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Title: Characterization and Cloning of a cDNA Encoding an Adipocyte-specific Membrane Protein

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The accumulation of excessive fat is a serious concern in both the livestock production and human health fields. Obesity is a condition of excessive energy storage in the form of body fat (triacylglycerols). The cellular basis for obesity is not yet understood but numerous factors have been suggested. Genetic factors and altered metabolism may be two cellular parameters that contribute to the excessive accumulation of fat. Adipocytes are responsive to extracellular signals, which have a dramatic effect on their metabolism implying that these metabolic responses may be the result of differences in the composition or responsiveness of adipocyte receptors.

The purpose of this research was to identify adipocyte-specific marker proteins and to determine if there are any differences in the expression of these proteins that may be

associated with the conditions of genetic obesity or leanness. Identification of adipocyte-specific markers should allow for a better understanding of adipocyte growth and development and determination of the adipocytes role in energy metabolism. A hybridoma line was produced which secreted a monoclonal antibody (LA-1) directed against a novel 64-kD protein unique to porcine adipocyte plasma membranes, having an undetermined function in the unique physiology of the adipocyte. This protein was found to be expressed in genetically lean adipocytes but not adipocytes derived from genetically obese sources. In order to elucidate the role of this unique adipocyte-specific plasma membrane protein, a porcine adipocyte cDNA library was produced. This library was screened with LA-1 and a cDNA clone isolated. This cDNA clone was used to study the expression of the gene responsible for this unique protein at the nucleic acid level. Northern blot analysis revealed a 5000- and a 7000-base pair species of poly (A+) RNA present in total RNA isolated from contemporary porcine adipose tissue. Determination of the nucleic acid sequence of the cDNA clone should allow for the determination of the actual identity and possible function of this adipocyte-specific protein and the possible role it may serve in regulating adipocyte growth and development.

Characterization and Cloning of a cDNA Encoding an Adipocyte-specific Membrane Protein

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TABLE OF CONTENTS

LIST OF FIGURES

Characterization and Cloning of a cDNA Encoding an Adipocyte-specific Membrane Protein

Chapter 1
Introduction

Introduction

The efficiency of animal production can be manipulated through numerous approaches, including nutritional, managerial, physiological and genetic manipulation. Improved efficiency in livestock production resides in the ability of the producer to increase the production of desirable products while simultaneously reducing the expense of inputs. When discussing meat animals such as cattle, swine and chicken, the inputs can be thought to be partitioned into either lean tissue, consisting of muscle and bone, and the fatty tissues, consisting of adipose tissue. Lean muscle tissue is considered a desirable product whereas fat is considered an undesirable product of growth. Consumer tastes have changed in the United States, with the preference being for leaner meats with less intramuscular marbling. The perception of the harmful effects of excessive animal fat in the diet has further increased the demand for leaner meat products. This change in consumer demand, along with the increased economic expense of producing fat, has played a major role in sparking interest in the manipulation and regulation of growth in animals. Improved growth in meat animals is often associated with a concomitant increase in the production of fat. This inability to significantly increase lean muscle mass without a concomitant increase in fat indicates that further research is

required to better understand the regulation of adipose tissue accretion.

Information on adipose tissue growth and development is quite limited in economically important animals, this possibly being attributable to a general lack of knowledge of adipose tissue regulation. Excess fat accumulation is not limited to livestock animals but is very prevalent in the human population as well. Obesity is a serious human health concern in the United States and has been linked to increased risk of hypertension, psychosocial dysfunction, respiratory disease, diabetes and certain orthopedic conditions. The exact cellular basis for obesity or its implication in premature mortality are unknown, but several adipocyte-specific factors have been proposed which may be very highly correlated to genetic forms of obesity. Further identification and understanding of the role that these proteins have in obesity should provide insights into the identification, and possible treatment and prevention of the obese condition. The porcine model of genetic obesity should prove superior to the rodent models currently available. Rodent models of obesity have traditionally been used as models of human obesity, but these models are typically complicated by major defects in carbohydrate and/or lipid metabolism. The genetically lean and obese pig models are free from these aberrations and may prove valuable models for human genetic obesity.

The purpose of this research was to allow the identification of a previously undescribed, but suspected adipocyte-specific protein. Identification was needed in order to determine its possible role in adipocyte growth and development. Identification of an adipocyte-specific protein will potentially enable the monitoring of the expression of the protein in response to nutritional, physiological, managerial, and genetic manipulation. It should be possible to distinguish between transcriptional and translational levels of regulation with probes specific at the nucleic acid level. This information should allow the elucidation of possible regulatory mechanisms responsible for the expression and function of this gene product. This information should increase our understanding of the mechanisms involved with obesity and adipocyte development in both agriculturally important animals as well as humans. Research was undertaken to identify the adipocyte-specific protein in swine and to produce nucleic acid probes to the gene encoding this particular protein.

Production of a porcine cDNA library and the cloning of the gene encoding the novel adipocyte-specific protein should provide a means of identifying the protein and those factors regulating its expression. Utilization of both the monoclonal antibodies and the cDNA probes should provide a means of determining if variations in the expression in response to genetic, ontogenic or dietary factors is at the level of transcription or translation. Use of the

developed probes should allow the assessment of the possible role this protein has in adipocyte physiology.

Literature Review

Adipose tissue accretion. Growth of adipose tissue involves both hyperplasia and hypertrophy of the adipocytes (Hirsch et al., 1989). Adipocyte hypertrophy, or increase in size, appears to occur more readily than does hyperplasia (Naslun et al., 1988). During periods of excessive fat storage, the adipocytes nearly always tend to be enlarged. The possibility that hypertrophy and hyperplasia are concomitant processes cannot be ruled out, but rather the inability to observe these occurrences may be due to the technological limitations of the present methods of studying adipose tissue growth and development (Jung et al., 1978). It is believed that severe obesity cannot be accounted for solely by adipocyte hypertrophy, since adipocytes containing proportionately large amounts of fat are not found. This implies that hyperplasia must occur during periods of excessive fat storage (Hirsch and Batchelor, 1976; Faust et al., 1978). During periods of food deprivation it was shown that adipocytes release their fat stores to the point where they are no longer morphologically recognizable as adipocytes and that upon refeeding the previously filled adipocytes rapidly refill (Miller et

al., 1983). This phenomenon suggests that in vivo, fully differentiated adipocytes upon depleting their fat stores do not to any appreciable degree dedifferentiate to become precursor cells again, but rather they remain fully differentiated adipocytes lacking a fat droplet. In contrast to the belief that mature adipocytes are committed and cannot divide, it has been shown that mature adipocytes are able to dedifferentiate and divide to produce progeny that can be induced to fill with triacylglycerols under appropriate conditions in vitro (Sugihara et al., 1986; Sugihara et al., 1987). This may imply that in vivo, the adipocyte may tend to stay committed and not dedifferentiate, and that the decrease in total body fat during periods of caloric restriction most likely is the result of a decrease in cell size and to a much lesser degree a decrease in cell number.

The specific triacylglycerol-storing function of adipose tissue indicates a unique structure and physiology among the cellular components of adipose tissue. Since adipose tissue is very responsive to extracellular signals it seems likely that the unique physiological functions of adipose tissue are reflected in the enzyme and receptor composition of the cell plasma membrane. Variations in the composition of the cellular enzymes and receptors may play a key role in regulating the level of triacylglycerol storage by the adipocyte. It is also postulated that the adipocyte may be directly involved in regulating feed intake of the animal by having a direct effect on satiety. Adipocyte size

appears to be a very prevalent factor in regulating maximum food intake. In a study with Osborne-Mendel rats (Faust et al., 1977) it was found that feeding of a high-fat diet resulted in hyperphagia and an initial increase in fat cell size followed by an increase in fat cell number. The increase in fat cell number or hyperplasia was restricted to certain depots. Lipectomy of young Osborne-Mendel rats decreased the total number of mature adipocytes that the animal possessed. Upon feeding the high-fat diet, the lipectomized rats increased the size of their adipocytes more rapidly than the sham-operated controls but, interestingly, ceased the hyperphagic feeding once the adipocytes had reached the normal maximum size. The adipocytes did not overfill nor was there an increase in the occurrence of newly formed adipocytes. Once the control group's adipocytes had reached the same level of filling as the lipectomized group they too ceased their hyperphagic feeding. From these types of experiments it can be speculated that adipose tissue, upon reaching some critical size and/or number of adipocytes, may produce a signal that regulates food consumption.

Adipocyte Precursor. Continued feeding of rodents leads to an initial increase in adipocyte size followed by an increase in adipocyte number. Radioisotopic studies monitoring the incorporation of radiolabelled nucleosides have indicated that newly filled adipocytes are from a population of cells which

contained newly synthesized nuclear DNA (Miller et al., 1984). This suggests that newly filled adipocytes are the result of hyperplasia, not the filling of existing cells, and are derived from an adipogenic stem cell line. The adipocyte is thought to be derived from an adipogenic precursor cell which has a fibroblastic morphology (Hausman et al., 1980). This adipogenic precursor may be from a precommitted line of cells or may be the product of a pluripotent stem cell (Amri et al., 1986). Adipose precursor cells show varying capacities for proliferation and differentiation when obtained from various anatomical locations (Djian et al., 1983; Djian et al., 1985). These differences may be due to the varying intrinsic capacities of the cells to respond to adipogenic stimuli. The difficulty in studying the adipocyte and its precursors is due to the lack of adipocyte-specific markers. It is essentially impossible to critically study the adipocyte, since current methodology relies on the morphologically characteristic accumulation of the fat droplet by mature adipocytes. Since adipocyte precursors do not contain a unilocular fat droplet and other non-adipocyte cell types can be induced to fill with triacylglycerols, it becomes necessary to find additional adipocyte-specific characteristics or markers that can be used to identify adipocytes independent of fat droplet accumulation. A monoclonal antibody has been produced to a 64-kD porcine adipocyte-specific plasma membrane protein (Killefer and Hu, 1990a). This immunological reagent detects the protein both in

intact cells and cell membrane preparations. This monoclonal antibody has yet to be used extensively in adipocyte precursor studies, but indications are that it can recognize a minor population (< 10%) of cells derived from the adipose tissue stromal-vascular fraction which is thought to contain preadipocytes and their stem cells. The production of nucleic acid probes to adipocyte-specific messages will be beneficial to the identification of adipocyte precursors and the study of adipocyte growth and development.

Antibody studies. Immunological reagents have been used to study unique antigenic components present in the adipocyte plasma membrane. Immunosorption of polyclonal sera prepared against rat (Pillion et al., 1979; Thompson and Abraham, 1979; Lee et al., 1986), cattle (Cryer et al., 1984) and sheep (Nassar, 1989) all have suggested the presence of adipocyte-specific plasma membrane proteins. A monoclonal antibody to a unique 64-kD protein present in the porcine adipocyte plasma membrane has been produced (Killefer and Hu, 1990a). The protein recognized by LA-1 was found only in the adipocyte plasma membrane when tested by Western blot analysis. The identity and function of this adipocyte-specific protein remain unknown and should be the focus of future research. The possible role of adipocyte-specific proteins in the adipocyte's unique physiology may be supported by the in vitro and in vivo rat

studies conducted by Flint et al., (1986). In these trials, polyclonal antibodies to rat adipocytes were found to have both stimulatory and cytotoxic effects on adipose tissue. This suggests the possible importance of the adipocyte-specific proteins in the physiology of the cell.

In vitro adipocyte culture. Adipocytes and preadipocytes can be isolated from adipose tissue through enzymatic digestion of the connective tissue matrix (Rodbell, 1964) and maintained as a primary culture in vitro. Mature adipocytes are very difficult to maintain in culture due to their fragility and buoyant nature. Primary culture of preadipocytes is complicated by the lack of adequate means of separating preadipocytes from the other cell types contained in the stromal-vascular fraction, this again emphasizing the need for additional means of recognizing adipocytes and preadipocytes. The majority of the in vitro culturing of preadipocytes has been accomplished through the use of established cell lines. Established cell lines are clones of a parent cell that have been immortalized. It must be recognized that immortalized cell lines may not truly represent the original parent cell and any conclusions should be drawn with this knowledge. The principal cell line used currently is the 3T3-L1 line (Green and Kehinde, 1974) and its derivatives. Differentiation of the preadipocytes in vitro is best controlled by using chemically defined, serum-free media (Gaillard et al., 1984;

Deslex et al., 1986; Deslex et al., 1987). IGF-I, insulin, glucocorticoids, thyroid hormone, sex steroids, prostaglandins, cytokines (Chapman et al., 1985; Ignotz and Massague, 1985; Vannier et al., 1985), and other as yet unidentified serum adipogenic factors (Faust et al., 1980; Roncari et al., 1983) all play important roles in the differentiation and filling of the preadipocyte. Whole serum was found to contain what is thought to be an inhibitor of preadipocyte differentiation. Other factors present in the serum may be derived from the adipocytes themselves and act as regulators of satiety or energy balance (Cook et al., 1987; Jewell et al., 1988).

Adipocyte-specific cellular products. Adipose tissue has been found to be the site of production of various unique or highly restricted cellular products. Adipose tissue has been shown to be the principal site of synthesis of the serine protease adipsin (Cook et al., 1987; Flier et al., 1987). This protein was found to be secreted into the circulation, suggesting its possible role as an endocrine signal in the regulation of energy storage, and to be diminished in rodent models of obesity. The adipocyte has also been implicated in the production of signalling molecules influencing systemic energy balance and reproduction (Faust et al., 1977). A hybridoma line has also been produced which secretes a monoclonal antibody against a 64-kD protein present only on the adipocyte plasma membrane as determined by

immunological assays (Killefer and Hu, 1990a). It has also been speculated that the unique physiology of the adipocyte may not be based on the presence of a unique protein but rather on a unique combination of more common gene products present during different developmental stages of the adipocyte (Zezulak and Green, 1985; Dani et al., 1989). Expression of adipocyte-specific genes may be tightly regulated by adipogenic factors (Distel et al., 1988; Bernlohr et al., 1985) resulting in either up or down regulation. The presence of proteins or combinations of proteins whose expression is restricted to the adipocyte further support the notion that adipose tissue is a unique tissue having a highly specialized and regulated physiology. Regulation of expression of adipocyte-specific proteins may play a key role in regulating adipocyte growth and development. A cDNA probe to the 64-kD protein (Killefer and Hu, 1990b) has been produced from an expression library representing the expressed genes of the mature porcine adipocyte. The production of a cDNA to this unique protein and its use as a nucleic acid probe should provide a much greater level of understanding regarding the function of this particular protein and possibly the role of the adipocyte in regulating energy storage and the development of obesity.

