as dynamic structures in response to insulin. Thus, along with IRK, p85, and IRS1, CMD were found to associate, in an insulin-dependent manner, with actin-containing cytoskeletal structures. Finally, the negative effect of cholesterol depletion on insulin signaling supports the view that CMD participate in the regulation of insulin signaling in liver.
REFERENCES:

FOOTNOTES:

1 The abbreviation used are: IRK, insulin receptor kinase; CMD, caveolin-1 enriched membrane domains; DRM, detergent resistant membranes; EGF, epidermal growth factor; IRS1, insulin receptor substrate 1; PM, plasma membranes; p85, regulatory subunit of PI3-kinase; PI3-kinase, phosphatidylinositol 3-kinase.

ACKNOWLEDGEMENTS

This work was supported by the Canadian Institutes for Health Research, and the National Cancer Institute of Canada. We appreciate the continuing generosity of the Cleghorn Fund at McGill University, and the Maurice Pollack Foundation of Montreal. We thank Louise Larose, Simon Wing and Amanda Parmar for the critical reading of the manuscript.
FIGURE LEGENDS:

Figure 1. Distribution of Caveolin-1 and Actin in Subcellular Fractions from Rat Liver.
Homogenate (H), microsomes (M), endosomes (EN), plasma membrane (PM) and Golgi (G) were purified from liver. Samples (50 µg of protein) were subjected to SDS-PAGE (6-12%), transferred to Immobilon-P membranes and immunoblotted with antibodies specific for caveolin-1 (panel a) and total actin (panel b).

Figure 2. Isolation of CMDs and DRMs/rafts from PM and Microsomes.
a) Plasma membranes and microsomes were prepared as described in Experimental Procedures. These cell fractions were then homogenized in presence of Triton X-100 (final concentration, 1%) and mixed with an equal volume of 80% sucrose (40% final concentration). Each preparation (4 ml) was placed at the bottom of a centrifuge tube and was overlaid with 4 ml of 30% and 4 ml of 10% sucrose and centrifuged at 29,000 x g for 21 hr. One ml samples were collected from the top (fractions 1 to 8) and proteins were concentrated by precipitation with TCA-deoxycholate. The 40% sucrose layer (fraction 9) and the pellet (re-suspended in 1 ml PBS) were also collected. Each of the eight concentrated fractions, and fraction 9 (50µg of protein/fraction) as well as the re-suspended pellet were subjected to SDS-PAGE (6-12%) and analyzed by immunoblotting with an anti caveolin-1 (panel a) and actin antibodies (panel b). A representative Western blot from one of 3 different experiments is shown. c) SDS-PAGE (6-12%) and silver staining of sucrose gradient fractions prepared from microsomes and PM (10 µg of protein).

Figure 3. Characterization of CMDs in Rat Liver PM
Rats were fasted overnight and liver PM was prepared as described in Experimental Procedures. PM preparations were re-suspended in TBS (pH 7.5) containing protease inhibitors and divided into three equal aliquots. These aliquots were homogenized in presence of 1% Triton X-100 (panel a), Na₂CO₃ (pH 11) (panel b), and the combination of 1% Triton X-100 and Na₂CO₃ (pH 11) (panel c). These homogenates were placed at the bottom of a centrifuge tube
and processed as described in Fig.2. Fractions 1-8 and fraction 9 correspond to the 10-30 \% sucrose gradient and the soluble proteins of the 40\% sucrose layer respectively. The pellet was re-suspended in 1ml of PBS. Each of the 9 fractions and the re-suspended pellet were subjected to SDS-PAGE (6-12\%) and analyzed by immunoblotting with anti caveolin-1 and actin antibodies. A representative immunoblot of the 3 different solubilization conditions is depicted. The volume of each fraction and the volume loaded for SDS-PAGE are shown at the bottom of the panel c. Bands were quantified by scanning densitometry and normalized to the total amount of caveolin-1 and actin in each fraction. The amount of caveolin-1 (panel d) and actin (panel e) are expressed as a percentage of the total amount in the original PM lysate (% total). Each point is the mean ± SE of three independent experiments. The protein content in each fraction is shown in panel f.

