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Expression of a 64 kD Adipocyte-Specific Plasma Membrane Protein in Genetically Lean but not Obese Porcine Adipocytes

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A monoclonal antibody (LA-1) to an adipocyte-specific plasma membrane protein (64 kD) was used to examine the differential expression of this protein in genetically lean and genetically obese pigs. Enzyme-linked immunosorbent assay (ELISA) implied the differential expression of the 64 kD protein in adipocyte plasma membranes having different genetic background. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of genetically lean, genetically obese, and contemporary subcutaneous adipocyte plasma membranes did not indicate any obvious qualitative differences in protein composition. Corresponding immunoblots utilizing LA-1 confirmed the presence of the 64 kD protein in contemporary and genetically lean adipocyte plasma membranes but absence in genetically obese adipocyte plasma membranes. LA-1 labelled intact adipocytes isolated from contemporary and genetically lean adipose tissue but did not react with isolated genetically obese adipocytes. The ability to bind to intact adipocytes indicates that the protein is exposed to the extracellular environment. The migration pattern of the protein was not affected by enzymatic deglycosylation by endoglycosidase-F suggesting that the protein is not highly, if at all, glycosylated. Presence of the 64 kD protein in genetically lean but not genetically obese adipocyte plasma membranes indicates the identification of a novel adipocyte-specific surface protein associated, either directly or secondary to the onset of obesity, with genetic predispositions for either genetically lean or obese body types in swine.

Key words: monoclonal antibody, ELISA, adipocyte plasma membranes, SDS-PAGE, protein composition

Obesity is a condition of excessive energy storage in the form of body fat (triacylglycerol). Although the exact effects of obesity on human health are unknown, undoubtedly obesity, when associated with elevated cholesterol levels, cardiovascular abnormalities, or diabetes, contributes to premature mortality. The cellular basis for obesity is not yet understood but numerous factors have been suggested. Several cellular parameters which may contribute to the level of triacylglycerol storage by adipocytes include genetic factors, altered metabolism, and defective thermogenesis [1].

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Rodent models of obesity have traditionally been used as models for human obesity, but these models are typically complicated by major defects in carbohydrate and/or lipid metabolism [2]. The genetically lean and obese pig models are free from these aberrations and may prove valuable models for human obesity [3].

In genetically lean and obese rat models dietary manipulation had very little effect on body composition, suggesting that genetic factors may be playing an intricate role in predisposing individual animals to either leanness or obesity. Genetically obese rats, when restricted to the caloric intake of their lean littermates, still became obese to a level similar to ad libitum fed obese rats [4]. The genetic contributions to obesity are further supported by human studies utilizing monozygotic twins. It was estimated that 80% of the contribution to obesity could be explained by genetics [5] and that the amount and distribution of fat were related to the closeness of genetic relationship [6].

Polyclonal antibodies have been used in an attempt to identify species-specific cell surface components by SDS-PAGE, immunoblotting, and immunoprecipitation [7,8]. These investigators suggested the possible existence of adipocyte-specific plasma membrane proteins. We have produced a monoclonal antibody (LA-1) which identifies a porcine adipocyte-specific plasma membrane protein [9]. This unique protein is expressed only on adipocytes from contemporary and genetically lean but not genetically obese pigs. This paper discusses the identification of the differential expression of this adipocyte-specific plasma membrane protein which is negatively associated with genetic predisposition for obesity.

MATERIALS AND METHODS

Animals and Cell Preparation

Three castrate male pigs were selected from each of three distinct groups of the genetically lean, genetically obese, and contemporary (typical market pig) phenotypes. Background and husbandry of the genetically lean and obese pigs used in this study were described previously [10]. All animals had approximate body weights of 30 kg at time of sampling. Adipose tissue samples were obtained through biopsy of dorsal subcutaneous fat [9]. Isolated adipocytes were prepared by collagenase digestion of the adipose tissue [11] and plasma membranes were purified on a self-forming Percoll gradient [12]. Purity of the preparations was monitored by using the 5′-nucleotidase assay [13]. No difference in purity could be detected among the strains of pigs (obese, 24.26 ± 0.56; lean, 21.72 ± 0.36 μM of inorganic phosphate released/μg/h). All studies were performed on three separate animals and replicated three times.

