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Presence of an Albumin-Like Protein on the Plasma Membrane of Adipocytes Isolated from Korean Cow

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The main function of adipocytes in various species is to store nutrient energy in the form of triglycerides, and this function may be closely related with hormonal signaling through the plasma membrane of adipocytes. Using SDS-PAGE, two-dimensional gel electrophoresis, and a membrane biotinylation technique, we have identified a 55KDa protein (55K protein) from the plasma membrane fraction of adipocytes, with an isoelectric point (pI) of 8.1–8.3. However, this 55K protein was not observed with a two-dimensional gel electrophoresis carried out on plasma membrane fractions prepared from the liver, heart, and kidney tissues. An analysis of the 12 amino acids sequence at the N-terminal of the 55K protein showed that it has a similar sequence to that of bovine serum albumin.

Keywords: Adipocyte, Albumin, 55K Protein, Plasma membrane, Two-dimensional gel electrophoresis.

Introduction

Free fatty acids are stored and released from adipocytes in response to the need for nutrient and the hormonal stimulation of the tissue. The increase in fuel storage that results from the exposure of adipocytes to insulin is mediated by a series of signaling events through the plasma membrane, which also leads to the regulation of protein synthesis and gene transcription (White and Kahn, 1994). The immediate consequences of some of the transducing mechanisms that have been defined to-date are not entirely clear. For example, the exposure of adipocytes to insulin results in an increase in intracellular pH (Klip *et al.*, 1988). It has been suggested that this effect is mediated by the activation of the Na^+/H^+ exchange. Alternatively, the effect of insulin on the intracellular pH could also have risen from the removal of cytosolic free fatty acids by esterification. The resulting alkalinization could function as a vital component of the tissue response to insulin, much as the pH changes incurred are involved in the regulation of fertilization, proliferation, and metabolism in other cellular contexts (Madshus, 1988).

On the other hand, the interaction of long-chain fatty acids with adipocytes is important for their uptake and metabolism as well as their involvement in the signaling processes. It has been demonstrated that albumin enhances prostaglandin E2 binding to the rat epididymal adipocyte membrane by modulating the affinity of prostaglandin E2 receptors (Luria and Rimon, 1986a; Cohen-Luria *et al.*, 1990). Furthermore, it has recently been observed that serum albumin played a direct role in the cellular up-take of long-chain fatty acids (Trigatti and Gerber, 1995). Albumin also increased the affinity of prostaglandin E2 binding to the native canine renal medullary membranes and enhanced the binding of the prostaglandin E2 to the prostaglandin receptors solubilized from these membranes. (Cohen-Luria and Rimon, 1988). A brief incubation of the adipocyte isolated membranes at 60°C caused an increase in prostaglandin E2 binding, which is similar to that obtained with albumin. The increase in the membrane binding capacity after a short heating of the membranes was concommitant with a substantial decline in the ability of albumin to induce a further increase in the binding capacity of the pretreated membranes. In contrast to the effect of albumin on prostaglandin E2 binding to the isolated membranes, albumin failed to alter prostaglandin E2 specific binding to intact adipocytes (Luria and Rimon, 1986b).

In the present study, we propose that an albumin-like protein is present on the plasma membrane of intact adipocytes.

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Materials and Methods

Isolation of adipocytes and preparation of the plasma membrane Adipose tissue (5 g) was taken from a Korean cow, and immediately placed into a buffered saline solution (0.15 M NaCl, 1 mM Hepes, pH 7.4) at 37°C. The adipocytes were then prepared by collagenase digestion in Krebs Ringer bicarbonate (KRB) buffer containing 25 mM Hepes, pH 7.4, 5.5 mM glucose, 2 mM acetate, 30 mg/ml BSA and 1.5 mg/ml collagenase. After a 1.5 h incubation at 37°C, the undigested material was removed by filtration through a polypropylene tube capped with chiffon. The separated adipocytes were washed with a digestion medium in the absence of collagenase. The cell volume and the number of adipocytes per gram of tissue were determined as described previously (Vernon *et al.*, 1981). The isolated adipocytes were disrupted by vigorous shaking in a lysing medium containing 2 mM Tris-HCl, pH 7.4, 2.5 mM ATP, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1 mM $KHCO_3$. After centrifugation for 30 min at 30,000 × *g* at 4°C, the pellet was resuspended with a cold sucrose extraction medium (0.25 M sucrose, 2 mM EGTA, 10 μM Tris-HCl, pH 7.4). Eight hundred μl of the resuspended pellet was gently applied to the top of the Percoll gradient (8 ml), and then centrifuged for another 15 min at 10,000 × *g* at 4°C. Using a syringe, approximately 2 ml of the gradient below the top band was collected and washed with 3 vol of cold Tris-buffered saline (TBS) buffer. The plasma membrane fraction of adipocytes was collected by centrifugation for 10 min at 30,000 × *g* at 4°C.

