Production of a Novel Monoclonal Antibody to 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 the unique identity of adipose tissues. 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 fluorescent enzyme-linked immunosorbent assay, immunoblotting, and indirect immunofluorescence. A monoclonal antibody (designated LA-1) with reactivity toward a porcine adipocyte plasma membrane component was used for further adipocyte characterization. LA-1 reacted with a species-specific 64-kDa 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.**

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Specific compositional differences in cellular plasma membranes which may confer cellular identity remain poorly understood. Biochemical and physiologic 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 physiologic 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 mass from 14 to 178 kDa (1–6).

Immunologic 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 (i.e., 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 biologic reagents for the study of adipocyte structure, metabolism, and development.

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 toward nonadipocyte-derived plasma membrane proteins is described. The antibody has proven useful for screening other cell types for the presence of this specific protein in fluorescent enzyme-linked immunosorbent assay, immunoblotting procedures, and immunofluorescence assay.

Materials and Methods

Plasma Membrane Fraction. Adipocytes were isolated by collagenase digestion according to the methods

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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 according to 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 with 100 μg (200 μl/mouse) of porcine dorsal subcutaneous adipocyte plasma membrane (APM) proteins emulsified in Freund's incomplete adjuvant (Sigma Chemical Co., St. Louis, MO). A second 200 μl/mouse i.p. injection (20 μg of APM protein in Freund's incomplete adjuvant) 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 fluorescent enzyme-linked immunosorbent assay (F-ELISA) methods (see below). A final booster injection (250 μl/mouse) of 5 μg of APM protein in sterile PBS was administered on Day 42 by i.p. injection. On Day 45, each mouse was sacrificed and the spleen removed using sterile techniques. Splenocytes from the hyperimmunized mice were chemically fused with Sp 2/O-AG 14 myeloma cells using a polyethylene glycol solution: 40% polyethylene glycol 1450 (Eastman Kodak Co., Rochester, NY), 10% polyethylene glycol 4000 (J. T. Baker Inc., Phillipsburg, NJ), 10% dimethyl sulfoxide (Sigma Chemical Co.), and 40% Dulbecco's modified Eagle's medium (Sigma Chemical Co.), 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 hypoxanthine-thymidine medium, and finally by Dulbecco's modified Eagle's medium-10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT). When medium in individual culture wells became acidic (yellow), those wells were tested for anti-APM antibody production by F-ELISA. Wells containing hybridomas which 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.

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% nonfat dry milk in PBS (350 μl/well) and incubated at 37°C for 30 min to prevent nonspecific protein binding. After the blocking step wells were washed once with PBS-Tween (PBS containing 0.05% Tween 20; Sigma Chemical Co.) with a 12-channel Mini-Fastwash manifold (3M Diagnostics, Mountain View, CA), and 50 μl of hybridoma culture fluid or mouse antiserum were added to appropriate wells. Plates were incubated at 37°C for 30 min 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.) followed by incubation at 37°C for 30 min. The plates were washed four times with PBS-Tween and 50 μl/well of substrate solution added. Substrate solution consisting of 4-methylumbelliferyl 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 was incubated at 37°C for 30 min. Fluorescence that developed was determined automatically in a 96-well fluorometer fitted with 365-nm excitation and 450-nm emission filters (3M Diagnostics).

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

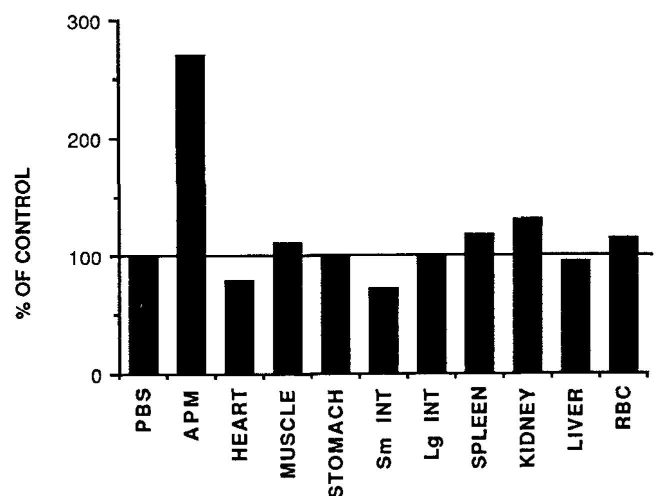

Figure 1. F-ELISA data comparing the relative cross-reactivities of LA-1 to the plasma membranes of 10 physiologically distinct tissue types (adipocyte, heart, skeletal muscle, stomach, small intestine, large intestine, spleen, kidney, liver, and erythrocyte). The relative cross-reactivities are shown as percentage of control (background) values. The control level of fluorescence is represented as 100%.