Genetic and dietary effects on obesity. The central defect(s) responsible for the onset or manifestation of obesity are unknown. This condition is characterized by the excessive

accumulation of energy in the form of triacylglycerols in adipose tissue. It has been estimated that the average adult human body possesses approximately 30 X 10^9 adipocytes, and that the average adipocyte contains approximately 0.5 μg of fat as a unilocular droplet (Hirsch et al., 1989). From these figures it can be estimated that the average adult human contains approximately 15 kg of fat accounting for 135,000 kcal of energy (9 kcal/g of fat). Earlier studies on the effects of genetics and diet suggested the presence of various types of obesity. It had been speculated that lean and obese individuals consumed diets differing greatly in their fatty acid compositions, which may contribute to the onset of obesity. It was shown that the fatty acid profiles of lean and obese individuals were very similar and that diet had little to do with the fatty acid composition of the triacylglycerols contained in the adipocytes of either obese or lean individuals (Berry et al., 1986). This then implies that obese individuals must either consume a greater number of calories, have lower metabolic rates or both. 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 obese rats fed ad libitum (Greenwood, 1985). Very few differences could be found in the metabolisms of genetically lean

and obese pigs (Mersmann et al., 1982), suggesting the possibility that the adipocytes are different between these two distinct phenotypes. The genetic contributions to obesity are further supported by human studies utilizing monozygotic twins. It was estimated that 80 percent of the contribution to obesity could be explained by genetics (Stunkard et al., 1986) and that the amount and distribution of fat were related to the closeness of genetic relationship (Bouchard et al., 1985). Adipsin mRNA levels were shown to be dramatically reduced in genetically (ob/ob and db/db) and chemically-induced (MSG-injected mouse) rodent models of obesity. Interestingly, adipsin mRNA was not found to be significantly reduced in pure overfeeding (cafeteria-fed) forms of obesity. The possible association of adipocyte-specific proteins in obesity is supported by our findings that the 64-kD protein appears to be present in genetically lean porcine adipocytes and absent in genetically obese porcine adipocytes (Killefer and Hu, 1990b).

Genetically lean and obese pigs. The genetically lean and obese pigs provide a model, other than rodent models, to study the physiology of obesity. This porcine model of obesity was developed by Hetzer (Hetzer and Harvey, 1967). Animals were selected for either thick (obese) or thin (lean) backfat thickness only, in a purebred Duroc population and a purebred Yorkshire population for approximately eighteen and fourteen

generations respectively. These two breeds are currently maintained as lines of obese pigs produced through the crossing of the obese Duroc X obese Yorkshire pigs and of lean pigs produced through the crossing of the lean Duroc X lean Yorkshire pigs. The effect of crossbreeding is documented (Bereskin et al., 1974; Bereskin and Harvey, 1986). The only selection parameter was backfat thickness and not rate of gain; as such the genetically lean and obese animals both tend to have lower rates of gain when compared to their contemporary counterparts (Pond et al., 1980; Mersmann et al., 1982; Steele et al., 1982; Tess et al., 1984). Postweaning growth rate of the obese pigs tends to be lower than that of the lean pigs (Mersmann et al., 1982). The current crossbred line of obese pigs is fatter and less muscular at maturity than the lean pigs (Ferrell and Cornelius, 1984; Tess et al., 1984, 1986). This divergence occurs very early in development, at four to eight weeks of age (Ferrell and Cornelius, 1984), and if body fat is adjusted for body weight this divergence can be detected as early as 100 days of gestation (McNamara and Martin, 1982; Stone et al., 1985). Dietary energy restriction (Davey et al., 1969) reduced body fat in both lean and obese pigs, but the reduction of body fat tends to be greater in lean pigs when subjected to severe dietary restriction (Mersmann and Leymaster, 1984). Growing obese pigs were found not to be hyperglycemic, -triglyceridemic, -cholesterolemic, or -insulinemic (Mersmann et al., 1982) and thus the pig model of obesity is not complicated by major defects in

carbohydrate or lipid metabolism as are most rodent models. The pig model expresses obesity early without extraneous metabolic or endocrine abnormalities (York, 1985), and may serve as an excellent, less complicated model for early onset human obesity.

Future of adipocyte research. The identification of adipocyte-specific markers, either genes or gene products, should greatly increase our understanding of adipocyte growth and development. The current limitations in adipocyte research methodology necessitate the finding of such markers. Identification of true preadipocytes, and potentially their pluripotent stem cells, may not be too distant once these new tools are implemented into adipocyte research. The use of nucleic acid probes may allow for the identification of adipogenic factors and determination of their roles in the regulation of adipocyte development and physiology. Understanding of the mechanisms regulating adipocyte growth and development may make possible the development of means to control adipose tissue growth and whole animal energy balance. The ability to regulate adipose tissue growth in livestock animals and humans alike could have dramatic economic and health benefits.

Chapter 2

Production of a Novel Monoclonal Antibody to the Porcine Adipocyte Plasma Membrane

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Abstract

The adipocyte plasma membrane is composed of specific intrinsic, integral and extrinsic proteins. The study of adipocyte development, morphology and metabolism has been limited by a lack of characterization of these proteins. It seems likely that the adipocyte plasma membrane possesses adipocyte-specific proteins which may be linked to adipose tissues' unique identity. To study the composition of the adipocyte plasma membrane, we produced a panel of monoclonal antibodies to purified adipocyte plasma membranes. Nineteen anti-adipocyte hybridoma cell lines were identified using fluorescence enzyme-linked immunosorbent assay (F-ELISA), immunoblotting and indirect immunofluorescence. A monoclonal antibody (designated LA-1) with reactivity towards a porcine adipocyte plasma membrane component was used for further adipocyte characterization. LA-1 reacted with a species-specific 64-kD protein expressed in adipocyte plasma membranes but not in hepatocyte, erythrocyte, skeletal muscle, heart, spleen, kidney, stomach, small intestine, or large intestine plasma membranes. The LA-1 antibody provides a specific probe for this adipocyte surface protein marker.

Introduction

Specific compositional differences in cellular plasma membranes which may confer cellular identity remain poorly understood. Biochemical and physiological studies of adipose tissue and, in particular, adipocyte biology have been restricted by a lack of reproducible reagents which can differentiate adipocytes from other cell types. The specific triacylglycerol-storing function of adipose tissue indicates a unique structure and physiology among the cellular components of adipose tissue. It seems likely that the unique physiological functions of adipose tissue are reflected in the enzyme and receptor composition of the cell plasma membranes. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of adipocyte plasma membranes isolated from different sources has resolved at least 5 - 23 glycoprotein and glycolipid components, which range in molecular weight from 14 - 178 kD (1-6).

Immunological reagents have been used to study some antigenic components present in adipocyte plasma membranes. Polyclonal sera have been prepared against adipocytes from rats (7-9), cattle (10), and sheep (11). Following adsorption with cells isolated from non-adipose tissue, the resulting sera reacted specifically with adipocyte plasma membranes (7-12). Although these polyclonal sera have been used to identify certain adipocyte-specific proteins, they have certain limitations (e.g.,

differing isotypes, affinities and specificities, variability among sera collected at different times, etc.). Thus, the preparation of monoclonal antibodies with specific reactivity to adipocyte-specific proteins is a logical step in developing defined biological reagents for the study of adipocyte structure, metabolism and development.

We felt that a monoclonal antibody specific for the porcine adipocyte plasma membrane could be produced. This report describes the production and initial characterization of a monoclonal antibody which reacts with an adipocyte plasma membrane component of swine. Cross-reactivity of the antibody towards non-adipocyte-derived plasma membrane proteins is described. The antibody has proven useful for screening other cell types for the presence of this specific protein in fluorescence ELISA, immunoblotting procedures, and in immunofluorescence assays.

Materials and Methods

Plasma Membrane Fraction

Adipocytes were isolated by collagenase digestion according to the methods of Rodbell (13). Erythrocyte cell ghosts and liver

homogenates were prepared according to the methods of Lodish and Braell (14), and Hertzberg (15) respectively. Kidney, spleen, large intestine, small intestine, stomach, skeletal muscle, and heart homogenates were prepared according to the method of Lo et al. (16). Plasma membrane-enriched fractions were prepared using a self-forming Percoll gradient as described by Belsham et al. (5). Protein content was measured by the method of Bradford (17) using bovine serum albumin as a standard. Plasma membrane proteins were stored in sterile phosphate buffered saline (PBS).

Production of Mouse Monoclonal Antibodies

Six-week-old Balb/c mice were injected intraperitoneally (IP) with 100 μg (200 μl/mouse) of porcine dorsal subcutaneous adipocyte plasma membrane (APM) proteins emulsified in Freund's incomplete adjuvant (FIA) (Sigma Chemical Co., St. Louis, MO). A second 200 μl/mouse IP injection (20 μg APM protein in FIA) was given on day 14. On day 28 blood samples were taken from each mouse, serum collected, and anti-APM antibody production was confirmed by F-ELISA methods (see below). A final booster injection (250 μl/mouse) of 5 μg APM protein in sterile PBS was administered on day 42 by IP injection. On day 45, each mouse was sacrificed and the spleen removed using sterile techniques. Splenocytes from the hyper-immunized mice

were chemically fused with Sp 2/O-AG 14 myeloma cells using a polyethylene glycol (PEG) solution: 40% PEG 1450 (Eastman Kodak Co., Rochester, NY), 10% PEG 4000 (J.T. Baker Inc., Phillipsburg, NJ), 10% dimethyl sulphoxide (Sigma Chemical Co., St. Louis, MO), and 40% Dulbecco's modified Eagle's medium (DME) (Sigma Chemical Co., St. Louis, MO) pH 7.2 (18,19). After the fusion procedure the cells were resuspended in hypoxanthine-aminopterin thymidine (HAT) medium (20) and plated into 96-well culture plates for selection of hybridomas. The cells were maintained in HAT until macroscopic colonies were observed and the myeloma controls were dead. HAT medium was then replaced with HT (hypoxanthine thymidine) medium and finally by DME-10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT). When media in individual culture wells became acidic (yellow), those wells were tested for anti-APM antibody production by F-ELISA. Wells containing hybridomas that showed a positive response in F-ELISA were minicloned and expanded according to the limiting dilution method. Subsequently, wells which contained single hybridoma colonies were subcloned a second time.

Fluorescent Enzyme-linked Immunosorbent Assay (F-ELISA)

An F-ELISA method was developed to detect the presence of anti-APM antibodies in culture fluid and to test cross-reactivity of

the antibodies. The APM fraction was diluted in PBS to a protein concentration of 2.5 μg/ml, and 50 μl/well of this preparation was adsorbed onto the well surfaces of black 96-well Microfluor plates (Dynatech Laboratories, Inc., Alexandria, VA) by overnight incubation at 4°C. Plates were then emptied and the wells "blocked" by the addition of 5% non-fat dry milk in PBS (350 μl/well) and incubated at 37°C for 30 minutes to prevent non-specific protein binding. After the blocking step wells were washed once with PBS-Tween (PBS containing 0.05% Tween 20; Sigma Chemical Co., St. Louis, MO) with a 12-channel Mini-Fastwash manifold (3M Diagnostics, Mountain View, CA), and 50 μl of hybridoma culture fluid or mouse antiserum was added to appropriate wells. Plates were incubated at 37°C for 30 minutes, and the wells were washed four times with PBS-Tween. Each well then received 50 μl of a 1:750 dilution of alkaline phosphatase-labeled goat anti-mouse IgG (whole molecule; Sigma Chemical Co., St. Louis, MO) followed by incubation at 37°C for 30 minutes. The plates were washed four times with PBS-Tween and 50 μl/well of substrate solution added. Substrate solution consisting of 4-methyl umbelliferyl phosphate prepared at a concentration of 25.6 μg/ml in diethanolamine buffer (10% diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) was added to each well and the plate incubated at 37°C for 30 minutes. Fluorescence that developed was determined automatically in a 96-well fluorometer fitted

with 365-nm excitation and 450-nm emission filters (3M Diagnostics, Mountain View, CA).

For the cross-reactivity studies, the APM coating proteins were replaced by either hepatocyte, erythrocyte, skeletal muscle, heart, spleen, kidney, stomach, small intestine, or large intestine plasma membranes. For controls, the hybridoma culture fluid was replaced by either normal mouse antiserum (for analysis of immune sera), fresh culture medium or PBS (for the analysis of hybridoma supernatants).

Immunohistochemistry

Isolated adipocytes were examined by an indirect immunofluorescence technique with anti-adipocyte hybridoma supernatant. Subcutaneous adipocytes were isolated as described and incubated with a 1:50 dilution of the hybridoma culture fluid in PBS for 30 minutes at 37°C, and washed three times with copious volumes of 37°C PBS. The adipocytes were then incubated with a 1:100 dilution of FITC-conjugated goat anti-mouse antibody (Sigma Chemical Co., St. Louis, MO) in the dark for 30 minutes at 37°C. Specimens were observed using a Zeiss epifluorescence microscope fitted with a 35-mm camera. Controls consisted of supernatants from F-ELISA-negative hybridomas and PBS.

Immunoblotting

The APM fraction, prepared in PBS, was mixed with an equal volume of sample buffer which contained 2% sodium dodecyl sulfate (SDS), 10% glycerol, 62.5 mM Tris-HCl buffer, pH 6.8, 5% 2-ß mercaptoethanol and .002% (w/v) bromophenol blue (Sigma Chemical Co., St. Louis, MO) and subjected to electrophoresis (12.5 µg protein/lane) through a 12% polyacrylamide slab gel according to the Laemmli method (21), using a Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, CA). Protein bands were electrophoretically transferred onto a nitrocellulose sheet (Sigma Chemical Co., St. Louis, MO., 0.45 µm pore size) using a Genie Blotter (Idea Scientific Inc., Corvallis, OR) according to the method of Towbin et al. (22) as modified by the manufacturer. Blotting was performed for 45 minutes. Tissue-specific cross-reactivity studies compared porcine hepatocyte, erythrocyte, skeletal muscle, heart, spleen, kidney, stomach, small intestine, and large intestine plasma membranes. Interspecies cross-reactivity studies compared chicken (abdominal), sheep (sternal), rat (inguinal), and porcine (backfat) APM. Samples were prepared as described above.

Nitrocellulose filters were "blocked" with 5% non-fat dry milk (NFDM) in PBS for 1 hour at 37°C using continuous shaking (all subsequent incubations were at 37°C with continuous shaking). Blocked filters were incubated with a 1:100 dilution of

hybridoma supernatant in 0.1% NFDM (in PBS) for 30 minutes and washed three times, five minutes per wash, with 0.1% NFDM in PBS. Filters were then incubated for 30 minutes with a 1:750 dilution of alkaline phosphatase-labelled goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) followed by three five-minute washes in PBS (no NFDM). The filters were exposed to the Vectastain ABC alkaline phosphatase substrate buffer (2 drops each of reagents 1, 2 and 3 into 10 ml of 100 mM Tris-HCl, pH 9.5; Vector Laboratories, Inc., Burlingame, CA) until color formation was detected (approximately 5-10 minutes). The reaction was stopped by rinsing the filters with copious volumes of distilled, deionized water. In some experiments, prestained molecular weight standards (Sigma Chemical Co., St. Louis, MO), concomitantly electrophoresed and transferred, were used as references. Corresponding gels were also stained with 0.1% Coomassie Blue R-250 in fixative (40% methanol, 10% acetic acid) and destained with 40% methanol/10% acetic acid to remove background staining.

Sub-isotype Determination of the Monoclonal Antibodies

Sub-isotyping of the monoclonal antibodies was performed using an ELISA kit (Miles Laboratories, Elkhart, IN). The manufacturer's instructions were followed except that the

fluorescence substrate was substituted in the final step of the assay. The sub-isotypes were tested for reaction to antisera specific for IgG1, IgG2, IgG2a, IgG2b, IgG3, IgM and IgA.

