

# Identification of Albumin-binding Proteins in Capillary Endothelial Cells

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**Abstract.** Isolated fat tissue microvessels and lung, whose capillary endothelia express in situ specific binding sites for albumin, were homogenized and subjected to SDS-gel electrophoresis and electroblotting. The nitrocellulose strips were incubated with either albumin-gold (Alb-Au) and directly visualized, or with [<sup>125</sup>I]albumin (monomeric or polymeric) and autoradiographed. The extracts of both microvascular endothelium and the lung express albumin-binding proteins (ABPs) represented by two pairs of polypeptides with major components of molecular mass 31 and 18 kD. The ABP peptides have pIs 8.05 to 8.75. Rabbit aortic endothelium, used as control, does not express detect-

able amounts of ABPs. The ABPs subjected to electrophoresis bind specifically and with high affinity ( $K_d = \sim 60 \times 10^{-9}$  M) both monomeric and polymeric albumin: the binding is saturable at  $\sim 80$  nM concentration and 50% inhibition is reached at 5.5  $\mu$ g/ml albumin concentration. Sulfhydryl-reducing agents  $\beta$ -mercaptoethanol and dithiothreitol do not markedly affect the ABPs electrophoretic mobility and binding properties. As indicated by cell surface iodination of isolated capillary endothelium followed by electroblotting, autoradiography, and incubation with Alb-Au, the bands specifically stained by this ligand are also labeled with radioiodine.

**S**ERUM albumin, in addition to its major role in securing the oncotic pressure of plasma and interstitial fluid, it is reputedly known to serve as carrier for free fatty acids, bilirubin, steroids,  $Ca^{2+}$ , amino acids and thyroid hormones (21, 22, 28, 29).

Recently, in experiments with albumin-gold (Alb-Au)<sup>1</sup> complex perfused in situ, it was demonstrated that capillary endothelium of murine heart, lung, diaphragm (9), skeletal muscle, and adipose tissue contains specific albumin-binding sites (ABS) mainly restricted to plasmalemmal vesicles. ABS seem to provide a mechanism for the transendothelial transport of albumin and the molecules it carries by a receptor-mediated process (9, 26, 27). In contradistinction, in the endothelia of arteries, arterioles, muscular venules, veins, endocardium, and fenestrated capillaries examined, ABS appeared to be poorly expressed, albumin being transported at relatively lower rate predominantly by a nonspecific fluid phase transport (9, 26, 27).

In an attempt to define the endothelial membrane proteins that bind albumin and may be instrumental in its carrier-mediated uptake and transcytosis, we tried to identify the

presence of such proteins in those endothelial cells which in situ bind specifically the Alb-Au complex. In this paper we report the identification by the ligand blotting technique (5) of albumin-binding proteins (ABPs) in endothelial cells and tissues which in vivo take up and transport albumin by a receptor-mediated process. A preliminary account of these findings was presented in (27).

## Materials and Methods

### Animals

Experiments were carried out on adult male animals: RAP mice 25–30 g weight, R rats weighing 150–200 g and New Zealand rabbits 2–3 kg body weight.

### Reagents

Special chemicals and supplies were obtained from the following sources: crystallized BSA from Miles Diagnostics (Kankakee, IL) and Serva (Heidelberg, FRG); polyethylene glycol (PEG) 20,000 from J. T. Baker Products (Phillipsburg, NY); tetrachloroauric acid, urea, sucrose, and white phosphorus from Merck (Darmstadt, FRG); iodogen from Pierce Chemical Co. (Rockford, IL); carrier free  $Na^{125}I$  from Institute of Atomic Energy (Otwock-Swierk, Poland); sodium deoxycholate from Koch-Light (Colnbrook, England); Pharmalyte 3–10 and Percoll from Pharmacia Biotechnology (Uppsala, Sweden); sorbitol, isoelectric focusing marker kit, FCS, histamine, heparin, endothelial cell growth supplement from Sigma Chemical Co. (St. Louis, MO); collagenase type I Worthington from Biochrom KG (Berlin, FRG); culture medium 199 from Gibco (Grand Island, NY); nitrocellulose membrane filters BA-85 0.45  $\mu$ m from Schleicher and Schuell (Dassel, FRG); low molecular weight standards from Merck or Pharmacia Biotechnology; and X-ray films from Eastman-Kodak (Rochester, NY), and Azo-Mures (Tg Mures, Romania). Monomeric (mAlb) and polymeric albu-