Preparation of plasma membrane fractions from various tissues (liver, kidney, heart) Tissues taken from a Korean cow were perfused *in situ* with 200 ml of Krebs-Ringer solution and fractionated by a modification of methods as previously described (Prpic *et al.*, 1984; Hong and Rhee, 1995). After their removal, each tissue was minced in a buffer containing 250 mM sucrose, 5 mM Hepes, pH 7.4, 1 mM EGTA, and 1 mM dithiothreitol (buffer A), homogenized at 4°C with a glass Potter homogenizer, and then centrifuged for 10 min at 500 × *g*. The supernatant was further centrifuged for 40 min at 36,000 × *g*, and the pellet was resuspended in buffer A containing 35% (v/v) Percoll and fractionated by centrifugation at 36,000 × *g* for 30 min. The upper low-density fraction was taken as the plasma membrane fraction, and was washed in 10 vol of a medium containing 120 mM KCl and 20 mM Hepes, pH 7.4.

SDS-polyacrylamide gel electrophoresis The plasma membrane fractions of various tissues were washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.2), and the protein concentration was measured using the Bradford method (Bradford, 1976). Electrophoresis was performed on 12% SDS-polyacrylamide gel according to the method of Laemmli (1970), using the Mini-Protean II system (Bio-Rad, Hercules, USA). The protein bands were visualized by a silver-staining kit.

Two-dimensional gel electrophoresis The plasma membrane fractions were further lysed with 2% Nonidet P-40 (NP-40, Sigma, St. Louis, USA) containing 8 M urea, 2% ampholine (pH 4–9, Sigma, St. Louis, USA), 5% two-mercaptoethanol, and protease inhibitors (CalBiochem, La Jolla, USA). The two-dimensional gel electrophoresis was performed using isoelectric focusing (IEF) for the first dimension followed by SDS-polyacrylamide gel electrophoresis for the second (O'Farrell, 1975). The conditions were as follows: the IEF was carried out on gels containing 3.5% polyacrylamide, 2% ampholine (pH 4–9), 2% NP-40 and 8 M urea at 400 V for 7 h, and then at 800 V for the last 1 h. A mixture composed of 0.1 M NaOH and 20 mM phosphoric acid was used as the electrode solution.

Avidin-biotin labeling of plasma membrane surface proteins Adipocytes and the other cells isolated from various tissues (liver, kidney, heart) were biotinylated with 10 ml of 0.5 mg/ml sulfo-NHS-biotin (Pierce Chemical Co., Rockford, USA), for 30 min at 4°C and lysed with 5 ml of 1% Triton X-100 in 10 μM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 1 μM pepstatin A, and 10 μM leupeptin for 30 min at 4°C, as described earlier (Hurley, 1985; Fujimoto, 1992). The extract was mixed with 300 μl of streptavidin (Gibco BRL, Gaithersburg, USA) conjugated to Sepharose 4B (Pharmacia, Sverige AB, Sweden) for 12 h at 4°C to recover the biotinylated proteins (Sargiacomo *et al.*, 1989). The recovered proteins, as well as the residual proteins, in the solution precipitated with cold acetone were subjected to the SDS-PAGE or two-dimensional gel electrophoresis.

N-terminal sequencing of 55K protein The 55K protein isolated and eluted from the two-dimensional gel electrophoresis was subjected to SDS-PAGE and electroblotted onto a PVDF membrane for 3 h at 200 V using 25 mM Tris, 192 mM glycine, 20% methanol buffer (pH 8.3) (Towbin *et al.*, 1979). After staining with Coomassie Blue, the blot was air dried, and then the 55K protein band was cut out. An amino-terminal amino acid sequence analysis of the 55K protein band was performed using an Applied Biosystems 473A protein sequencer. The amino-terminal sequences were compared to an NBRF-PIR database, as described in Yoon *et al.* (1997).