Results and Discussion

Nineteen hybridoma culture supernatants were identified which contained anti-APM monoclonal antibodies. Each of the 19 colonies was expanded and subsequently stored frozen in liquid nitrogen. Cross-reactivity studies using F-ELISA detected differences between the assayed tissue types, suggesting the presence of antigenically unique, adipocyte-specific cell surface proteins (Figure 1.1). A monoclonal antibody line, designated LA-1, was identified which distinguished adipocytes from the various tissue types assayed (Figures 2 & 3) on the basis of the presence or absence of a particular protein. Sub-isotyping sera indicated that LA-1 was of the IgM isotype.

Subcutaneous backfat (original antigen source) expressed the antigenic protein recognized by LA-1. The nine physiologically distinct tissue types tested did not express the protein (Figure 1.3), even when all sources were adjusted to equivalent, or doubled, protein concentrations. LA-1 recognized the APM protein only from porcine and not rat, sheep or chicken sources (Figure 1.4). Control sera did not react with this protein.

The antigen identified by LA-1 has an apparent molecular weight of 64,000 Daltons (64-kD), as determined by comparison to SDS-PAGE molecular weight protein markers (Figure 1.3). The true molecular weight of the protein recognized by LA-1 may not be as indicated by SDS-PAGE due to the possible interference of migration patterns by carbohydrate moieties. Comparisons of protein profiles in Coomassie Blue-stained SDS-PAGE gels (Figure 1.2) indicated numerous differences in plasma membrane protein composition among various porcine tissues.

LA-1 also bound intact, isolated adipocytes, as shown by immunofluorescence (Figure 1.5). Both isotype matched and FITC-conjugated goat anti-mouse antibody, singly or combined, failed to stain any adipocytes (data not shown). The observed fluorescence was uniform over the surface of the cells. LA-1 also reacted with adipocyte cell ghosts and plasma membrane fragments as judged by immunofluorescence.

In this work, we sought to develop a specific and reproducible biological reagent which would enable us to identify unique adipocyte plasma membrane proteins. To accomplish this, we produced monoclonal antibodies against purified adipocyte plasma membranes. One particular hybridoma was selected (designated LA-1) and partially characterized. The LA-1 antibody was shown to be of the IgM isotype and was produced in relatively high quantities by this hybridoma.

To screen for the presence of a unique protein in the adipocyte plasma membrane we developed an F-ELISA method. The F-ELISA data (Figure 1.1), indicated that this protein was expressed by adipocytes but not by the nine other physiologically distinct tissue types. Immunoblots of these tissue proteins probed by LA-1 (Figure 1.3) confirmed these predictions. The antigen identified by LA-1 has a molecular weight of approximately 64 kD under reducing conditions, as determined by SDS-PAGE and immunoblot analysis (Figures 2 & 3). Only mature adipocytes expressed the protein identified by LA-1. Adipocytes from dorsal and ventral subcutaneous, as well as perirenal depots contained the 64-kD protein when immunoblotted with LA-1 (data not shown). Coomassie Blue stained SDS-PAGE gels (Figure 1.2) emphasized the disparity in plasma membrane composition between the various tissue types.

Intact, isolated adipocytes were also readily labelled by LA-1 as indicated by indirect immunofluorescence (Figure 1.5). Uniform staining of intact adipocytes along with the intensity and size of the recognized bands in the immunoblots suggests a relative abundance of this unique protein in the adipocyte. Presence of the antigen in both intact adipocytes and plasma membranes suggests that the protein is either extrinsic or integral. Neither presence of the protein in the intracellular pool nor its physiological function has yet been determined, but both will be subjects of future studies.

Species-specific polypeptides on the adipocyte plasma membrane have been demonstrated by polyclonal antibodies (6,9,10). These species-specific antigens had molecular weights of 124 kD, 92 kD and 59 kD for the rat, 87 kD for cattle, and 56 kD, 47 kD and 37 kD for the chicken. None of the antigens was cross-immunoprecipitated by non-homologous antibodies. LA-1 also shows apparent species-specificity due to its lack of cross-reactivity with adipocyte plasma membranes isolated from rat, sheep and chicken (Figure 1.4). The mono-specificity and reproducibility of LA-1, should prove useful for more detailed analyses of adipose tissue development and physiology.

The cross-reactivity assessments of LA-1 to other tissue types were chosen to be representative of the cellular diversity present in the body. The indications are that LA-1 recognizes a protein whose expression may be restricted to adipogenic cell types. Molecular characterization of this protein should provide further information as to the basis for the cell-type restriction of this protein.

We have produced a monoclonal antibody that identifies a protein unique to the porcine adipocyte plasma membrane. Monoclonal antibodies such as LA-1 provide defined and reproducible reagents for the study of cellular composition, and the physiological roles that these particular proteins have in the identity of their respective cell types. The protein, recognized as unique to adipocytes by LA-1, must be studied further to assess

its possible role as a targeting protein (receptor) or its functional role as a tissue specific trans-membrane carrier.

Acknowledgements

We wish to thank Dr. G. M. Banowetz for his expert assistance in the production of the monoclonal antibodies and review of this manuscript. Supported in part by the NIH Biomedical Research Support Grant RR07079 and Agricultural Research Foundation Grant 3208. Tech. paper no. 8775, Oregon State Exp. Station.

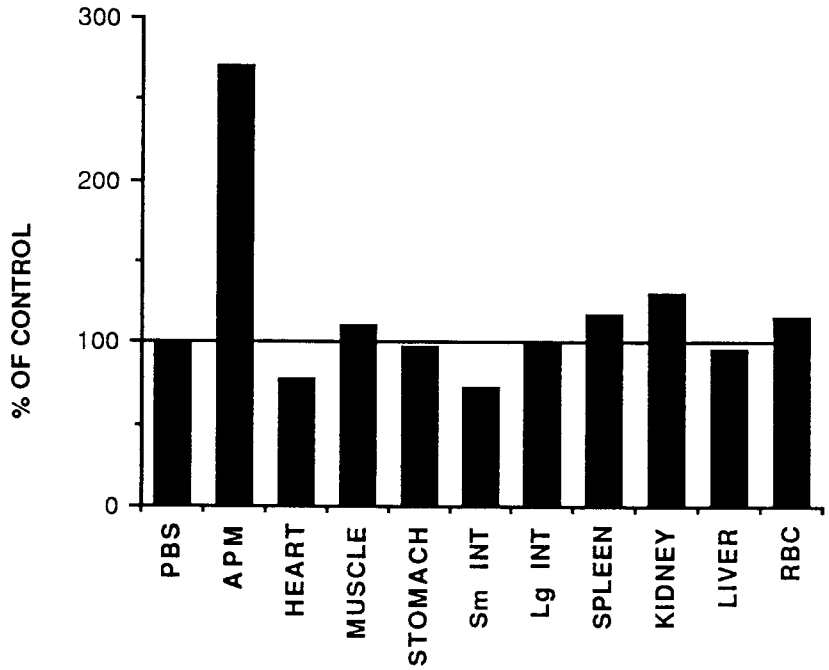

FIGURE 1.1. F-ELISA data comparing the relative cross-reactivities of LA-1 to the plasma membranes of ten physiologically distinct tissue types (adipocyte, heart, skeletal muscle, stomach, small intestine, large intestine, spleen, kidney, liver, and erythrocyte). The relative cross-reactivities are shown as percent of control (background) values. The control level of fluorescence is represented as 100%.

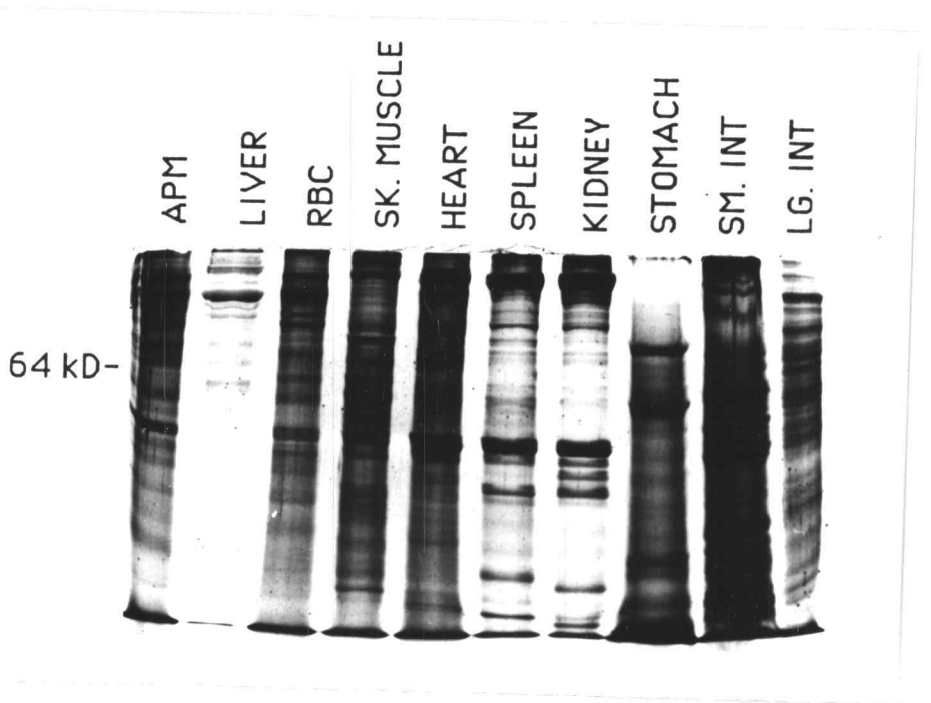

FIGURE 1.2. Coomassie Blue R-250-stained SDS-PAGE of subcutaneous adipocyte, hepatocyte, erythrocyte, skeletal muscle, heart, spleen, kidney, stomach, small intestine, and large intestine plasma membranes (12.5 μg of protein per lane) run on a 4% stacking, 12% separating gel at 200 V, 4°C for 45 minutes.

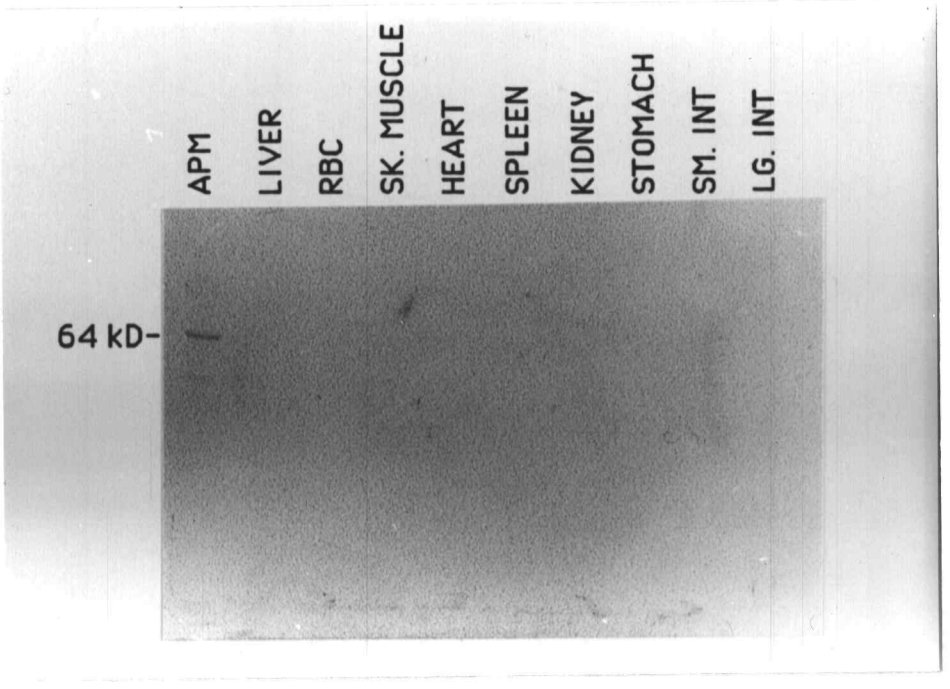

FIGURE 1.3. Western blot of subcutaneous adipocyte, hepatocyte, erythrocyte, skeletal muscle, heart, spleen, kidney, stomach, small intestine, and large intestine plasma membranes immunoblotted with LA-1. Protein amounts of 12.5 μg/lane were loaded onto a 12% SDS-PAGE gel, separated and transferred to nitrocellulose.

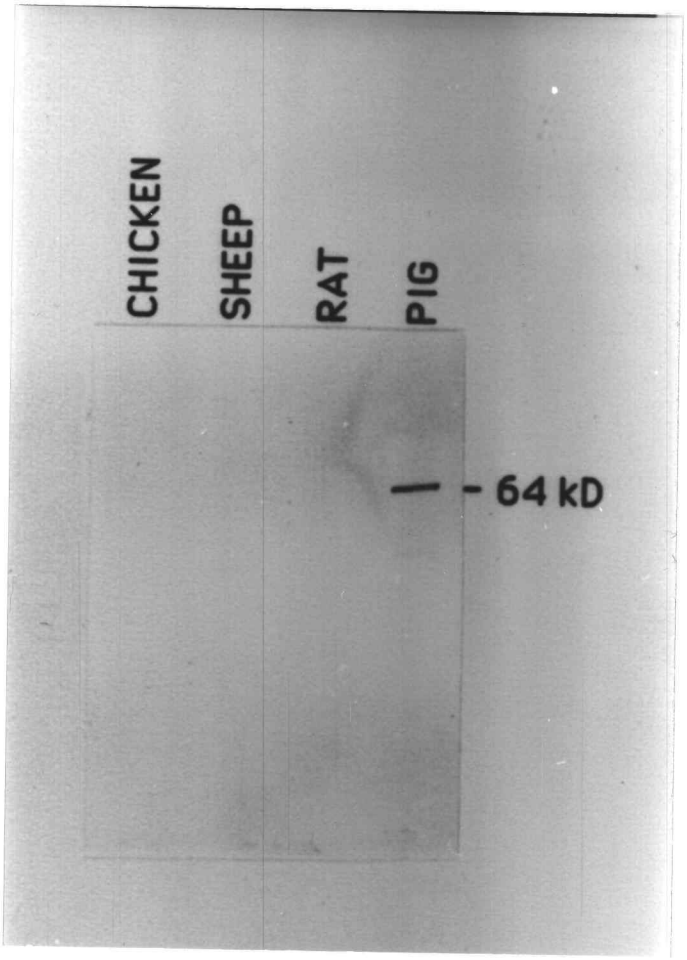

FIGURE 1.4. Western blot of subcutaneous APM proteins isolated from chicken, sheep, rat and pig. Protein amounts of 5 μg/lane were loaded onto a 12% SDS-PAGE gel, separated, transferred to nitrocellulose and immunoblotted with LA-1.