Figure 4. Effect of MßCD on the Integrity of the Hepatocyte PM and Clathrin-dependent internalization.

a) Primary hepatocytes were incubated with the indicated concentrations of MßCD for 1 hr at 37°C. Cell surface proteins were biotinylated as described in Experimental Procedures. Hepatocytes were then lysed and PI3-kinase was immunoprecipitated from these lysates using an antibody against to p85. Immunoprecipitates were boiled in Laemmli sample buffer, and subjected to SDS-PAGE. The gel proteins were transferred to immobilon-P membranes, and probed with streptavidin-HRP for detection of the pool of p85 labeled with biotin. b) Uptake of \(^{125}\text{I}\)-Transferrin (Tf) into hepatocytes: Primary hepatocytes were pre-incubated in the presence or absence of 2 mM MßCD for 1 hr at 37°C, and \(^{125}\text{I}\)-Tf or \(^{125}\text{I}\)-Tf plus unlabeled Tf were then added and hepatocytes were further incubated for 15 or 60 minutes. The level of intracellular transferrin was determined as indicated in Experimental Procedures. The results are expressed as the mean ± SE of three separate experiments. c) IRK internalization: Primary hepatocytes were incubated with or without 2 mM of MßCD for 1 h at 37°C followed by addition of insulin.
(100nM) for the indicated times. Cell surface proteins were biotinylated and lysed as indicated in Experimental Procedures. IRK was immunoprecipitated from these lysates using an antibody to the α subunit of IRK. Immunoprecipitates were processed as indicated above and immobilon-P membranes were probed with an anti IRK antibody (α960) or with streptavidin-HRP for detection of the total amount of IRK or PM-associated IRK, respectively. The depicted experiment is one of 2 similar ones.

**Figure 5. Effect of 2 mM MβCD on CMD in hepatocytes.**

Primary hepatocytes were pre-incubated in presence (○) or absence (●) of 2 mM of MβCD for 1 hr at 37 °C, followed by homogenization with 1% Triton X-100 and sucrose gradient flotation analysis as described in Experimental Procedures. The amount of protein loaded onto the gradient was normalized within each experiment. Fractions were subjected to SDS-PAGE (6-12%) and analyzed by immunoblotting with an anti caveolin-1 (Cav-1) antibody. The level of caveolin-1 was quantified by scanning densitometry and normalized to the total amount of caveolin-1 in each fraction. a) The amount of caveolin-1 is expressed as a percentage of the total amount in the original PM lysate (% of total). A representative Immunoblot at the bottom of the bar graph is shown. Each point is the mean of three independent experiments. b) The content of Caveolin-1 in MβCD treated cells is expressed as a percentage of control hepatocytes. Each bar is the mean ± SEM of three independent experiments. *p < 0.05.

**Figure 6. Insulin-stimulated Caveolin-1 and Actin Redistribution in PM Sub-Compartments.**

After an overnight fast rats received a single iv dose of insulin (1.5 µg/100g BW) and were killed at the noted times thereafter. Liver PM were prepared and homogenized with 1% Triton X-100 as described in Experimental Procedures. The amount of protein loaded onto the gradient was normalized within each experiment and the preparations were processed as described in Fig.2. Lipid raft (▲), pellet (○) and total PM (●) fractions were subjected to SDS-PAGE (6-12%) and
analyzed by immunoblotting with anti caveolin-1 (panel a) or total actin (panel b) antibodies. The level of caveolin-1 and actin in each fraction was quantified using scanning densitometry. The results were plotted as a percentage of the value in basal animals (no insulin treatment). Each point is the mean ± SE of three to seven independent experiments. *, P < 0.01; #, P < 0.05 (vs. basal, by Student's t test).

**Figure 7. Insulin Stimulates the Association of CMD in DRM/rafts with the PM-Cytoskeleton**

After an overnight fast rats received a single iv dose of insulin (1.5 µg/100g BW) and were killed at the noted times thereafter. a) Endosomes were prepared as described in Experimental Procedures. Samples (50µg of proteins) were subjected to SDS-PAGE (6-12%), transferred to PVDF membranes and blotted with antibodies against the insulin receptor (IRK) or caveolin-1 (Cav-1). A representative immunoblot of two independent experiments is shown. b) PM preparations, derived from insulin-treated rats (5 minutes), were split into two equal aliquots. One aliquot was incubated with 1% Triton X-100, and the other with a combination of 1% Triton X-100 and Na₂CO₃ (pH 11) as described in Fig. 3. PM fractions were isolated as described in Figure 2, subjected to SDS-PAGE (6-12%) and analyzed by immunoblotting with anti caveolin-1 and actin antibodies. A representative immunoblot of three independent experiments is shown.