Western blot analysis of the 55K protein with a polyclonal antibody raised against bovine serum albumin Bovine serum albumin and the 55K protein eluted from the two-dimensional gel were loaded into a 12% SDS-polyacrylamide gel, and then electroblotted onto a PVDF membrane for 45 min at a current density of 0.8 mA/cm^2 using 25 mM Tris, 192 mM glycine, 20% methanol buffer (pH 8.3). The membrane was blocked by PBS containing 5% nonfat dry milk, and then incubated with a rabbit polyclonal antibody raised against bovine serum albumin (Sigma, USA) at 5 μg/ml for 1 h at room temperature. After washing with PBS-Tween 20 (0.5 %) for 30 min with changes occuring at every 10 min, the membrane was incubated with a HRP-labeled secondary antibody (Amersham Life Science, Buckinghamshire, UK). The protein band(s) was visualized by using the ECL Western blot detection kit (Amersham Life Science, Buckinghamshire, UK) and then being exposed to Kodak X-ray films (Hwang, 1998).

Results

Comparison of proteins on the plasma membrane fractions of various tissues by SDS-PAGE and two-dimensional electrophoresis analyses The proteins present in the plasma membrane fractions isolated from the

liver, heart, kidney, and adipocytes were subjected to SDS-polyacrylamide gel electrophoresis (Fig. 1). The silver-stained protein patterns of the various tissue on the gel were not significantly different, although less protein was observed on the plasma membrane fraction for adipocytes (lane 3). The plasma membrane proteins of various tissues were also isolated by two-dimensional electrophoresis (Fig. 2). As seen in Fig. 1, less protein was also observed for the adipocyte membrane fraction (top left panel). A specific protein was observed in the adipocyte fraction with a relative molecular mass of 55kDa (indicated by arrow), and thus named as the 55K protein. The isoelectric point of this 55K protein appeared to be around 8.1–8.3. The resolution for isolating proteins using the two-dimensional electrophoretic analysis does not seem strong enough to separate one protein from another. This is probably due to the intrinsic physicochemical properties of the membrane proteins. However, the 55K protein was not observed at similar positions on the two-dimensional gels of the liver (top right), kidney (bottom left), and heart (bottom right).

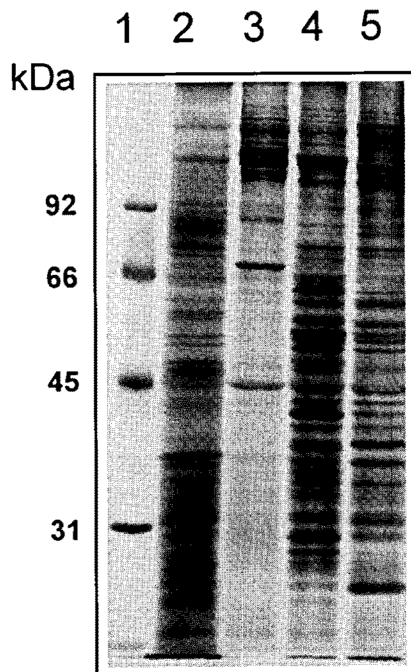

Fig. 1. SDS-polyacrylamide gel electrophoresis of the Plasma Membrane fractions (PMs) isolated from various tissues in the Korean cow. The total amount of proteins of each plasma membrane fraction (5 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis. Lane 1, molecular weight standard proteins; Lane 2, PM of liver tissue; Lane 3, PM of adipocytes; Lane 4, PM of kidney tissue; Lane 5, PM of heart tissue. Electrophoresis was carried out on 12% polyacrylamide gel, and protein bands were visualized using a silver staining method.

Biotinylation of plasma membrane proteins of the adipocyte and analysis by two-dimensional electrophoresis To verify further the presence of the 55K protein in the plasma membrane of the adipocyte, we selectively biotinylated the cell surface of the cultured adipocytes with a membrane-impermeable reagent, sulfo-NHS-biotin (Hurley *et al.*, 1985). Biotinylated proteins were recovered with immobilized streptavidin and subjected to a two-dimensional electrophoresis (Fig. 3). The proteins prepared by this biotinylating method should represent only the plasmalemmal proteins exposed to the exterior surface of the adipocytes. The intensity of the protein staining shown in Fig. 3 is considerably weaker, compared with the protein staining shown in Fig. 2. This phenomenon is probably due to the loss of the total protein during the recovery after biotinylation and/or after a certain fraction of the proteins observed in Fig. 2 are intracellularly exposed. However, the 55K protein is clearly visible in Fig. 3 (indicated by arrow), demonstrating that it is present on the plasma membrane of the adipocyte, and that a certain domain of the protein is exposed to the exterior surface of the adipocyte.