Enzyme-Linked Immunosorbent Assay (ELISA)

Binding of monoclonal antibody (LA-1) to contemporary, genetically lean, and genetically obese adipocyte plasma membranes (APM) was determined by using an ELISA method. APM were prepared and the assay conducted as described earlier [9]. Binding of LA-1 to the APM was detected by a secondary goat-antimouse alkaline-phosphatase-conjugated antibody. Control level of absorbance (100%, O.D. = 0.20) was defined as the absorbance produced by reaction of LA-1 with obese APM. Isotype-matched primary control monoclonal antibody and the secondary antibody were used as controls. Staining was not observed with the alkaline-phosphatase-conjugated secondary antibody alone or in conjunction with the isotype-matched control.

Electrophoresis and Immunoblotting

APM proteins were separated on 12% polyacrylamide and either stained with Coomassie Brilliant Blue R-250 (CBB) or transblotted onto a nitrocellulose membrane [9]. Immunoblotting was performed with LA-1 and visualized by using a goat-antimouse alkaline phosphatase conjugate.

Deglycosylation

Hydrolysis of contemporary APM proteins was performed by using endoglycosidase-F (Endo-F), (Sigma Chemical Co., St. Louis, MO). The Endo-F reactions were conducted at 37°C for 24 h under the following conditions: 0.25 M sodium acetate (pH 5.0), 200 milliunits enzyme, 20 mM EDTA, 10 mM β-mercaptoethanol, 50 μg test protein in a total volume of 100 μL [14]. Predigested APM, digested APM, and digestion supernatant were all immunoblotted with LA-1.

Immunohistochemistry

Intact, isolated adipocytes were prepared by collagenase digestion from fresh fat samples. Viability of porcine adipocytes isolated by using this technique was demonstrated by sensitivity of glucose transport to insulin and adrenergic agonists [15]. The isolated adipocytes were incubated with LA-1 (diluted 1:100 in PBS) and subsequently incubated with a goat-antimouse FITC conjugate (diluted 1:20 in PBS) and observed by using an epifluorescence microscope [9]. These conditions were shown to be effective in staining contemporary porcine adipocytes [9]. Isotype-matched primary control antibody and the goat-antimouse FITC conjugate were used both singly and together as negative controls.

RESULTS AND DISCUSSION

ELISA

Figure 1 illustrates the differences in binding of LA-1 to contemporary, genetically lean, and genetically obese APM. These results indicate differing degrees of response of LA-1 to the APM from the three sources. Such differences might be attributed to differences in the purity of the two APM preparations or to varying amounts of contaminants which might interfere with binding of the LA-1 antigen to the microtiter plate surface. It seems likely, however, that recovery of the 64 kD protein from the three APM preparations is not a significant contributing factor since ELISA performed by using adipose tissue homogenate preparations gave similar results (data not shown). Thus results shown in Figure 1 imply the possibility of either quantitative or qualitative differences in the levels of expression of the 64 kD protein which may be associated with genetic predisposition for obesity. Contemporary pigs have been selected for leanness, but less intensively when compared to the genetic model. Contemporary pigs, thus, are phenotypically more closely related to the genetically lean animal than the obese. This may explain in part the presence of the common 64 kD protein. Since all of the APM were coated at equal total protein concentrations and there were obvious differences in the levels of 64 kD protein present, this allows us to suggest that the contemporary APM has a much higher level of 64 kD protein present as a percentage of the total APM protein. The genetically lean and genetically obese lines of animals have the same parental genetic background

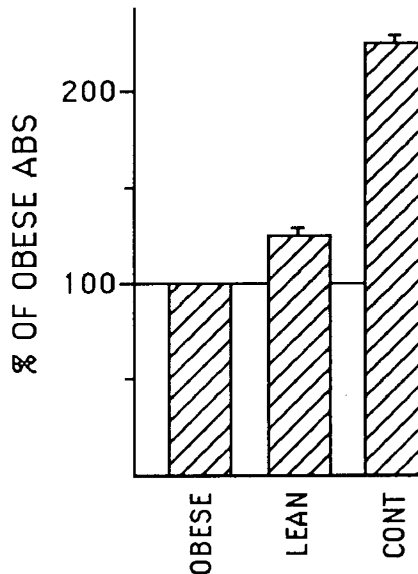

Fig. 1. Enzyme-linked immunosorbent assay data showing the relative levels of binding (absorbance) of LA-1 to contemporary (CONT), genetically lean, and genetically obese APM. Each well of the microtiter plate was coated with 1 μg APM overnight. The data are expressed as percent absorbance of obese APM (100%, O.D. = 0.20).

and were selected for approximately 18 generations for either low (lean) or high (obese) backfat thickness [16]. The similarity in genetic background suggests that one possible explanation is an alteration in the genetic code resulting in the differences in physiology.