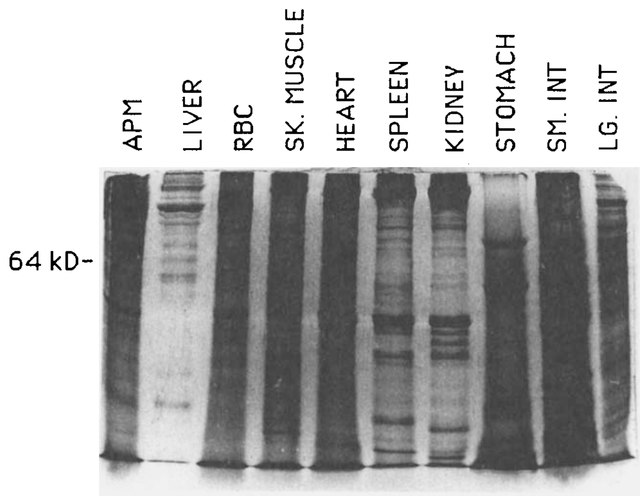

Figure 2. Coomassie Blue R-250 stained SDS-PAGE of subcutaneous adipocyte, hepatocyte, erythrocyte, skeletal muscle, heart, spleen, kidney, stomach, small intestine, and large intestine plasma membranes (12.5 μg/lane) run on a 4% stacking, 12% separating gel at 200 V, 4°C for 45 min.

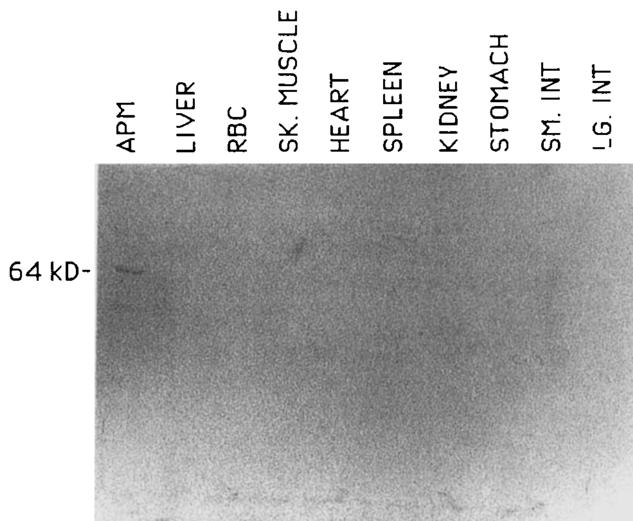

Figure 3. Western blot of subcutaneous adipocyte, hepatocyte, erythrocyte, skeletal muscle, heart, spleen, kidney, stomach, small intestine, and large intestine plasma membranes immunoblotted with LA-1. Protein concentrations of 12.5 μg/lane were loaded onto a 12% SDS-PAGE gel, separated, and transferred to nitrocellulose.

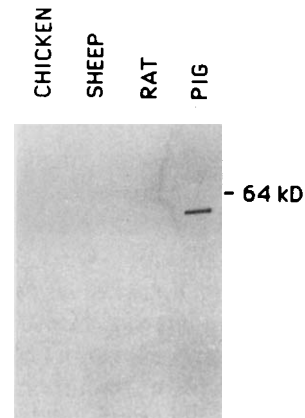

Figure 4. Western blot of subcutaneous APM proteins isolated from chicken, sheep, rat, and pig. Protein concentrations of 5 μg/lane were loaded onto a 12% SDS-PAGE gel, separated, transferred to nitrocellulose, and immunoblotted with LA-1.