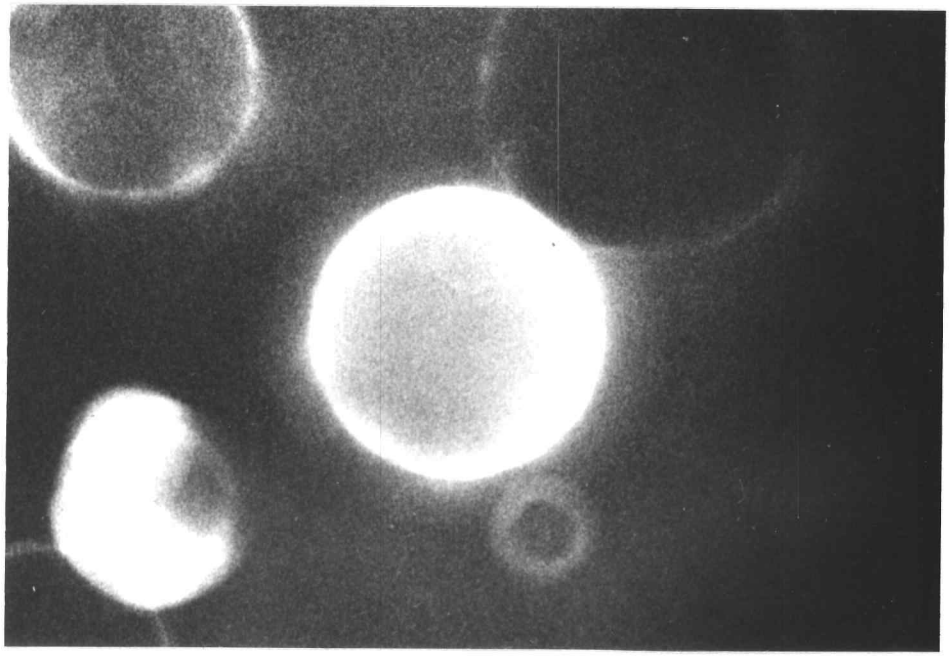

FIGURE 1.5. Indirect Immunofluorescence of isolated subcutaneous adipocytes by LA-1 showing a uniform staining pattern consistent with an even distribution of the antigen. The results indicate that the protein is exposed to the extracellular environment. Magnification X200.

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Chapter 3

Expression of a 64-kD Adipocyte-specific Plasma Membrane Protein in Genetically Lean But Not Obese Porcine Adipocytes

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Abstract

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 backgrounds. 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 glycosylated, if at all. 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.

Introduction

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 or defective thermogenesis (1).

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 obese rats fed ad libitum (4). The genetic contributions to obesity are further supported by human studies utilizing monozygotic twins. It was estimated that 80 percent 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). We hypothesized that genetically lean and genetically obese adipocytes differed in their expression of the adipocyte-specific protein. This unique protein was found to be 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 (Oregon State University reference herd) 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 using the 5'-nucleotidase assay (13). No difference in purity could be detected among the strains of pigs (obese, 24.26 $\pm$ 0.56; lean, 21.72 $\pm$ 0.36 μM of inorganic phosphate released/μg/hr). 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 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 using 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 using a goat-antimouse alkaline phosphatase conjugate.

Deglycosylation

Hydrolysis of contemporary APM proteins was performed using endoglycosidase-F (Endo-F), (Sigma Chemical Co., St. Louis, MO). The Endo-F reactions were conducted at 37°C for 24 hours under the following conditions: 0.25 M sodium acetate (pH 5.0), 200 milliunits enzyme, 20 mM EDTA, 10 mM ß-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 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 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 2.1 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 using adipose tissue homogenate preparations gave similar results (data not shown). Thus, results shown in Figure 2.1 imply the possibility for 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 intensely 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 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 (Figure 2.2). 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 (Figure 2.3). 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 levels of the protein in its APM, but these levels were still detectable by immunoblotting (Figure 2.3).

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 (Figure 2.4). 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 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 due to the unavailability of adequate serum samples.

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. We found that isolated adipocytes from the genetically lean pig were stained by LA-1 (Figure 2.5) as were isolated adipocytes from the contemporary pig (9). Interestingly, LA-1 was unable to stain any of the isolated genetically obese adipocytes (Figure 2.5), and the number and intensity of cells stained from the genetically lean source was much lower than that stained from the contemporary source. Damage to the adipocyte plasma membrane during collagenase digestion may be an 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.

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 has 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.

Acknowledgements

We thank Dr. H.J. Mersmann for providing the genetically lean and obese adipose tissue. Research was supported in part by the NIH Biomedical Research Support Grant RR07079, the Agricultural Research Foundation Grant 3208, and the USDA, ARS Cooperative Agreement 58-6125-7-19. Tech. paper no. 8926, Oregon Agricultural Experiment Station.

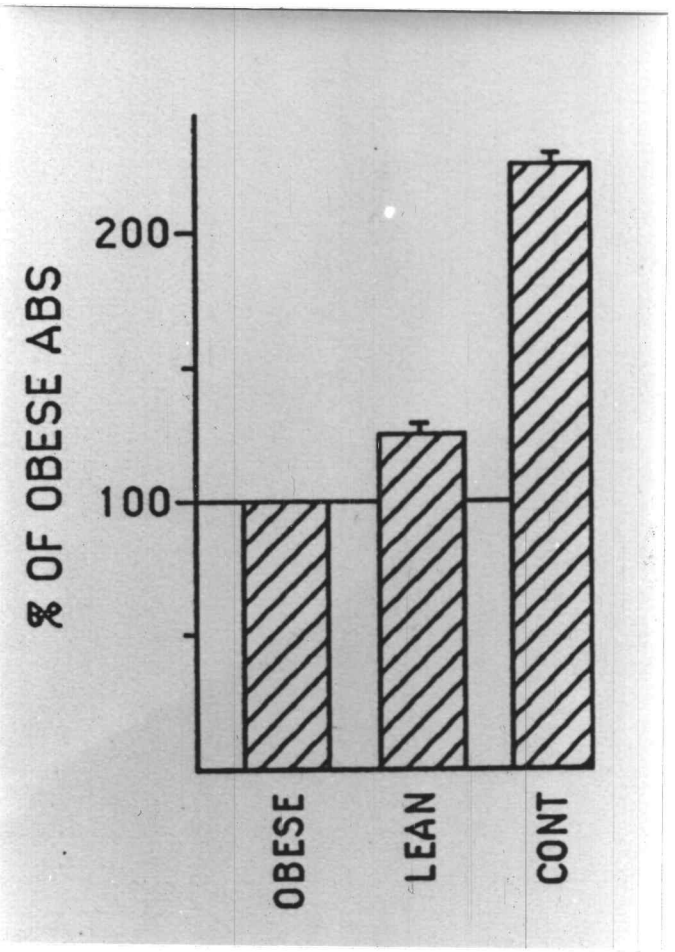

Fig. 2.1. Enzyme-linked immunosorbent assay data showing the relative levels of binding (absorbance) of LA-1 to contemporary (CONT), genetically lean, and genetically obese APM. Each well of the microtiter plate was coated with 1 μg APM overnight. The data are expressed as percent absorbance of obese APM (100%, O.D.= 0.20).

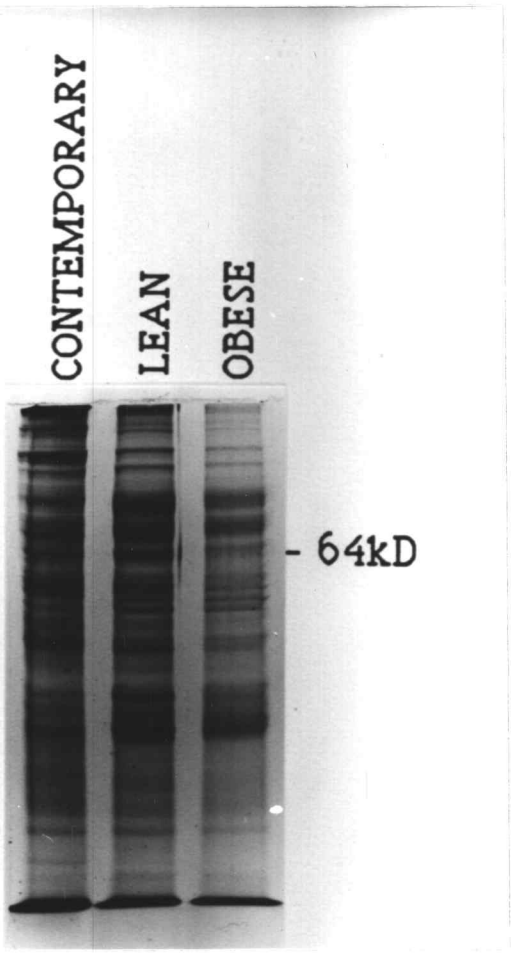

Fig. 2.2. Coomassie Brilliant Blue R-250 (CBB)-stained SDS-PAGE of contemporary, genetically lean, and genetically obese APMs. 12.5 μg/lane run on a 4% stacking, 12% separating gel at 200 V, 4°C for 45 minutes. No apparent differences in the protein banding patterns were noticed among the three tissue sources.

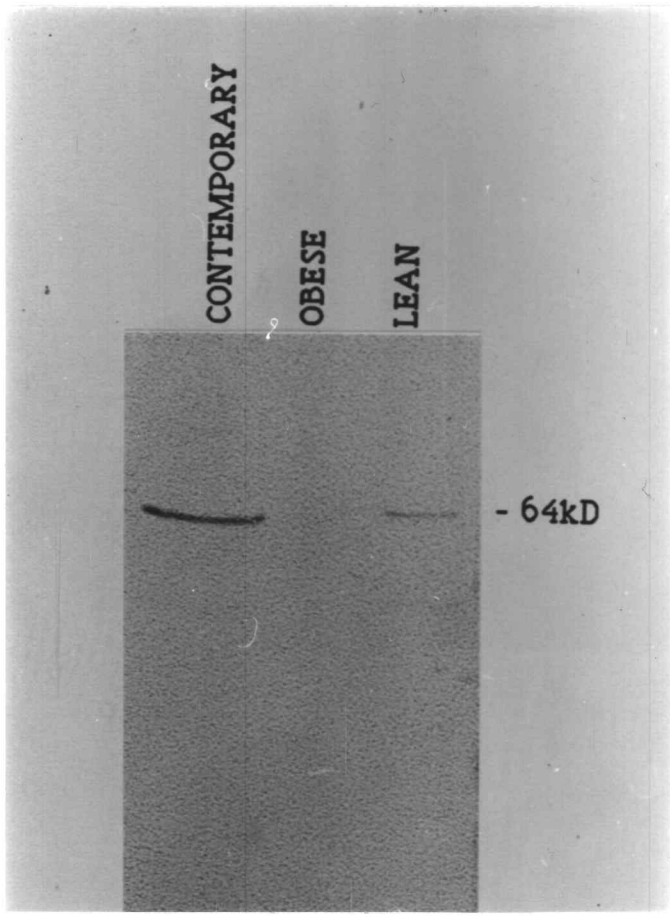

Fig. 2.3. Western blot of contemporary, genetically lean, and genetically obese APM immunoblotted with LA-1. Protein concentrations of 12.5 µg/lane were used for contemporary and genetically lean APMs, and 25 µg/lane was used for the genetically obese APM. Only the contemporary and genetically lean APM contained the protein recognized by LA-1. The genetically obese APM failed to show the protein despite a doubling of the total protein content.

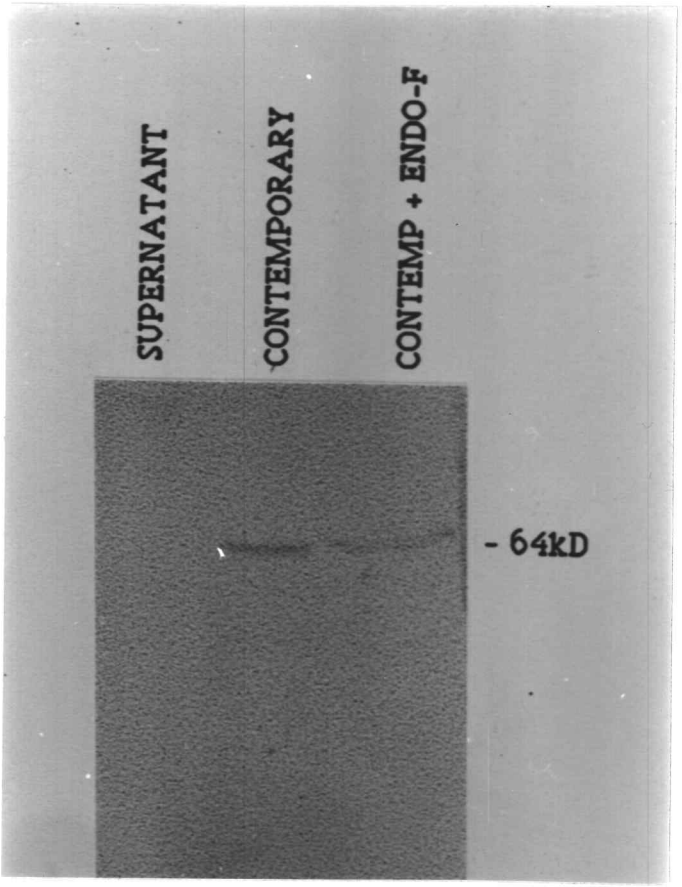

Fig. 2.4. Effects of enzymatic deglycosylation of contemporary APM by endoglycosidase-F. Contemporary APM was digested with Endo-F and the APM pellet and supernatant were subjected to SDS-PAGE and immunoblotting with LA-1. The digested materials were compared to the control (undigested) contemporary APM.

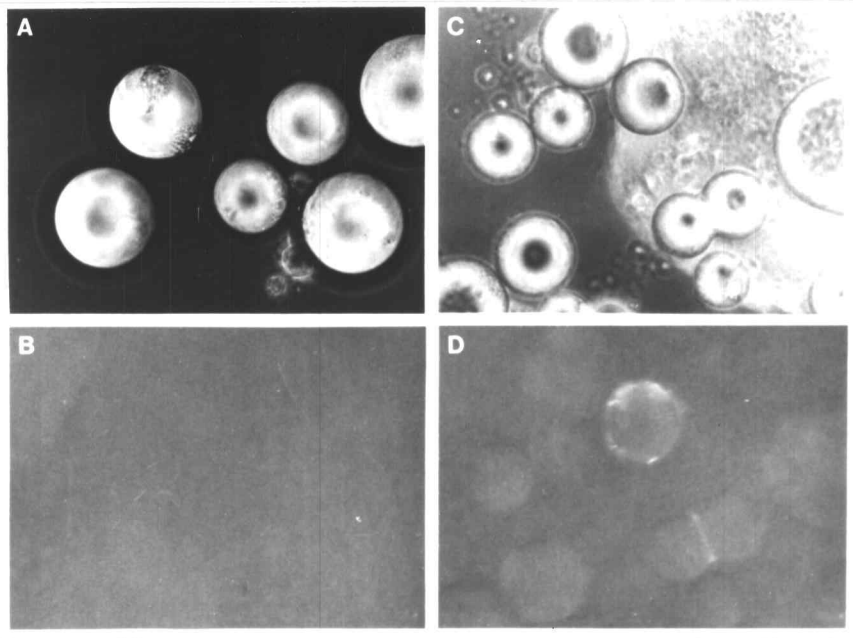

Fig. 2.5. Photomicrographs of isolated porcine adipocytes from genetically lean and genetically obese subcutaneous dorsal fat depots. All cells were isolated from adipose tissue by collagenase digestion and incubated with LA-1 (1:100) and then goat-antimouse FITC conjugated second antibody (1:20). (A) 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 X250.