Identification of the N-terminal 12 amino acids of 55K protein The sequence of the 12 residues at the amino-terminal of the 55K protein was analyzed by a protein sequencer (Applied Biosystems Precise Sequencer). The N-terminal sequence comparisons further revealed that the 55K protein has a high homology to that of the bovine serum albumin (Liao *et al.*, 1980), human serum albumin (Savva *et al.*, 1998), and the GP70 (albumin-like glycoprotein) (Onozuka *et al.*, 1995), as shown in Table 1. In order to investigate further homology between the 55K protein and albumin, we performed a Western blot analysis using a polyclonal antibody raised against the bovine serum albumin (Fig 4). The antibody cross-reacted with bovine serum albumin, giving a strong signal at the 66 kDa position (lane 3). However, no signal was detected on the Western blot for the 55K protein (lane 4). This result demonstrates that the antigenic determinants of 55K protein are not similar to those of the bovine serum albumin, although a strong homology exists between the N-terminal regions of the two proteins.

Discussion

From this study, two major conclusions can be made: (i) the 55K protein is present on the plasma membrane of adipocytes, but is not observed to be present on the plasma membranes of other tissues including heart, liver, kidney; (ii) the N-terminal region of the 55K protein contains a strong sequence homology to the N-terminal region of albumin and albumin-related proteins (Table 1). However, a polyclonal antibody raised against the bovine serum albumin (BSA) did not cross-react with the 55K protein on

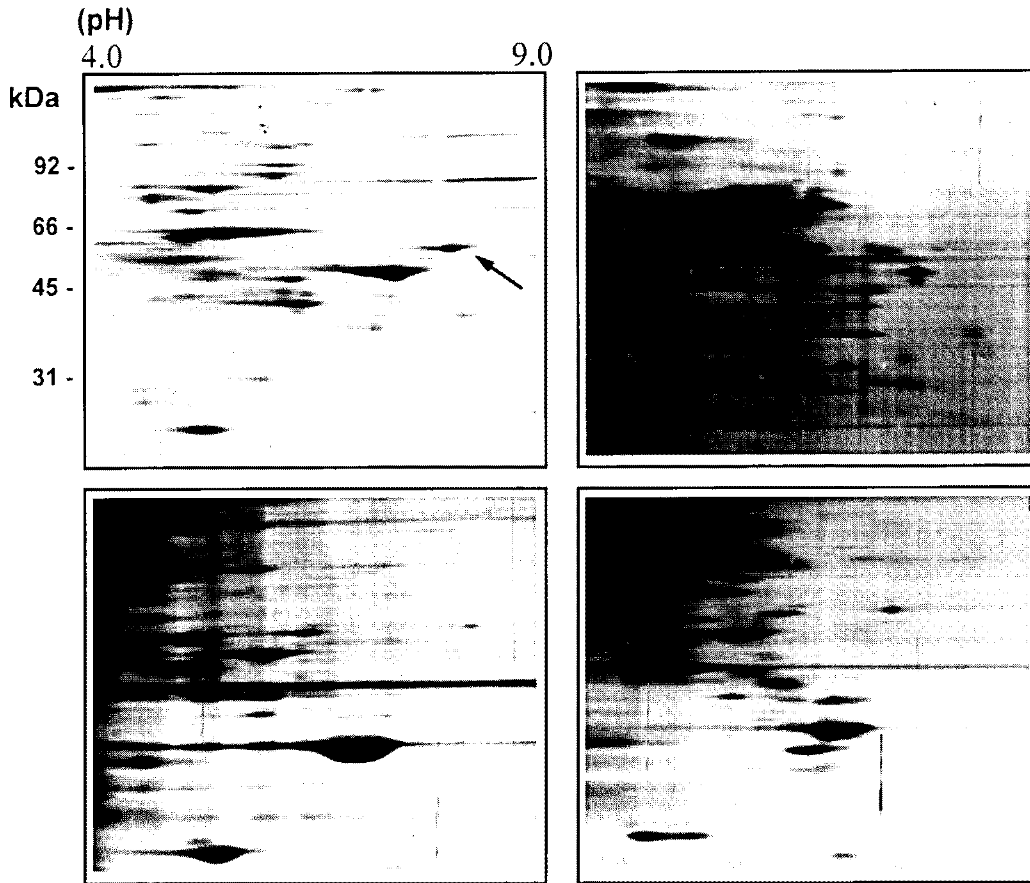

Fig. 2. Two-dimensional gel electrophoresis of Plasma Membrane fractions (PM) of various tissues: adipocyte (top left), liver (top right), heart (bottom left), and kidney (bottom right). The total amount of proteins of each plasma membrane fraction (5 μg) were separated by IEF, using gels containing 3.5% polyacrylamide, and protein spots were visualized using a silver staining method. The arrow (top left) indicates the 55K protein.