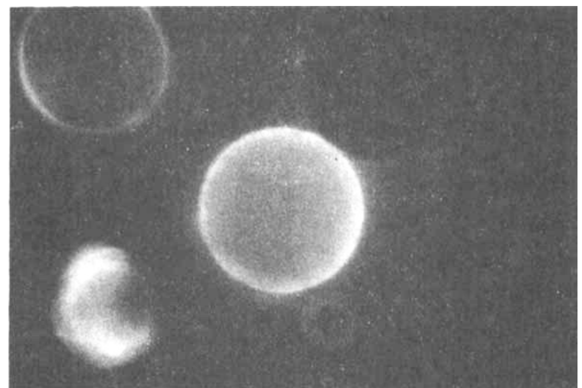

Figure 5. Indirect immunofluorescence of isolated subcutaneous adipocytes by LA-1 showing a uniform staining pattern consistent with an even distribution of the antigen. The results indicate that the protein is exposed to the extracellular environment (original magnification ×200).

immune sera), fresh culture medium, or PBS (for the analysis of hybridoma supernatants).

Immunohistochemistry. Isolated adipocytes were examined by an indirect immunofluorescence technique with antiadipocyte hybridoma supernatant. Subcutaneous adipocytes were isolated as described and incubated with a 1/50 dilution of the hybridoma culture fluid in PBS for 30 min 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 fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Sigma Chemical Co.) in the dark for 30 min 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 sample buffer which contained 2% SDS, 10% glycerol, 62.5 m*M* Tris-HCl buffer (pH 6.8), 5% 2-β mercaptoethanol, and 0.002% (w/v) bromophenol blue (Sigma Chemical Co.) and subjected to electrophoresis (12.5 μg of 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 (0.45-μm pore size; Sigma Chemical Co.) 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 min. Tissue-specific cross-reactivity studies compared porcine hepatocyte, erythrocyte, skeletal muscle, heart, spleen, kidney, stomach, small in-

testine, 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% nonfat dry milk (NFDM) in PBS for 1 hr 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 supenatant in 0.1% NFDM (in PBS) for 30 min and washed three times, 5 min/wash, with 0.1% NFDM in PBS. Filters were then incubated for 30 min with a 1/750 dilution of alkaline phosphatase-labeled goat anti-mouse IgG (Sigma Chemical Co.) followed by 3- to 5-min washes in PBS (no NFDM). The filters were exposed to the Vectastain ABC alkaline phosphatase substrate buffer (two drops each of reagents 1, 2, and 3 into 10 ml of 100 m*M* Tris-HCl pH 9.5; Vector Laboratories, Inc., Burlingame, CA) until color formation was detected (approximately 5–10 min). 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.), 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.

Subisotype Determination of the Monoclonal Antibodies. Subisotyping 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 subisotypes were tested for reaction to antisera specific for IgG1, IgG2, IgG2a, IgG2b, IgG3, IgM, and IgA.

Results

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 (Fig. 1). A monoclonal antibody line, designated LA-1, was identified which distinguished adipocytes from the various tissue types assayed (Figs. 2 and 3) on the basis of the presence or absence of a particular protein. Subisotyping 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 (Fig. 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 (Fig. 4). Control sera did not react with this protein.

The antigen identified by LA-1 has an apparent molecular mass of 64,000 daltons (64 kDa), as determined by comparison to SDS-PAGE molecular weight protein markers (Fig. 3). Comparisons of protein profiles in Coomassie Blue-stained SDS-PAGE gels (Fig. 2) indicated numerous differences in plasma membrane protein composition among various porcine tissues.

LA-1 also bound intact, isolated adipocytes as shown by immunofluorescence (Fig. 5). Both isotype-matched and fluorescein isothiocyanate-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.

Discussion

In this work, we sought to develop a specific and reproducible biologic reagent that 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 (Fig. 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 (Fig. 3) confirmed these predictions. The antigen identified by LA-1 has a molecular mass of approximately 64 kDa under reducing conditions, as determined by SDS-PAGE and immunoblot analysis (Figs. 2 and 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-kDa protein when immunoblotted with LA-1 (data not shown). Coomassie Blue-stained SDS-PAGE gels (Fig. 2) emphasized the disparity in plasma membrane composition between the various tissue types.

Intact, isolated adipocytes were also readily labeled by LA-1 as indicated by indirect immunofluorescence (Fig. 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 sug-

gests that the protein is either extrinsic or integral. Neither presence of the protein in the intracellular pool nor its physiologic 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 masses of 124 kDa, 92 kDa, and 59 kDa for the rat, 87 kDa for cattle, and 56 kDa, 47 kDa, and 37 kDa for the chicken. None of the antigens was cross-immunoprecipitated by nonhomologous 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 (Fig. 4). The monospecificity 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 physiologic 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 transmembrane carrier.