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Chapter 4

Cloning of a cDNA Encoding an Adipocyte-specific Protein

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Abstract

The specific derivation of adipocytes and the factors that regulate adipogenesis are unknown. Studies seeking to identify the origins of the adipocyte and the factors which regulate their development are complicated by the lack of techniques which can clearly identify the adipocyte and its precursors. Many cell types can be clearly identified by the presence of cell-specific proteins, but the presence of such markers for the adipocyte have not been investigated. Presently the most accepted method for detecting adipocyte (adipocyte-like) cells is the morphological characteristic of intracellular triacylglycerol accumulation. The problem associated with this technique stems from the ability of other cell types to fill with triacylglycerols when placed under appropriate conditions. Not until recently have adipocyte-specific proteins been identified. These particular proteins or the mRNAs which encode them may prove to be important markers for identifying adipocytes and their precursors. This study describes the cloning of the putative cDNA encoding the 64-kD porcine adipocyte-specific plasma membrane protein and the identification of a 5 kbp and a 7 kbp species of poly (A+) RNA derived from porcine adipose tissue.

Introduction

Adipocytes are characterized by their unique triacylglycerol storage function and can be recognized through histological staining of the lipid droplet or enzymatic characterization of the cell (2,10). Current technology is limited by the inability of these techniques to (i) identify adipocyte precursor cells which do not contain the characteristic lipid droplet (9) and (ii) distinguish true adipocytes from morphologically similar adipocyte-like cells. Although G3PDH and LPL are extensively used as markers in adipose tissue development studies, these enzymes are not unique to adipocytes. Consequently, these markers can not be used to distinguish adipocytes from other cell types. White adipose tissue cells have distinct characteristics which distinguish this tissue from other cell types: (i) possession of enzymes required for triacylglycerol synthesis, (ii) ability to store triacylglycerols, (iii) presence of receptors and pathways to allow hormonally-regulated hydrolysis and release of stored triacylglycerols. It is the unique combination of these characteristics which lend the specialized physiology to the adipocyte. The complex nature of these processes and the fact that other cell types may contain some of these characteristics (5,14) make the identification of a single trait, unique to the adipocyte, an attractive goal. No enzyme of triacylglycerol metabolism has been found to be totally restricted to adipocytes (16). Although adipose tissue has

recently been found to be the primary site of synthesis of the serine protease adipsin (6), adipsin has been found in sciatic nerve tissue and in the bloodstream, where it is postulated to function as a regulatory signal produced by adipose tissue. Since adipsin (i) is not exclusively restricted to adipocytes and (ii) is a secreted peptide, it should prove quite difficult to use assays designed to detect the presence of the protein to identify adipocytes. Northern blot analysis of porcine adipose tissue RNA failed to indicate the presence of adipsin in the pig, whereas rat adipose tissue RNA samples confirmed the presence of adipsin in the rat. We have identified a novel 64-kD protein (12) which appears to be restricted to porcine adipocytes and can serve as an immunological marker for identifying mature adipocytes. Similar to adipsin (7), expression of the 64-kD protein (13) appears to be impaired in models of obesity.

The porcine adipocyte has proven to be an underutilized cell type for the production of cDNA probes. There are no probes produced from porcine adipose tissue currently available, although one such probe to porcine adipose tissue fatty acid synthase has purportedly been produced but has not yet been made available (S. Clarke, personal communication). Adipose tissue from other species has been used for the production of cDNA probes, but they have not been found to cross hybridize with porcine mRNA. In particular, goose and rat fatty acid synthase cDNA probes were found not to bind to RNA isolated

from porcine tissues (3). The unavailability and potential usefulness of porcine adipocyte cDNA probes necessitates their production. This study describes the production of a porcine adipocyte cDNA library utilizing an expression system to allow identification of a probe to the 64-kD porcine adipocyte-specific plasma membrane protein.

Materials and Methods

Adipose Tissue

Animals consisted of two 40-kg castrate male contemporary pigs fed a standard corn-soy bean meal diet from the Oregon State University herd. Adipose tissue was obtained through surgical biopsy of one-gram samples of dorsal subcutaneous fat (13). The samples were wrapped in foil and immediately frozen in liquid nitrogen. The samples were transported to the laboratory and stored at -80°C until processed.

Isolation of total RNA

Total porcine adipocyte cellular RNA was isolated according to the method of Chomczynski and Sacchi (4) with minor modifications. Briefly, 0.5 gram of adipose tissue, free of skin and muscle, was wrapped in foil and cooled in liquid nitrogen. The foil wrapped tissue was then powdered by crushing with a pre-chilled pestle. The powdered adipose tissue was then transferred into 10 ml of ice cold denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, ph 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). The sample was immediately homogenized on ice using a Brinkmann Polytron set at power level 7 for 30 seconds. The following solutions were added to the homogenized sample with vigorous shaking after each addition: (a) 1 ml of 2 M sodium acetate, pH 4.0, (b) 10 ml phenol (DEPC-water saturated), (c) 2 ml of a 49:1 chloroform : isoamyl alcohol mixture. After the addition of all reagents the mixture was shaken vigorously for 15 seconds and then placed on ice for 15 minutes. The sample was transferred to a 50-ml polypropylene centrifuge tube and the sample separated at 12,000 X g for 15 minutes at 4°C. The aqueous phase was transferred to a clean 50-ml polypropylene centrifuge tube and 10 ml isopropanol (equal volume) was added. Contents were mixed well, and the RNA was allowed to precipitate for 1 hour at -20°C. The RNA was pelleted by centrifugation at 12,000 X g for 15 minutes at 4°C. The total RNA pellet was

washed twice with ice-cold 75% ethanol and resuspended in sterile DEPC-water. The total RNA was quantified by reading its absorbance at 260 nm and stored at -80°C.

Isolation of Poly (A+) RNA

Poly (A+) RNA was isolated from porcine adipocyte total RNA by oligo (dT)-cellulose chromatography as described by Aviv and Leder (1). Briefly, 5 mg of denatured total porcine adipocyte RNA was passed over a 1-ml oligo (dT)-cellulose column. The poly (A+) RNA was eluted from the column and run over a second fresh oligo (dT)-cellulose column. To the eluate 0.1 volume 3 M sodium acetate and 2.2 volumes ethanol were added, and the poly (A+) RNA was allowed to precipitate overnight at -20°C. The poly (A+) RNA was quantified and stored as described above.

Production of the porcine adipocyte expression cDNA library

A cDNA library representing the porcine adipocyte poly (A+) RNA was constructed using 5 μg of poly (A+) RNA and the ZAP II cloning vector (15) as described by the manufacturer (Stratagene, Inc., La Jolla, CA). The first strand was synthesized with Moloney-

Murine Leukemia Virus Reverse Transcriptase using the poly (A+) RNA as the template and a poly (T)-containing primer:

5'....GAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTT3'

Xho I poly (dT)

The GAGA region protects the Xho I restriction site which is used in the unidirectional cloning of the finished double stranded cDNA into the vector. The first strand was synthesized in the presence of dATP, dGTP, dTTP and 5-methyl dCTP. The presence of the methylated dCTP ensured that the resulting cDNA was protected from cleavage by the restriction enzymes used in the subsequent cloning procedure. The second strand was synthesized using DNA polymerase I, and the ends of the cDNA were either filled in or nibbled back with T4 DNA polymerase. EcoR I adaptors having the following structure were added to the blunt termini:

5'AATTCGGCACGAG3'

3' GCCGTGCTC5'

EcoR I

The adaptor has a 9-mer and a 13-mer set of complementary strands. The 9-mer was kinased to allow its ligation to the cDNA EcoR I ends, whereas the 13-mer was dephosphorylated to inhibit it from ligating to the other cohesive ends. After ligation was completed and ligase removed, the 13-mer was kinased to make this EcoR I site available for ligation. At this point the cDNA containing the linker arms was restriction enzyme-digested with

Xho I to produce the Xho I cohesive end. The small linker fragment and the cDNA were size-fractionated on a Sepharose CL-4B column and the larger cDNA population pooled and precipitated. The cDNA was unidirectionally ligated into the ZAP XR vector arms and packaged in vitro. The library was amplified in the PLK-F' (mcrA-, mcrB-) host strain to allow the DNA to be replicated without digestion of the hemi-methylated DNA. Once the library was passed through PLK-F', it was no longer hemi-methylated and other host strains could be utilized. At this point a cDNA library representing contemporary porcine adipose tissue poly (A+) RNA had been constructed. The next process was to screen the library to identify the clone(s) representing the 64-kD protein.

Recombinant phage library screening

The amplified recombinant phagemid library was used to infect the XL1-Blue host strain. In the presence of IPTG/X-gal, blue plaques were produced with nonrecombinants and white plaques with recombinants due to cloning of the insert cDNA into the lacZ gene of the recombinant phagemid. This cloning system allows for both immunological and nucleic acid screening of recombinants. The Zap II cloning vector has promoter sequences (e.g., the ß-galactosidase promoter) that are functional in XL1-Blue

and are positioned to allow the transcription of cDNA insert sequences that are expressed as fusion proteins. The presence of appropriate fusion proteins were detected immunologically using LA-1 (12). XL1-Blue allows the determination of the ratio of recombinants to nonrecombinants and the immunological screening of recombinant phagemids for expression of fusion proteins.

Recombinant phagemid (3 X 10^4 pfu) were combined with 600 µl of XL1-Blue cells (O.D. 600 = 0.5) and incubated for 15 minutes at 37°C. Following the incubation, 7.5 ml of top agar was added to the cell/phagemid mixture. The top agar was then poured onto a prewarmed LB plate (150 mm), allowed to harden and incubated at 42°C until the plaques just became visible (3 hours). Phage were transferred to IPTG treated nitrocellulose filters for 4 hours at 37°C (to allow induction of the fusion protein) and then removed and washed 4 X 15 minutes with TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) at room temperature.

The filters were incubated for 1 hour at room temperature in 50 ml of blocking solution (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.0% BSA) to block any remaining protein binding sites. The filter was transferred to 10 ml fresh blocking solution containing 100 µl of LA-1 and allowed to incubate for 1 hour at room temperature. After the incubation in antibody solution the filter was washed 4 X 5 minutes in TBST. The filter was then incubated

for 1 hour at 37°C with an anti-mouse alkaline phosphatase conjugate (diluted 1:5000 in fresh blocking solution) to detect the presence of LA-1 reacting with its appropriate fusion protein. The filter was again washed 4 X 5 minutes in TBST followed by a final wash in TBS to remove residual Tween-20. Immunodetection was performed using 20 ml of fresh developing solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 6 mg nitro blue tetrazolium, 3 mg 5-bromo-4-chloro-3-indoyl phosphate). The filter was incubated in the dark until the desired spots developed (usually within 1 hour). The developed filter was then rinsed with TBS and color development stopped by immersing the filter in a stop solution (20 mM Tris-HCl pH 2.9, 1 mM EDTA). The filter could then be stored dry in the dark.

Positive plaques were cored from the original plate and the phagemid particles eluted into SM buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$). These eluates were retitered and rescreened two additional times to ensure clonality.

Preparation of the cDNA probe

The ZAP II cloning vector was designed to allow in vivo excision and recircularization of any piece of DNA cloned into the lambda vector to form the pBluescript phagemid containing the insert. This subcloning step occurs during the simultaneous

infection of the host cell (XL-1 Blue, 200 µl of O.D.600 = 1.0) by the lambda vector (200 µl containing approximately 1 X 10^6 pfu) and f1 bacteriophage R408 (1 µl containing >1 X 10^6 pfu/ml) which creates a circularized phagemid that does not contain the sequences normally associated with lambda and has a functional f1 origin as is found in bacteriophage. The pBluescript containing the insert cDNA was packaged and used to infect new XL1-Blue host cells for the production of double-stranded phagemids. Phagemid DNA was purified using the rapid boiling mini prep procedure (11) and used for restriction enzyme mapping to determine the insert size.

The size of the contained inserts was determined by restriction enzyme mapping of the double-stranded DNA purified from the colonies after excision. Double stranded DNA from five putative 64-kD protein colonies that immunoreacted with LA-1 were digested with EcoR I, Xho I or both to determine the size of the contained insert and analyzed on a 1% agarose gel. Insert size was determined to range from 1300 b.p. to 1500 b.p., with no variation between clones containing the larger insert. The DNA was linearized with Xho I for the production of probes.

Linearized DNA representing the larger insert was labelled with digoxigenin-11-dUTP (dgg) according to the methods recommended in the GENIUS kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The dgg-labelled probe was used to probe Northern blots of porcine adipocyte total RNA and poly

(A+) RNA. RNA was prepared as described earlier and electrophoretically separated on a horizontal, 1.2% agarose gel (1.2% agarose, 0.66 M formaldehyde, 1X MOPS, 10 μg ethidium bromide). After electrophoretic separation the gel was photographed, and the RNA was transferred to MagnaGraph membrane (Micron Separations Inc.) by 16-hour capillary transfer in 10X SSC. When the transfer was complete the membrane was baked for two hours at 80°C to permanently immobilize the RNA to the support. Northern blot analysis was performed according to the GENIUS kit with the following modifications: Prehybridization was performed for 5 hours at 37°C using a modified hybridization solution consisting of 5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 50 mM Tris-HCl pH 8.0, 50% deionized formamide, 5% blocking agent. The prehybridization solution was replaced with 5 ml fresh hybridization buffer containing 250 ng of freshly denatured dgg-labelled probe. Hybridization proceeded overnight at 37°C. All reagents, except for the final color development buffer (buffer 3) were treated with 0.1% diethyl pyrocarbonate and allowed to stand at 68°C overnight prior to use.

Results and Discussion

Isolation of porcine adipocyte RNA

Total RNA was isolated from fresh contemporary porcine adipocyte biopsy samples. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (4) produced yields of approximately 500 μg total RNA/gram adipose tissue. The products appeared to be quite free of contamination when the 260 nm : 280 nm absorbance ratios were compared, routinely giving values of ≥1.85. The method was modified to process only one-half the amount of tissue as compared to the method described in the literature. This condition was tested and selected to increase the yield and quality of total RNA isolated from adipose tissue. This modification was dictated by the nature of the tissue being processed. Adipose tissue has a very high content of hydrophobic components (e.g., triacylglycerols), which may possibly have a saturating effect on the phenol and chloroform if used in the original ratios. Total RNA samples were also prepared from genetically lean and obese porcine adipose tissue using the described methods. The yields of total RNA for lean (100 μg/gram) and obese (20 μg/gram) were quite low and were most likely due to the age and quality of the frozen samples. It was suspected that much of the RNA had been

degraded and that which was left was of low quality. The isolation of poly (A+) RNA by oligo (dT)-chromatography of contemporary porcine total RNA resulted in approximately a 1% recovery with a 260 nm : 280 nm ratio of approximately 2.0. The 1% recovery represents the proportion of poly (A+) RNA to the total RNA of the original sample. This poly (A+) RNA was used for the production of a contemporary porcine adipocyte cDNA library.