**Figure 8. Effect of Insulin on IRK, p85 and IRS1 Distribution in PM Sub-fractions.**

After an overnight fast rats received a single iv dose of insulin (1.5 µg/100g BW) and were killed at the noted times thereafter. Liver PM sub-compartments were isolated as indicated in Fig. 2, subjected to SDS-PAGE (6-12%), and analyzed by immunoblotting with an anti IRK or PY antibodies (panel a). A representative immunoblot of three independent experiments is shown. b) Immunoisolation of caveolin-enriched membranes: PM preparations were homogenized in Na₂CO₃ (pH 11). Homogenates were centrifuged; the pellets were re-suspended in TBS (pH 7.5), and incubated with magnetic beads coated with an antibody specific for caveolin-1 or IgG (negative control) as described in Experimental Procedures. Immunoisolated caveolin-1
containing structures were subjected to SDS-PAGE (6-12%) and analyzed by immunoblotting with anti caveolin-1 and IRK antibodies. A representative immunoblot of two independent experiments is shown. c) Lipid rafts (▲), pellets (○) and total PM (●) fractions were subjected to SDS-PAGE (6-12%) and analyzed by immunoblotting with anti IRS1 or p85 antibodies. The levels of these proteins were quantified using scanning densitometry. Upper panel: Representative immunoblot. Lower panel: Levels of IRS1 and p85 were plotted as a percentage of the value in non-insulin treated (basal) animals. Each point is the mean ± SE of three to seven independent experiments (*, P < 0.01; #, P < 0.05 [vs. basal, by Student’s t test]).

Figure 9. Effect of MβCD on insulin-induced IRK tyrosine-phosphorylation, Akt-Ser473 phosphorylation and glycogen synthesis.

Hepatocytes were incubated with or without MβCD (1 mM or 2 mM) for 1 hr at 37°C followed by insulin (100nM) for the indicated times. Lysates were prepared and equal amounts of protein (50 µg) were subjected to SDS-PAGE and transferred to Immobilon-P membranes. Membranes were immunoblotted with α960 and αPY (panel a); αAkt, and αAkt-Ser473 (panel b) and a specific antibody against Akt2 (panel d). The immunoblots shown in panel a & b are representative of two independent experiments with similar result. Results in panel c are the mean ± ½ the range in two independent experiments. Symbols: Control (●), 1 mM MβCD (○), 2 mM MβCD (▲). d) 2 independent experiments are shown. e) Serum starved hepatocytes were pre-incubated in the absence or presence of MβCD (1 mM or 2mM), subsequently MβCD was removed from the media and cells were further incubated with 15 mM [U-¹⁴C] glucose in presence or absence of insulin (100 nM) for 2 h at 37°C. Glycogen synthesis was determined as described in Experimental Procedures. The results are expressed as the means ± SE of an experiment performed in triplicate (*p < 0.01).
Figure 1

a

WB: Caveolin-1

b

WB: Actin
Figure 2

(a) PM: WB caveolin

(b) PM: WB actin

(c) Total Raft}

<table>
<thead>
<tr>
<th>Fraction</th>
<th>PM</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pellet
a  
**WB:**  

<table>
<thead>
<tr>
<th>Gradient</th>
<th>Frac. 9</th>
<th>Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>20</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>40</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>60</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>80</td>
<td>10</td>
<td>30</td>
</tr>
</tbody>
</table>

b  

**Na$_2$CO$_3$ (pH 11)**  

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Cav-1</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>


c  

**Triton X-100 (1 %) plus Na$_2$CO$_3$ (pH 11)**  

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Cav-1</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

**Figure 3**

**d**  

**Caveolin-1 content (±SD) (% of total)**  

**e**  

**Actin content (±SD) (% of total)**  

**f**  

**Protein content (±SD) (µg)**
Figure 4

(a) Transferrin uptake (cpm / ug of protein)

Time (min)         15      15     60      60
MBCD (mM)      0       2          0        2

(b) Biotinylation and IP with αIRK

WB: Streptavidin

MBCD (mM)          0           0.5       1.0        2.0        5.0  10.0

(c) Biotinylation and IP with αp85

WB: Streptavidin

Insulin (min)      0      5     15    0      5     15
MBCD (mM)          -      -     -       2      2     2

IRK (α subunit)
IRK (β subunit)
IRK
Figure 5

(a) 

Caveolin-1 (% of total)

Fraction 1 2 3 4 5 6 7 8 9 pellet

control

2 mM MBCD

(b) 

Caveolin-1 (% of control)

Fraction 4 & 5 9 Pellet

* * *
**Endosomes**

![Image of Endosomes with WB: IRK and Cav-1](image)

**Insulin (min)** 0 2 15

**PM: 5' insulin**

**Treatment:**
- Triton X-100 (1%)
- Triton X-100 (1%) plus Na₂CO₃ pH 11

![Image of PM: 5' insulin with WB: Cav-1](image)
Figure 9

(a) Treatment:
- Control
- 1 mM MβCD
- 2 mM MβCD

Insulin (min) 0 2 15

(b) Treatment:
- WB: α960
- WB: PY
- WB: Akt
- WB: Akt-Ser473

Insulin (min) 0 2 15

(c) Akt-Ser473 (% 2 min insulin)

Insulin stimulation (min)

(d) WB: Akt
- Control
- 2 mM MβCD

Insulin (min) 0 2 15

(e) Glycogen synthesis (arbitrary units)

MβCD (mM) 0 1.0 2.0

Control

Insulin