Electrophoresis and Immunoblotting

There were very few differences in the protein profiles of the contemporary, genetically lean, and genetically obese APM as indicated by CBB staining of the SDS-PAGE (Fig. 2). Although all samples were loaded at equal levels of total protein, there appear to be some minor quantitative but no noticeable qualitative differences. It is possible that the obese APM preparation contained some interfering factors which could lead to an overestimation of total protein content in this sample. If this were the case, it might explain the slight apparent decrease in the amount of protein contained in the obese lane. To compensate for this possible condition, we loaded double (25 μg/lane) the estimated obese APM protein for immunoblotting. Immunoblotting of the three APM sources indicated major differences in the levels of expression of the 64 kD protein recognized by LA-1. The contemporary APM expressed high levels of the protein with the genetically lean APM showing much lower levels. The genetically obese APM showed no expression of the protein at equivalent (data not shown) or doubled levels of total APM protein (Fig. 3). This suggested that the genetically obese adipocyte either did not express the protein in its APM to any appreciable level or that the protein had been modified to the extent that it could no longer be detected by LA-1. The genetically lean adipocyte had reduced

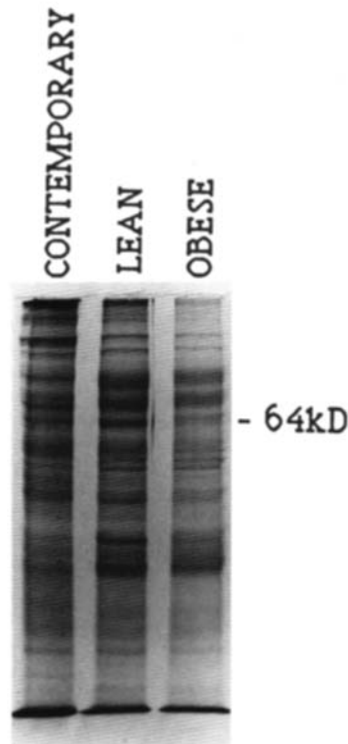

Fig. 2. Coomassie Brilliant Blue R-250 (CBB)-stained SDS-PAGE of contemporary, genetically lean, and genetically obese APMs; 12.5 μg/lane run on a 4% stacking, 12% separating gel at 200 V, 4°C for 45 min. No apparent differences in the protein banding patterns was noticed among the three tissue sources.

levels of the protein in its APM, but these levels were still detectable by immunoblotting (Fig. 3).

Deglycosylation

Because the two physiologically lean adipose sources expressed this protein while the genetically obese adipose source did not, it appeared appropriate to compare 64 kD characteristics to those of the serine protease adipsin for which a similar expression pattern has been proposed [17]. Adipose tissue has been shown to be the principal site of synthesis of adipsin [17,18] which is then secreted into the circulation. Adipsin levels were found to be diminished in rodent models of genetic obesity. Circulating levels of adipsin were not found to be significantly altered in models of diet-induced obesity, suggesting a possible correlation of adipsin with the condition of genetic obesity [17]. Adipsin was found to exist in two forms, both extensively glycosylated in the natural state, having molecular weights of 37 and 44 kD. Deglycosylation of both forms of the protein yields products having a molecular weight of 25.5 kD. We were interested in determining if the 64 kD protein, recognized by LA-1, was also highly glycosylated. Upon digestion with Endo-F, we were not able to detect any changes in the migration pattern of the protein (Fig. 4). This led us to the conclusion that the 64 kD protein was not highly glycosylated. A second possibility is that the epitope recognized by LA-1 is at least partially composed of a carbohydrate