Production of a porcine adipocyte cDNA library

In order to further identify and characterize the adipocyte-specific 64-kD membrane protein and the factors regulating its expression requires the detailed study of the gene encoding the protein. Toward this end we have constructed a porcine adipocyte cDNA library from adipose tissue expressing the 64-kD protein. This cDNA library contained approximately 2 X 10^6 recombinants with less than 1% of the library as non-recombinants as determined by white/blue color selection on IPTG/X-gal (Figure 3.1). Amplification of the library resulted in a titer of approximately 10^9 pfu/ml.

Immunoscreening of the cDNA library

The cDNA library was screened with LA-1 (12), a monoclonal antibody produced against a unique 64 kD porcine adipocyte plasma membrane protein. Immunodetection was performed using the alkaline phosphatase, picoBLUE screening kit supplied by the manufacturer (Stratagene Inc., La Jolla, CA). The insert cDNA was cloned into the vector in such a manner that its expression was under the control of the lacZ promoter found in ZAP II. Expression of the insert cDNA results in the production of a fusion protein (insert protein + ß-galactosidase) which can be detected by a primary antibody directed against it. Transcription from the lacZ promoter is under tight control and must be induced by the presence of IPTG. This ability to regulate lacZ expression and thus expression of the insert cDNA makes it possible to avoid problems of lethality which often results when foreign genes are expressed. The result is that the foreign cloned genes are only expressed under specific conditions. Approximately 5 X 10^5 plaques were screened with LA-1 ultimately resulting in 6 clones thought to carry the putative gene for the 64 kD protein. Elution of the phagemids and rescreening for a total of three rounds continued to give positive plaques (Figure 3.2).

Determination of insert cDNA size

It was estimated that an insert of approximately 1.5 kbp would be required to encode a protein of 64 kD. To determine the size of the cloned inserts the phagemids were first rescued as single stranded phage and used to infect new XL1-Blue host cells where they were converted to double stranded pBluescript. Selection for host cells infected by pBluescript was performed on LB/ampicillin plates, since ampicillin resistance is provided by pBluescript. Bacteria that were not transformed by pBluescript or were infected by the R408 helper phage alone, used in single stranded phagemid rescue, do not possess ampicillin resistance, since the ampicillin resistance genes are present only on the pBluescript. Double infection of the XL1-Blue host cell by pBluescript and R408 helper phage was avoided by initially plating the transformed XL1-Blue cells on LB/ampicillin at 42°C for 16 hours and then replating the developed colonies on new LB/ampicillin plates at 37°C. The R408 helper phage gene II product is heat labile and is inactivated at 42°C, stopping its replication; pBluescript on the other hand, is able to replicate, and subsequent replating results in the ability to remove coinfection. Infection by R408 can be detected by its migration at approximately 6 kbp double stranded DNA on an agarose gel.

The double-stranded pBluescript was purified using a plasmid boiling mini preparation (11) of overnight cultures. The

plasmid DNA was analyzed by digestion with either EcoR I, Xho I or both. These particular restriction enzymes were chosen because they were used in the unidirectional cloning of the cDNA into the ZAP II vector and cut the pBluescript vector once each. Xho I was particularly useful, since it is a rare cutting enzyme and was found to cut the transformed pBluescript DNA only once at the 5' end of the insert cDNA, resulting in linearization of the molecule. The linearized pBluescript without any insert DNA should migrate at 2.95 kbp. It was estimated from the Xho I-linearized vector that the insert was approximately 1.4 kbp in size and that excision of the insert by double digestion with Xho I and EcoR I resulted in the production of the 2.95 kbp pBluescript and cleavage of the insert DNA into multiple resolvable fragments of approximately 7 kbp and 4 kbp (Figure 3.3). The size of the insert cDNA corresponds quite well with the predicted size of a gene encoding a 64 kD protein. The presence of multiple EcoR I restriction sites in the insert cDNA prompted us to use the linearized form of the entire pBluescript vector containing the insert cDNA as the template for labelled probe production.

Production of digoxigenin-labelled probe

Our probe was produced using the nonradioactive GENIUS kit (Boehringer Mannheim, Indianapolis, IN). Probe DNA was

labelled by random primed incorporation of digoxigenin-labelled deoxyuridine-triphosphate (dgg). The dUTP is linked to the steroid hapten digoxigenin via a spacer arm. Upon hybridization of the dgg-labelled probe to its target nucleic acid, either DNA or RNA, the hybrids can be detected using an enzyme-linked immunoassay incorporating an anti-dgg alkaline phosphatase conjugated primary antibody and subsequent color formation. Approximately 1 μg of linearized probe DNA (Figure 3.4) was random primed with dgg giving a final yield of dgg-labelled probe DNA of approximately 250 ng. Sensitivity of the probe to homologous DNA was estimated at 0.5 pg.

Northern blot analysis of porcine adipocyte RNA

Porcine adipocyte total RNA was isolated using the acid guanidinium isothiocyanate-phenol-chloroform extraction procedure of Chomczynski and Sacchi (4). This procedure was found to produce high quality RNA in a short amount of time. Total RNA was isolated from contemporary, and genetically lean and obese porcine adipose tissue. Northern blot analysis of the total RNA (50 μg total RNA/lane) did not distinguish any particular RNA species which reacted with the dgg-labelled probe. Dot blot analysis indicated a weak signal in contemporary porcine adipocyte total RNA. Isolation of poly (A+) RNA from

contemporary total RNA (approximately a 1% yield) was performed. Northern blot analysis of 5 μg poly (A+) RNA indicated the presence of two species of poly (A+) RNA which hybridized to the probe (Figure 3.5) having estimated sizes of 5 kbp and 7 kbp respectively. The presence of two poly (A+) species may indicate that post-transcriptional processing of a larger message is occurring. The 5 kbp species was more predominant and may indicate that it represents a more active or more labile gene product, thus requiring more message. The low amount of message, as indicated by the level of signal in relation to the amount of poly (A+) RNA loaded, may indicate that the message is quite rare in adipocytes. The necessity for poly (A+) RNA as compared to total RNA for detection also supports this contention. It was not possible to obtain great enough quantities of genetically lean and obese poly (A+) RNA to make precise determinations as to the existence of the message. As indicated earlier, it was believed that the genetically lean and obese adipose tissue samples were not appropriate for the isolation of quality RNA due to sample collection and storage conditions.

Conclusion

A cDNA library was produced in the ZAP II expression vector system representing the contemporary porcine adipocyte. A clone was identified by immunological detection with a monoclonal antibody directed against a porcine adipocyte-specific plasma membrane protein. The vector was rescued as a double stranded DNA phagemid and the insert determined to be approximately 1.5 kbp in size. Linearization of the vector and its subsequent labelling with digoxigenin-dUTP produced a nonradioactive probe which was found to function well in Northern blot analysis. Poly (A+) RNA was found to be necessary for the detection of the hybridizing poly (A+) RNA species. Two bands were detected in Northern blot analysis of contemporary porcine adipocyte poly (A+) RNA representing a 5 kbp and a 7 kbp species of poly (A+) RNA. The exact pattern of expression of these messages in genetically lean and obese tissues has yet to be determined. Further characterization and sequencing of these messages should provide insight into the identity and function of the 64-kD porcine adipocyte-specific plasma membrane protein.

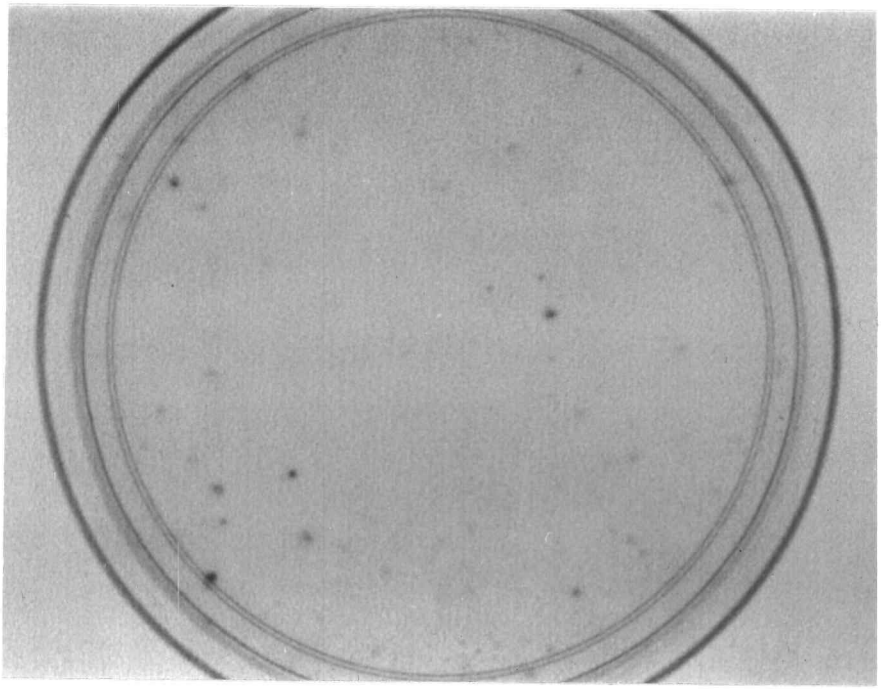

FIG. 3.1 **Determination of recombinants to nonrecombinants.** The number of recombinants to nonrecombinants was determined by the ratio of white to blue plaques respectively. Color selection was performed by infection of 200 µl of XL1-Blue cells (O.D.600 = 0.5) with 20 µl of a 1:1000 dilution of the porcine adipocyte library and subsequent growth in NZY top agar supplemented with 2.5 mM IPTG and 16.6 mM X-gal. The percentage of nonrecombinants is approximately 1%.

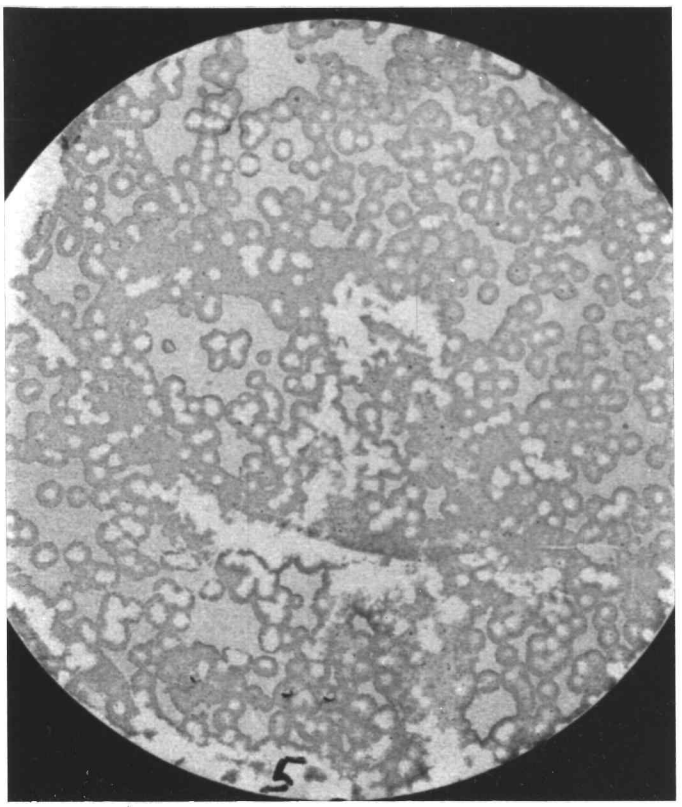

FIG. 3.2 **Immunoscreening the cDNA library with LA-1.** Clones that were determined to be positive by immunoscreening with LA-1 were isolated and recloned two additional times. A homogeneous population of positive clones was produced; homogeneity was indicated by uniform staining of clonal plaques.

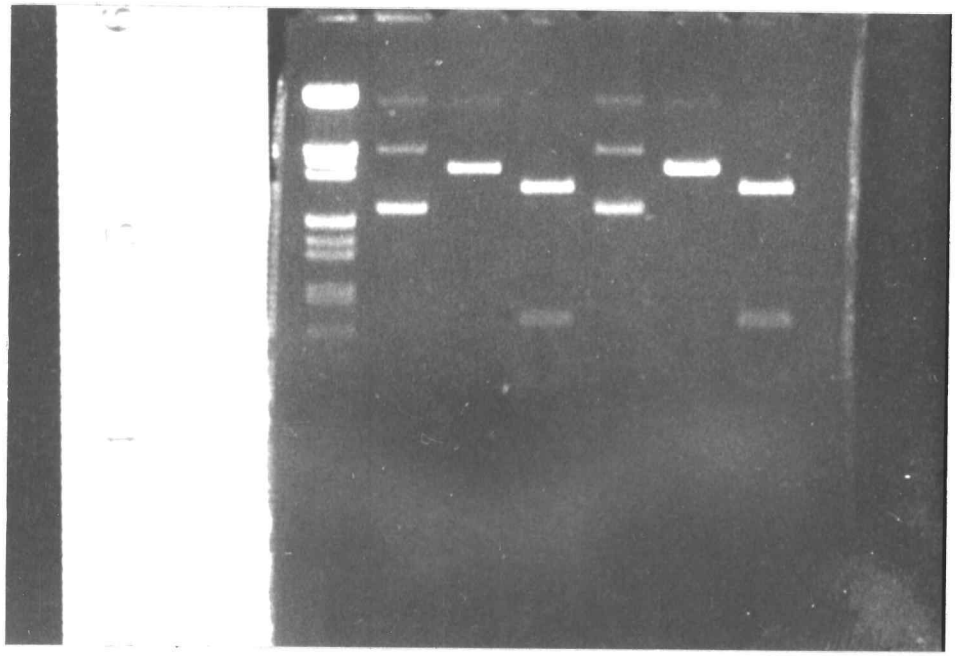

FIG. 3.3 **Determination of cDNA insert size by restriction enzyme mapping.** Double-stranded DNA pBluescript was rescued from the ZAP II vector containing the cDNA insert of interest as described under "Materials and Methods". Approximately 1 μg of DNA was digested and loaded per lane on a 1% agarose gel. Lane: 1, Hind III/EcoR I digest of DNA; lane 2, undigested clone 52A; lane 3, Xho I-digested 52A; lane 4, Xho I/EcoR I-digested 52A, lane 5, undigested clone 52B; lane 6, Xho I-digested 52B; lane 7, Xho I/EcoR I-digested 52B. It appears that 52A and 52B have the same restriction enzyme patterns and most likely carry the same insert DNA. The linearized fragment has a size of approximately 4.4 kbp, giving the insert an estimated size of approximately 1.4 kbp (pBluescript alone is approximately 2.95 kbp). The insert cDNA appears to possess several internal EcoR I restriction sites.