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1. Czech MP, Lynn WS. The plasma membrane of isolated fat cells. I. Identification of trypsin-sensitive membrane peptide by sodium dodecyl sulfate polyacrylamide gel electrophoresis. J Biol Chem **248**:5081–5088, 1973.
2. Trosper T, Levy D. Characterization of the surface protein components in adipocyte plasma membranes. Biochemistry **13**:4284–4290, 1974.
3. Giacobino JP, Chmelar M. Comparison of plasma membranes and endoplasmic reticulum fractions obtained from whole white adipose tissue and isolated adipocytes. Biochim Biophys Acta **406**:68–82, 1975.
4. Kawai Y, Spiro RG. Fat cell plasma membranes. I. Preparation, characterization and chemical composition. J Biol Chem **252**:6229–6235, 1977.
5. Belsham GJ, Denton RM, Tanner MJA. Use of a novel rapid preparation of fat-cell plasma membranes employing Percoll to investigate the effects of insulin and adrenaline on membrane protein phoshorylation within intact fat-cells. Biochem J **192**:457–467, 1980.
6. Tume RK, Lee SR, Cryer A. A comparison of the polypeptide composition of plasma membranes prepared from the white adipose tissue and adipocytes of the mouse, rat rabbit, ox and chicken by a Percoll self-forming gradient procedure. Comp Biochem Physiol **80B**:127–134, 1985.
7. Pillion DJ, Grantham JR, Czech MP. Biological properties of antibodies against rat adipocyte intrinsic membrane proteins. J Biol Chem **254**:3211–3220, 1979.
8. Thompson K, Abraham S. Identification of mouse mammary adipose cells by membrane antigens. In Vitro **15**:441–445, 1979.
9. Lee SR, Tume RK, Cryer J, Cryer A. Studies on the expression of adipocyte- specific cell surface antigens during the differentiation of adipocyte precursor cells in vitro. J Dev Physiol **8**:207–226, 1986.
10. Cryer A, Gray BR, Woodhead JS. Studies on the characterization of bovine adipocyte precursor cells and their differentiation in vitro, using an indirect-labelled-second-antibody cellular immunoassay. J Dev Physiol **6**:159–176, 1984.
11. Nassar AH. Development, characterization and application of polyclonal antibodies against ovine adipocytes for body fat reduction by passive immunization [Thesis]. Corvallis, OR: Oregon State University, 1989.
12. Flint DJ, Coggrave H, Futter CE, Gardner MJ, Clarke TJ. Stimulatory and cytotoxic effects of an antisera to adipocyte plasma membranes on adipose tissue metabolism in vitro and in vivo. Int J Obes **10**:69–77, 1986.
13. Rodbell M. Metabolism of isolated fat cells. I. Effect of hormones on glucose metabolism and lipolysis. J Biol Chem **239**:375–380, 1964.
14. Lodish HF, Braell WA. Methods for the study of the synthesis and maturation of the erythrocyte anion transport protein. Methods Enzymol **96**:257–267, 1983.
15. Hertzberg EL. Isolation and characterization of liver gap junctions. Methods Enzymol **98**:501–510, 1983.
16. Lo C, August TR, Liberman UA, Edelman IS. Dependence of renal (Na^+ + K^+)-adenosine triphosphatase activity on thyroid status. J Biol Chem **251**:7826 -7833, 1976.
17. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem **72**:248–254, 1976.
18. Oi VT, Herzenberg LA. Immunoglobulin-producing hybrid cell lines. In: Mishell BB, Shiigi SM, Eds. Selected Methods in Cellular Immunology. San Francisco: WH Freeman and Co., p351, 1980.
19. Trione EJ, Krygier BB, Banowetz GM, Kathrein JM. The development of monoclonal antibodies against the cytokinin zeatin riboside. J Plant Growth Regul **4**:101–109, 1985.
20. Littlefield JW. Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. Science **145**:709–710, 1964.
21. Laemmli UK. Cleavage of structural proteins during the assembly of bacteriophage T. Nature **227**:680–685, 1970.
22. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA **76**:4350–4354, 1979.