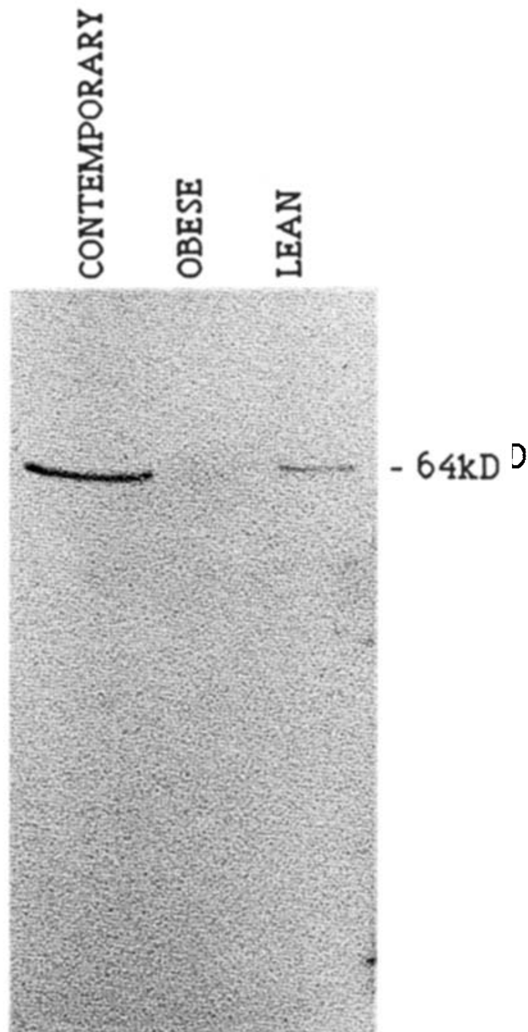

Fig. 3. Western blot of contemporary, genetically lean, and genetically obese APM immunoblotted with LA-1. Protein concentrations of 12.5 μg/lane were used for contemporary and genetically lean APMs, and 25 μg/lane was used for the genetically obese APM. Only the contemporary and genetically lean APM contained the protein recognized by LA-1. The genetically obese APM failed to show the protein despite a doubling of the total protein content.

moiety. If this were the case we would not be able to detect the deglycosylated protein by immunoblotting with LA-1. This is not the most likely case since the conditions of digestion were chosen to allow complete enzymatic digestion. It has not yet been determined if the 64 kD protein is present in varying levels, if at all, in the contemporary, genetically lean, and genetically obese circulation.

Immunohistochemistry

We performed an immunohistochemical staining of intact, isolated adipocytes to determine if the protein detected by LA-1 is exposed to the extracellular environment.

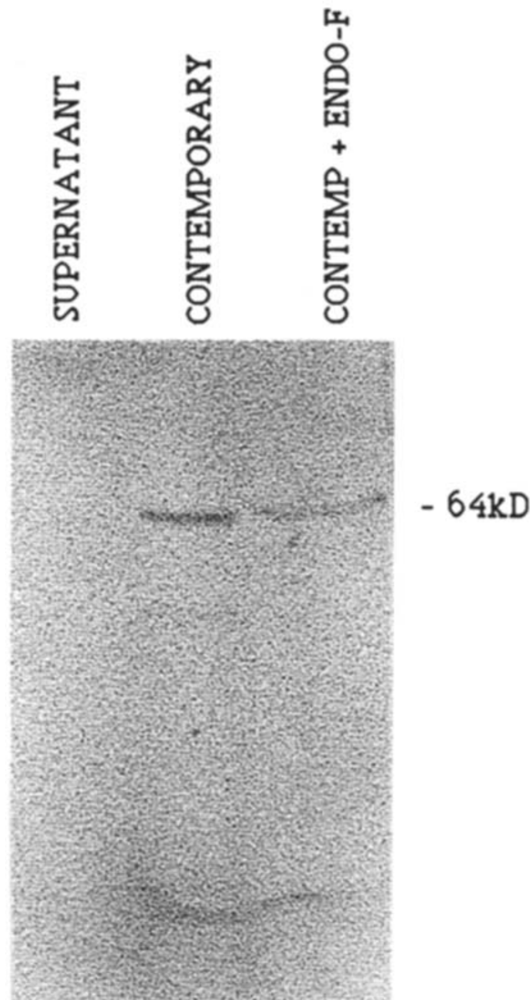

Fig. 4. Effects of enzymatic deglycosylation of contemporary APM by endoglycosidase-F. Contemporary APM was digested with Endo-F and the APM pellet and supernatant were subjected to SDS-PAGE and immunoblotting with LA-1. The digested materials were compared to the control (undigested) contemporary APM.