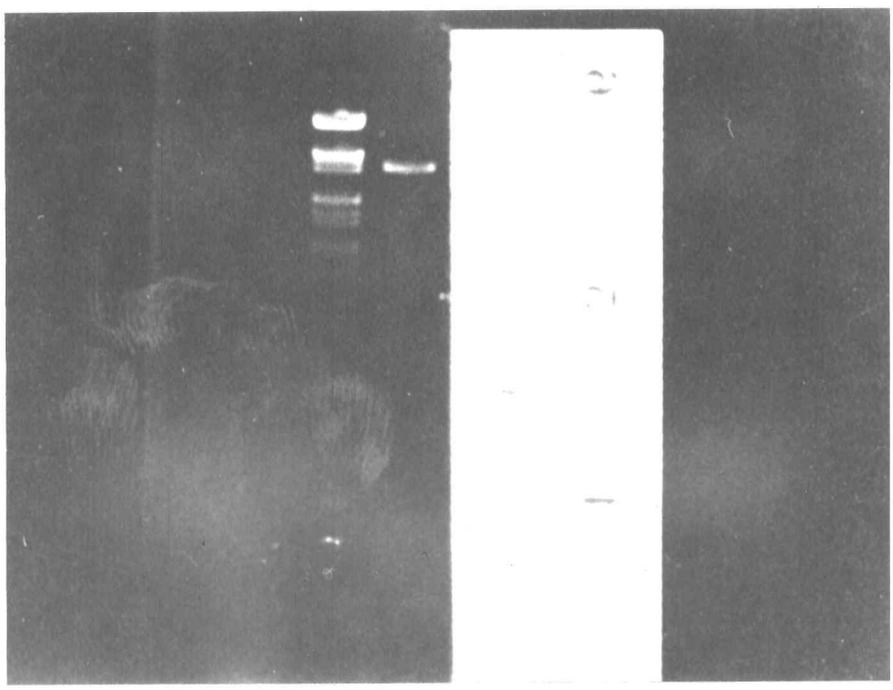

FIG. 3.4 **Linearization of the pBluescript/insert cDNA by Xho I.** The pBluescript was incubated at 42°C for 16 hours and replated to remove the R408 helper phage, which will migrate at 6 kbp. Isolation of the phagemid DNA and subsequent linearization with Xho I revealed a single band of approximately 4.4 kbp. Lane 1, Hind III/EcoR I digest of lambda DNA; lane 2, 2.5 μg 52A linearized with Xho I. This linearized cDNA was used for the production of a digoxigenin-labelled probe. Note the absence of the R408 helper phage band.

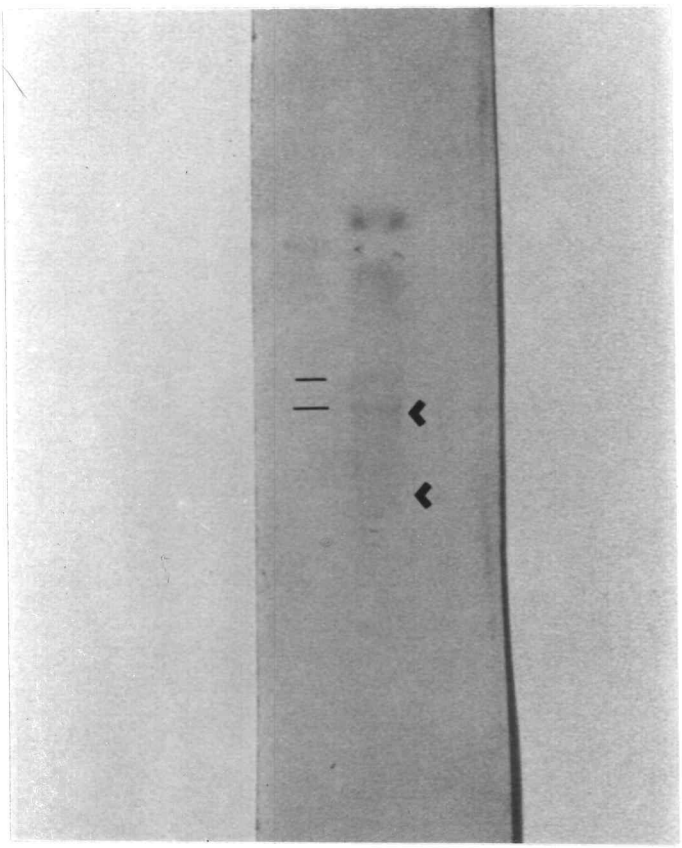

FIG. 3.5 **Northern blot analysis of contemporary porcine adipose tissue poly (A+) RNA.** Poly (A+) RNA was isolated from contemporary porcine adipose tissue and probed by a digoxigenin-labelled cDNA probe representing the adipocyte-specific 64 kD membrane protein. A total of 5 µg of poly (A+) RNA was loaded on the gel, separated and transferred to MagnaGraph membrane. The blot was probed with 250 ng labelled probe in a volume of 5 ml for 16 hours. Immunodetection of the probe reveled two bands of approximately 5 kbp and 7 kbp. Arrows indicate the position of the 28 S and 18 S ribosomal RNAs.

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Chapter 5

General Summary

General Summary

Adipocytes are a unique cell type having the characteristic function of synthesis, storage and hormonally regulated hydrolysis of triacylglycerols. This particular set of characteristics distinguishes the adipocyte from all other cell types, but the complexity of these characteristics makes it difficult to use them to easily identify adipocytes. Other cell types are known to possess one or more, but not all, of the adipocyte characteristics, which further complicates adipocyte identification. The traditional means of identifying adipocytes involves histological staining of the characteristic unilocular triacylglycerol droplet, but this is not a truly accurate means of identification since other cell types can accumulate triacylglycerols under appropriate conditions. A more desirable means of identifying the adipocyte would involve the identification of an adipocyte-specific marker which is preferably present on the extracellular surface of the cell. This set of studies described the identification of such a marker and the production of reagents which can be used to study its expression.

A panel of monoclonal antibodies was produced to the porcine subcutaneous adipocyte plasma membrane (APM). A hybridoma line designated LA-1 was identified which secreted a monoclonal antibody directed against a 64-kD protein present in the APM. Further characterization revealed that the expression of

the protein (at least the portion containing the antigenic epitope) was restricted to adipocyte plasma membranes and in particular, APMs derived from swine. Immunohistological staining of intact, isolated adipocytes with LA-1 showed that the 64-kD marker protein was expressed on the extracellular surface of the adipocyte. Two characteristics make the 64-kD protein a highly desirable marker for identifying mature adipocytes: (i) It is unique to adipocytes and (ii) it is expressed on the cell surface, allowing detection without necessitating destruction of the cell.

The onset of obesity appears to arise from many sources, including nutritional, environmental, physiological and genetic factors. It appears that genetics can predispose individuals to either a lean or obese phenotype. We therefore set out to determine if there were any differences in the expression of the 64-kD protein in relation to genetic predisposition to obesity or leanness. Since a line of genetically lean and obese swine had been produced, and we had identified a porcine adipocyte marker, LA-1 was used to study the expression of the 64-kD protein in these cases of genetically predetermined obesity or leanness. Interestingly it was found that the expression of the 64-kD protein was restricted to adipocytes of swine of a genetically lean phenotype. The adipocytes of genetically obese animals did not express the marker protein to any identifiable level. The significance of this finding has yet to be determined. It is interesting to speculate that the lack of expression or absence of

the 64-kD protein in adipocytes may somehow be involved in predisposing individuals to obesity.

In order to better understand and characterize the 64-kD protein, efforts were made to develop a nucleic acid probe representing the gene encoding the protein. Development of such a probe would allow sequencing of the gene encoding the protein, thus determining the amino acid sequence of the protein. We hope that this will lead to the elucidation of the physiological function of the protein and allow the study of its expression in response to numerous factors. A cDNA library was produced in the ZAP II expression vector, representing the expressed genes in porcine adipose tissue. The use of an expression vector permitted the use of LA-1 in the identification of the cDNA encoding the 64-kD fusion protein. It was predicted that an mRNA species of approximately 1.5 kbp would be required to encode a 64-kD protein. A cDNA clone, which contained a 1.4 kbp insert was identified by LA-1. This cDNA was linearized and random primed to produce a digoxigenin-labelled probe. The probe was hybridized to poly (A+) RNA derived from porcine adipose tissue and was found to hybridize to a 5 kbp and a 7 kbp species. The expression of both of these messages was difficult to detect, with the larger message being the minor species. The sequence, identity and function of this 64-kD adipocyte-specific protein has yet to be determined. This probe should prove useful in the study of adipocyte growth and development in the swine and the

possible role of the 64-kD protein in obesity. The use of this nucleic acid probe should also prove beneficial in the study of expression and regulation of the 64-kD porcine adipocyte-specific plasma membrane protein.

These findings make it likely that similar markers can be found for other tissues and species. The approaches used in this research make it possible to identify proteins of interest and to potentially determine their physiological roles in the cell. The development of nucleic acid probes to these proteins of interest makes it possible to study the regulation of their expression to a much finer degree. These approaches should prove very beneficial to the study of overall animal growth and development and its regulation.

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APPENDIX

Appendix: Details of Laboratory Protocols

Plasma Membrane Isolation from Porcine Adipose Tissue

Equipment:

For Fat Biopsy:

1. Knife and/or scalpels
2. transport medium (37°C)
3. biopsy gun
4. anesthetic (eg Biotal)
5. ethanol, 70%
6. Topazone

In Lab:

1. 37°C waterbath
2. gyrotory shaker (37°C)
3. tray warmer
4. scalpels, forceps, scissors, slicer
5. balance and weugh boats
6. polypropylene or siliconized 50 ml Erylenmeyer flasks
7. 95:5 (O_2:CO_2)
8. chiffon (5" X 5" square/flask)
9. polypropylene rod
10. siliconized large bore blunt needle and 30 ml syringe
11. polypropylene centrifuge tubes (50 ml)
    " " " (30 ml)
    " " " (20 ml)
    polycarbonate centrifuge tubes (20 ml)
12. vortex
13. test tube racks
14. misc.

Warm appropriate baths and solutions.
Try to have everything ready well in advance, this is a long procedure (15-17 hours). Use sterile technique when possible.

Adipose Cell Isolation

Procedure:

1. Slice tissue 0.65 mm thick or mince finely.
2. Add 15 ml KRB-glu-BSA (37°C), 3 ml buffer/ gram tissue
3. Add 45 mg collagenase to flask (3 mg/ml buffer)
   (total of 6000 units/5 grams tissue)
4. Swirl flask to dissolve enzyme
5. Gas medium (gas phase only!) with 95:5 for 10 minutes, stopper.
6. Add 5 g tissue (1 g tissue yields approx. 1 ml cells).
7. Incubate two hours at 37°C in gyrotory water bath at setting #2.
8. Filter through chiffon into polypropylene tube with cap. Squeeze cells through chiffon with polypropylene rod and an additional 2 ml of KRB.
9. Let set at 37°C, adipocytes will rise to top.
10. Remove infranatant with siliconized needle and syringe. Discard infranatant (infranatant contains S-V cells).
11. Slowly add 10 ml KRB (2-3 ml/g tissue) and cap tube, gently invert to wash cells. (discard infranatant)
12. Wash 2 more times.
13. Remove infranatant and resuspend in sucrose extraction medium (10 ml).
14. Now have isolated adipocytes.

(Note: Step 13 may be omitted if proceeding directly with lysis of the adipocytes. Proceed with step 1 of Fat Cell Ghost Preparation).

Fat Cell Ghost Preparation

1. Remove infranatant from cell suspension.
   Add 10 ml warm lysing medium (2 ml/g tissue)
2. Vigorously shake the capped tube to lyse the cells (15 seconds). For very small adipocytes you may repipette through a small gauge needle to aid in lysis of the cells.
3.* Centrifuge at 400 x g for 1 minute at room temperature.
4. Aspirate infranatant into a cold polycarbonate test tube.
5. Repeat lysis procedure 2 times more adding 1/2 volume warm lysing medium (1/2 volume of "step 4" infranatant).
6. Centrifuge at 4°C, 30,000 x g (16 K rpm in Beckman JA-17) for 30 minutes.
7. Decant and discard supernatant and wipe the test tube wall free of lipid (best to use finger and a Kimwipe).
8. Now have pellet.

* May be omitted and still get good yield.

Plasma Membrane Isolation

1. To pellet ("step 8" above) add 1-2 ml sucrose extraction medium (cold), vortex to mix.
2. Combine all tubes.
3. Gently add 700 - 800 µl of the pellet suspension to the top of Percoll gradient (8 ml). (Use a clear polycarbonate tube that fits the Beckman JA-20.1 rotor)
4. Centrifuge at 10,000 X g for 15 minutes at 4°C (11K rpm) to stratify.
5. Remove infranatant leaving approximately 2 ml below the top band (we removed 6 ml from each tube) and discard.
6. Add contents of two tubes into one polypropylene tube (JA-17).

7. Wash with 3X volumes of NaCl-based medium (cold).
8. Centrifuge at 10,000 X g for 5 minutes, 4°C (JA-17, 15K rpm).
9. Now have plasma membrane pellet, discard supernatant.
10. Mix all together in one tube.
11. Resuspend pellet in NaCl medium + Strep-Penn.
12. Run protein analysis.
13. Dilute to desired concentrations (eg 1 mg/ml).
14. Can be stored at 4°C, long term storage may require addition of 0.02% sodium azide, freezing may be possible.

Preparation of Media for Plasma Membrane Isolation

A. 3000 ml **KRB-HEPES buffer** (enough for 100 g tissue)
20 flasks x 15 ml/flask = 300 ml Incubation Medium

10 ml wash x 20 flasks x 3 x 4 = 2400 ml
+ 600 ml spare = 3000 ml

Content:			100 ml ml	3000 ml ml	3000 ml grams
.770	M	NaCl	15.80	474.0	21.33
.770	M	KCl	.63	18.9	1.085
.770	M	KH_2PO_4	.16	4.65	0.487
.770	M	$MgSO_4$-$7H_2O$	.16	4.65	0.883
.770	M	$NaHC_3$	3.32	99.6	6.444
.3	M	Glucose	10.00	300	16.20
H_2O			49.45	1483.5	2400
.275	M	$CaCl_2$-H_2O	.48	14.4	0.582
HEPES (MW=238.3) (final conc = 20 mM)					14.3

Adjust pH to 7.4

Strep-Penn Solution
(final concentration = 100 µg/ml)

1.0 ml	30 ml	30 ml

Gas 10 min before adding BSA with 95:5 (CO_2:O_2)

20% BSA Fraction V
(only added to incubation medium!)

Incubation	Washing	**gassed
360 ml	2040 ml	
+	+	
90 ml BSA	510 ml DDH_2O	
450 ml	2550 ml**	

B. **Lysing Medium** 400 ml

	1000 ml	400 ml	
2.5 mM ATP (2Na + $2H_2O$) MW=587.2		1.4680 g	0.5872 g
2.5 mM $MgCl_2$-$6H_2O$ (MW=203.3)	0.508	0.203	
0.1 mM $CaCl_2$-$2H_2O$ (MW=147.02)	0.0147	0.006	
1.0 mM $KHCO_3$ (MW=100.12)	0.100	0.040	
2.0 mM Tris Base (MW=121.1)	0.242	0.097	

Bring to volume with DDH_2O
Adjust pH to 7.6

*ATP not required if preparations are not used for enzyme activity studies.