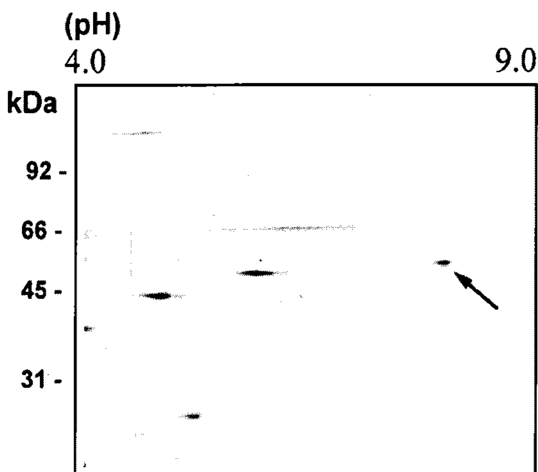

Fig. 3. Two-dimensional gel electrophoresis of the plasma membrane fraction of the adipocyte. In this experiment, the plasma membrane fraction was specifically isolated by a biotinylation method, in which a membrane impermeable reagent, sulfo-NHS-biotin, was used. Proteins recovered by immobilized streptavidin were subjected to IEF and SDS-polyacrylamide gel electrophoresis under the same conditions as in Fig 2. The arrow indicates the 55K protein.

Table 1. Comparison of the amino acid sequences of the N-terminal region of the 55K protein with the published sequences of albumin, alloalbumin, and 70 kDa albumin-like glycoprotein (GP70).

Protein	Amino-terminal sequence	
55K protein	D T H A S E I A H R F K	(1-12)
bovine serum albumin	D T H K S E I A H R F K	(1-12)
human serum albumin	D A H K S E V A H R F K	(2-13)
human alloalbumin	D A H K S E V A H R F K	(25-36)
canine albumin	E A Y K S E I A H R F K	(1-12)
GP70	E A H K S E I A H R F K	(1-12)

the Western blotting analysis, demonstrating that the 55K protein is not a degraded product or a post-translationally modified form of albumin. In addition, the isoelectric point (pI) of the 55K protein (8.1–8.3 as shown in Fig. 2) is somewhat different from that of the bovine serum albumin, which ranges from 5.2–5.6. In relevance to our study, an α-fetoprotein isolated from rat tissue also showed a striking homology to the corresponding regions of the rat, bovine, and human albumin (Lios *et al.*, 1980),

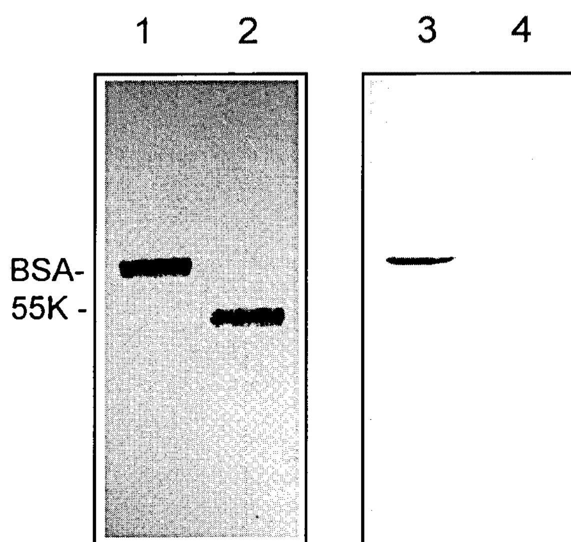

Fig. 4. A Western blot analysis of the 55K protein using an antibody raised against bovine serum albumin (BSA). The 55K protein eluted from the two-dimensional gel (as shown in the top left panel of Fig. 2) and BSA were loaded into a 12% SDS-polyacrylamide gel, and then electroblotted onto a PVDF membrane. The left panel (lanes 1 & 2) represents the silver-staining of BSA and the 55 K protein, respectively, and the right panel (lane 3 & 4) is the Western blot analysis of BSA and the 55K protein, using a polyclonal antibody raised against BSA.

although these two proteins function is a somewhat different way.

At present, it cannot be concluded from our results whether the 55K protein and albumin are similar in their functional properties. However, the Western blotting data showed that these two proteins are completely different from each other, at least in terms of the organization of the antigenic determinants, suggesting little or no functional similarity. Regardless of the mechanism involved in the localization of the 55K protein and its functional properties, our study demonstrates that the 55K protein is present on the plasma membrane of the adipocyte, possibly in a tissue-specific manner. The preparation of antibodies against a peptide corresponding the amino-terminal region of the 55K protein and against the whole protein, and the isolation of its cDNA, are currently underway to perform further characterization.

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