We found that isolated adipocytes from the genetically lean pig were stained by LA-1 (Fig. 5) as were isolated adipocytes from the contemporary pig [9]. Interestingly, LA-1 was unable to stain any of the isolated genetically obese adipocytes (Fig. 5), and the number and intensity of cells stained from the genetically lean source were much lower than those stained from the contemporary source. Damage to the adipocyte plasma membrane during collagenase digestion may be a possible explanation for the differences in reactivity to LA-1, but this situation probably is not significant since porcine adipocytes isolated using these techniques still retain their responsiveness to insulin and adrenergic agonists [15]. This finding corresponds with the different levels of binding of LA-1 to the 64 kD protein shown in the ELISA and immunoblot data.

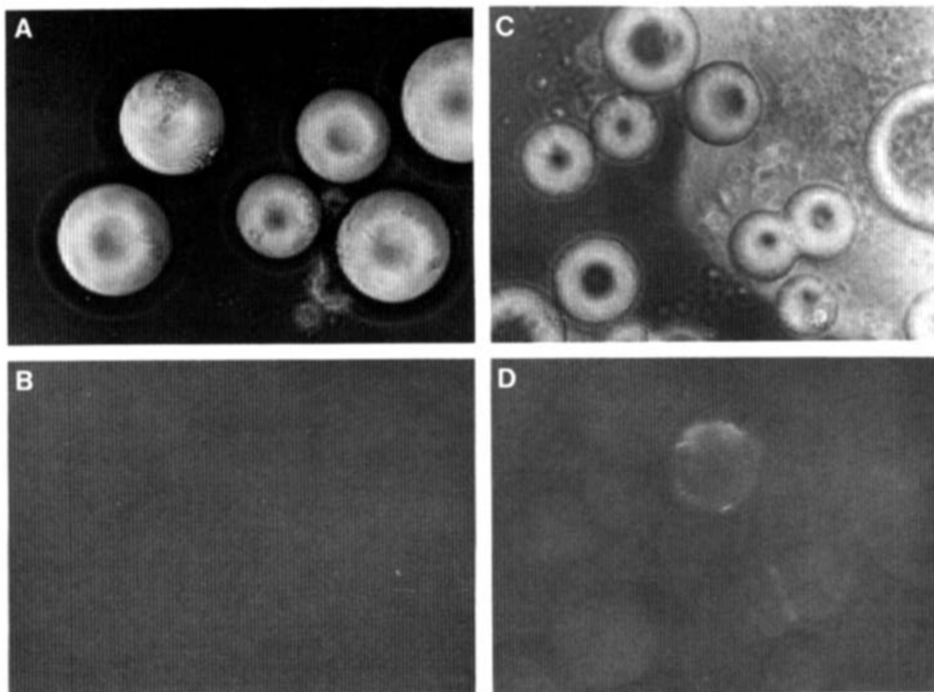

Fig. 5. Photomicrographs of isolated porcine adipocytes from genetically lean and genetically obese subcutaneous dorsal fat depots. All cells were isolated from adipose tissue by collagenase digestion and incubated with LA-1 (1:100) and then goat-antimouse FITC-conjugated second antibody (1:20). **A:** Genetically obese adipocytes photographed under phase contrast. **B:** Indirect immunofluorescence of genetically obese adipocytes (same field as A). **C:** Genetically lean adipocytes photographed under phase contrast. **D:** Indirect immunofluorescence of genetically lean adipocytes (same field as C). Note that no staining was noticed in the genetically obese cells and that there were varying levels of staining in the genetically lean cells. Original magnification ×250.

The protein recognized by LA-1 may possibly be intimately involved with the physiological effects produced by genetic obesity or leanness. The functional role of the protein and any possible involvement in the obese condition have yet to be determined. It is not known whether the expression of this protein, in this case, may be a primary factor inhibiting the onset of obesity or whether the protein is a secondary product of the physiological state of the cells.

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