C. **Transport Medium** (500 ml) (=.154 M NaCl + 20 mM HEPES + Strep-Penn) used for transport and tissue mincing.

	500 ml	
NaCl		4.5 g
10,000 µg/ml Strep-Penn	5.0 ml	
HEPES	2.38 g	

Bring to volume with DDH_2O
Adjust pH to 7.4

D. **NaCl-based Medium:** 500 ml, pH 7.4

	500 ml	1000 ml
.15 M NaCl	4.384 g	8.768 g
10 mM Tris/HCl	.788 g	1.576 g
1.0 mM EGTA	.191 g	0.382 g
Strep-Penn	5.0 ml	10.0 ml
HEPES (20 mM)	2.383 g	4.767 g

Bring to volume and pH

E. **Sucrose Extraction Medium:** 500 ml, pH 7.4

final wash	(20 flasks x 10 ml)	=	200
resuspension	(20 x 2 ml)	=	40

For Percoll Solution (30 tubes)
(30 x 8 ml = 240 ml)

7 Percoll
:
1 Sucrose
:
32 Sucrose Extraction Medium
40

==> 240 / 40 = 6
32 x 6 = 192 ml Extraction Med

	192
Extra Medium	68
	500 ml

For 500 ml:	500 ml	1000 ml
0.25 M Sucrose	42.788 g	85.576 g
0.10 mM Tris/HCl	0.788 g	1.576 g
2.0 mM EGTA	0.381 g	0.762 g

Bring to volume
Adjust ph to 7.4

F. **Sucrose-Tris-EGTA,** 20 ml

2 M Sycrose	13.692 g	
80 mM Tris/HCl		0.2520 g
8 mM EGTA	0.0600 g	
Bring to volume		

G. **Iso-Osmotic Percoll Solution,** 260 ml

7:	Percoll	45.5 ml	
1:	Sucrose-Tris-EGTA	6.50 ml	
32:		Sucrose-based Extr. Med.	208 ml
		260 ml	

NOTE: Keep Percoll stock solution (100%) sterile!

On day of isolation KRB, Transport and Lysing mediums should be warmed to 37°C. All others kept at 4°C.

Monoclonal Antibody Production:

Equipment List:

Immunization of mice

1 ml tuberculin syringes	Becton Dickinson #5602
23 and 25# needles	BD #5145 and #5124
Freund's Incomplete Adjuvant	Sigma #F5506
RIBI Adjuvant	RIBI

Cell Fusion

96-well microtiter plates	BD #3072
1-ml disposable pipettes	VWR #53300-068
5-ml ""	VWR #53300-421
10-ml ""	VWR #53300-523
15-ml conical centr. tubes	VWR #21008-623
Hybridmax, DMEM	Sigma
Fetal Bovine Serum	Hyclone
HAT and HT supplements	Sigma
Scissors	
Forceps	
Petri dishes, sterile	

ELISA Screening

96-well plates	Costar #3590
Pipettors and tips, 50 µl and 100 µl	
Phosphate Buffered Saline (PBS)	
Goat anti-mouse-AP	Sigma #A5153

4-Methylumbelliferal phosphate	Sigma #M8883
Diethanolamine buffer	

Cloning of Hybridomas

96-well plates	BD #3072
24-well plates	BD #3847
50-ml flasks	BD #3018

Hybridoma Production (Fusion), all procedures done in sterile hood

Harvesting Myeloma Cells:

1. Discard old culture fluid from flasks
2. Add 10 ml DME-0 to flasks and "RAP" cells loose
3. Transfer cells to a 15 ml sterile centrifuge tube (1 flask/tube)
4. Centrifuge at 400 x g, 10 min, RT
5. Decant supernatant
6. Add 10 ml DME-0, repipette to suspend cells
7. Centrifuge as in "step 4" and decant, repeat for a total of 3 times*
8. Place pellet into 37°C incubator until ready for use

* also keep 1 ml of cell suspension as negative control
-to the 1 ml, add 4 ml DME-0 and put into 37°C incubator

Isolating Splenocytes:

Materials: (per animal)

1. CO_2 killing jar
2. Beaker of 95 % ETOH
3. Sterile Hood
4. Sterile: 5-ml & 10-ml pipettes, petri dishes, 15-ml conical centrifuge tubes (2), #23 needles (2), 30-ml DME-0, round bottom 50-ml tube
5. Sterile instuments in 250-ml Erlynmeyer flask w/ 95% ETOH (2 rat tooth forceps, 1 smooth forcep, 1 scissors)

Method:

1. Kill mice with CO_2 and put into beaker of 95% EtOH (5-10 sec.)
2. Put 5 ml DME-0 into Petri dish
3. Place mouse onto alcoholed paper towels in a left side up position
4. Using 2 rat toothed forceps, carefully tear skin layer open (not muscle layer)
5. Dip tips of forceps in EtOH and carefully open muscle layers in same manner
6. Dissect out spleen with smooth forceps and scissors
   - place spleen into Petri dish containing 5 ml DME-0
   - place instruments into Erlenmeyer containing EtOH
7. Using 2 #23 needles (new ones for each fusion), tease the spleen apart into very fine parts
8. Pipette spleen cells into a 15-ml conical tube
   -let set to allow large pieces to settle
9. Pipette the cell suspension back into a new 15-ml tube and bring volume up to 10 ml with DME-0
10. Centrifuge at 400 x g, 10 minutes, RT
11. Pour off supernatant
12. Add 10 ml DME-0 and resuspend splenocytes

13. Centrifuge at 400 x g, 10 minutes, RT (repeat for a total of 3 times)
14. Pour off supernatant from final wash
    - Compare amount of splenocytes and amount of myeloma cells
    - Want equal amounts (or more splenocytes if necessary)
    - Can approximate volumes by resuspending and pelleting the appropriate amount of the suspension
15. Combine the splenocytes and myeloma cells, then mix
16. Centrifuge at 400 x g, 10 minutes, RT
17. Pour off supernatant and place pellet into 37°C incubator until ready for fusion (it is best to fuse very quickly)
18. Follow fusion protocol

Fusion of Myeloma Cells and Splenocytes

Materials (per fusion):

1. Myeloma and Splenocyte pellet in 50-ml round bottom tube
   - from "step 17" of "Isolating Splenocytes"
2. 50-ml beaker to use as test tube holder
3. Eppendorf Repipettor on setting 1 (with 2.5 ml tip and yellow tip)
   - need two sets
   - this combination delivers 0.05 ml/click (20 clicks = 1 ml)
4. 10-ml pipettes (2)
5. 50% PEG (make sure pH is approximately 7)
6. 9 ml DME-0
7. 7 ml HAT
8. 96-well culture plates and covers (3)
9. Digital timer

Procedure:

(from the start of adding PEG @ 25'00" to the finish @ 14'00" the fusion sequence takes 11 minutes)

		START	END
1.	Add 1 ml PEG over 1 minute	25'00"	24'00"
2.	Stir 1 min longer using same tip	24'00"	23'00"
3.	Let sit 1 min	23'00"	22'00"
4.	Stir in 1 ml DME-0 over 1 min	22'00"	21'00"
5.	Stir 1 min	21'00"	20'00"
6.	let sit 1 min	20'00"	19'00"
7.	Stir in 1 ml DME-0 over 1 min	19'00"	18'00"
8.	let sit 1 min	18'00"	17'00"
9.	Stir in 7 ml DME-0 over 3 min	17'00"	14'00"
10.	let sit 1 min	14'00"	13'00"
11.	Centr. @ 400 x g, 10 min, RT	13'00"	3'00"
12.	Pour off DME-0, add 30 ml HAT - resuspend fused cells	3'00"	0'00"

13. Plate out into 3, 96-well culture plates (100 µl/well)
    - leave some myeloma cells for control (12 wells)
14. Add 100 µl of HAT/well
15. Place plates into 37°C incubator (4% CO_2)

General Procedure for Running ELISA

1. Coat the black microfluor plate(s) with 100 µl/well of the coating agent (e.g., 2.5 µg/ml APM) and incubate @ 37°C for 2 hours or overnight @ 4°C.

2. Empty the plate and add 350 µl/well of blocking solution (i.e., 1% BSA, 1% OVA, 0.1% Tween-80, 1% Non-Fat Dry Milk). Incubate @ 37°C for 30 minutes.

3. After incubation period empty the plate and wash once with PBS-Tween 20 (0.05%). Add 50 µl of test solution (eg culture fluid)/well. Incubate @ 37°C for 30 minutes.

4. Empty the plate and wash 3-4 times with PBS-Tween 20 (0.05%). Pound dry on a paper towel and pop all bubbles.

5. Add 50 µl/well of goat anti-mouse-alkaline phosphatase conjugate diluted 1:750 in PBS. Incubate @ 37°C for 30 minutes.

6. Empty the plate and wash 3-4 times with PBS-Tween 20 (0.05%). Pound dry on paper towels and pop all bubbles.

7. Add 50 µl/well of enzyme substrate 4-methyl umbelliferyl phosphate to each well.

8. Incubate @ 37°C in the dark. Read plate at designated time (e.g., 20 minutes).

Immunodetection of antigens on nitrocellulose using alkaline phosphatase (AP)

STEP	TIME	PROCEDURAL NOTES	
1.	≥ 30 min	PBS + 5% NFDM	Blocking, can use O.N. @ 4°C
2.	≥ 30 min	PBS + 0.1% NFDM + antibody (1:100)	Primary antibody, rocking
3.	3 x 5 min	PBS + 0.1% NFDM	Washes
4.	30 min	PBS + 0.1% NFDM + 0.100 ml/10 ml secondary antibody	Goat anti-mouse-AP, rocking
5.	3 x 5 min	PBS	Washes
6.	≥ 5 min	100 mM Tris-HCl pH 9.5 + 2 drops 1/10 ml + 2 drops 2/10 ml + 2 drops 3/10 ml	Color reaction **
7.	4 x 1 min	DDH_2O	Stop color reaction

**Make up the color developing solution immediately prior to use. Do not allow the blot to over-develop.
After the final washing, photograph the blot for a permanent record since colors may fade.
This procedure was adopted from the literature supplied in the Vectastain ABC Kit (Vector Laboratories, Inc., 30 Ingold Road Burlingame, CA 94010-9976 USA. (800)-227-6666

Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction
(Reference: same title as above, Piotr Chomczynski and Nicoletta Sacchi, Analytical Biochemistry 162:156-159, 1987)

General:

1. All clean metal and glassware including Pasteur pipettes were:
   - covered with foil
   - baked 4 hours @ 200°C
   - autoclaved for 30 minutes

2. Sterile disposable plastic and glassware were used directly from the package

3. Eppendorf tubes were autoclaved open in a pre-baked beaker

4. Plastic, glass and other items which cannot be baked or autoclaved were treated with 0.1 N NaOH for at least 15 minutes and rinsed 4X with DEPC-treated water

5. Gloves were worn at all times

6. All lids for baked glassware were treated with DEPC in water (0.1%); allowed to stand 8 hours at RT and autoclaved on their respective bottles.

Reagent Preparation:

All reagents were prepared with DEPC-treated water (DEPC-water):
Use a baked, autoclaved 1-L reagent bottle
Add 900 ml glass-distilled (deionized) water
Add Diethyl Pyrocarbonate (DEPC) to 0.1%
Shake well and let stand 8-16 hours at RT
Autoclave for 45 minutes

For all aqueous solutions use DEPC-treated water and prepare in baked, autoclaved glassware
**Tris buffers should not be further DEPC-treated (do not mix Tris and DEPC)

1. Denaturing Solution (Sol'n D)

4 M guanidinium thiocyanate (GIT)
25 mM sodium citrate
0.5% sarcosyl
0.1 M 2-mercaptoethanol

To make 200 ml:

	MW	Grams
GIT	118.2	94.56
sodium citrate ($3Na + 2H_2O$)	294.1	1.47
Na-sarcosyl (from a 10% sol'n)		10.0 ml

Bring to 190 ml with DEPC-water

Adjust pH to 7.0 (use NaOH or HCl)
Bring volume to **198.5** ml with DEPC-water

Store in brown bottle @ RT (stable for 3 months if w/out 2-mercaptoethanol)

Solution D (working solution): is prepared by adding 0.36 ml 2-mercaptoethanol/50 ml stock (stable 1 month @ RT)

2. **2 M Sodium Acetate, pH 4.0** (for 100 ml)

	MW	Grams
Na-acetate (3 H2O)	136.1	27.2 g
DEPC-water		40 ml

Adjust pH to 4.0 with glacial acetic acid
Bring to volume, store @ 4°C

3. **Phenol** (DEPC-water saturated)
for 100 ml

Preparation of 500 g phenol (BM Biochemica)
- melt @ 65°C in water bath
- add an equal volume of DEPC-water
- shake thoroughly
- cover with N_2 gas (to prevent oxidation)
- allow to separate O.N. @ 4°C
- remove aqueous phase
- decant phenol into 3-5oo ml bottles
- cover with N_2 gas
- good several months @ 4°C

4. **CHISAM** (49:1, v:v; chloroform-isoamyl alcohol)

Chloroform	98 ml
Isoamyl alcohol	2 ml

5. **Isopropanol** (2-propanol)

6. **Ethanol**, 75% in DEPC-water

7. **SDS**, 0.5%

Sodium Dodecyl Sulfate (lauryl sulfate)	0.5 g
DEPC-water	to 100 ml

Procedure: (for 0.5 g adipose tissue)

Sample preparation: place sample in foil, freeze in liquid N_2 and crush with a prechilled pestle. Pour sample into a 50-ml disposible tube containing 10 ml Sol'n D and proceed with processing.

1. Tissue is homogenized in 10 ml of cold Sol'n D, polytron setting of #7, 30 seconds (fresh samples that were snap frozen are best)

2. Transfer homogenate to a new 50-ml screw-cap test tube
   - attempt to remove any large unhomogenized fragments

3. Add 1.0 ml of 2 M sodium acetate, pH 4.0
   - mix

4. Add 10 ml phenol
   - mix

5. Add 2.0 ml CHISAM
   - mix

6. Shake vigorously for 15 seconds
   - place on ice for 15 minutes

7. Transfer to a treated, autoclaved 50-ml polypropylene (pp) round bottom centrifuge tube with cap for centrifugation

8. Centrifuge @ 12,000 x g for 15 minutes, 4°C
   - lower phenol-chloroform phase: DNA + proteins
   - upper aqueous phase: RNA

9. Transfer aqueous phase to a new pp tube
   - add 10 ml ice cold isopropanol (equal volume), mix
   - let sit for 1 hour @ - 20°C to ppt RNA

10. Centrifuge @ 12,000 x g for 15 minutes, 4°C
    - RNA forms a white pellet at bottom of tube

11. Decant supernatant and discard

12. Transfer RNA pellet to an autoclaved Eppendorf tube
    - add 0.5 ml of 75% EtOH (cold) and suspend pellet to aid in transfer

13. Add an additional 0.5 ml of 75% EtOH (cold) to the pellet for a total volume of 1 ml
    - vortex

14. Centrifuge @ 15,000 rpm in a microfuge for 10 minutes @ 4°C

15. Remove the supernatant and <u>wash the pellet twice</u> with 75% EtOH
    - vortex after the addition of EtOH
    - use at least 0.8 ml EtOH/50-100 μg RNA

16. Centrifuge @ 15,000 rpm in a microfuge for 10 minutes @ 4°C

17. Dry the RNA pellet briefly (10-15 minutes)
    - do not dry completely

18. Dissolve RNA pellet in 0.5% SDS or 1 mM EDTA, pH 7
    - approximately 50-100 µl
    - incubation @ 60°C for 10-15 minutes may aid in dissolving

19. Quantitate the RNA (260 nm absorbance)

20. Store RNA @ -80°C