A preliminary report of this work was presented at the Fourth World Congress for Microcirculation, Tokyo, Japan, July 26–31, 1987, and published in its Proceedings (see reference 27), and at the EMBO-INSERM Workshop "Cell Polarity," Bendor, France, September 6–11, 1987.

1. *Abbreviations used in this paper:* ABP, albumin binding proteins; ABS, albumin-binding sites; Alb-Au, albumin-gold complex; MEs, microvascular endothelial cells; [<sup>125</sup>I]Alb, radioiodinated albumin; PVP, polyvinylpyrrolidone.

min (pAlb) were prepared from commercial available BSA by gel filtration on Sephadex G-200. Bovine immunoglobulin G, albumin-free, was kindly prepared by Dr. G. Szegli (Dr. I. Cantacuzino Institute, Bucharest, Romania) and by V. Partenie in our laboratory. All other chemicals used were of analytical grade.

## Tracers

**Gold Conjugates.** Alb-Au, bovine immunoglobulin G-gold (IgG-Au), and polyethyleneglycol-gold (PEG-Au) complexes were prepared as previously described (9). The tracers were diluted so as to give a concentration corresponding to  $A\ 1.0\ \text{cm}/515\ \text{nm} = 1.0$ .

**Radiiodinated Albumin.** Monomeric and polymeric albumins were radioiodinated by using iodogen ( $10\ \mu\text{g}/100\ \mu\text{g}$  of protein) in the presence of  $200\ \mu\text{Ci}\ \text{Na}^{125}\text{I}$  (7). Unbound iodine was removed by chromatocentrifugation (34) and extensive dialysis against distilled water (4–5 changes) and Dulbecco's PBS (2–3 changes). The specific radioactivity obtained was  $0.14\text{--}1.0\ \text{mCi}/\text{mg}$  of protein.

## Experimental Procedure

Based on the previous *in situ* detection of specific ABS expressed by certain capillary endothelia (9, 26, 27) we tried to identify the putative ABPs in capillaries isolated from rat adipose tissue and the lung, the capillaries of which are provided with ABS. As controls, we used freshly isolated rabbit aortic endothelial cells which *in vivo* appeared not to express detectable amounts of ABS.

In addition, adipocytes, reportedly provided with ABS (2) were also examined.

As general protocol, the cells or tissue extracts were subjected to homogenization, solubilization, gel electrophoresis and electroblotting on nitrocellulose membrane. The transfer strips were incubated with either Alb-Au,  $^{125}\text{I}$  monomeric albumin,  $^{125}\text{I}$  polymeric albumin and other ligands or reagents required for various control experiments.

## Cell Isolation

**Microvascular Endothelial Cells (MEs).** MEs were isolated from rat epididymal fat pads according to (3) and (35). Since *in situ* neither arterioles nor venules larger than  $20\ \mu\text{m}$  showed a significant affinity for Alb-Au (26, 27), we consider that, although working on microvascular segments, the emerging findings virtually reflected the ligand interactions with the endothelium of capillaries and small postcapillary venules. MEs were freshly used either as suspensions or seeded on plastic Petri dishes precoated with 1% gelatin. The culture medium 199 was supplemented with 15% FCS, 2 mg/ml histamine, 100  $\mu\text{g}/\text{ml}$  heparin, 20  $\mu\text{g}/\text{ml}$  endothelial cell growth supplement, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. In addition to their aspect in phase contrast microscopy and electron microscopy, the endothelial cells were identified by the presence of factor VIII-related antigen (14), angiotensin converting enzyme (4) and by their ability to incorporate acetylated low density lipoprotein (33). Suspensions of MEs were devoid of adipocytes but contained up to 15% pericytes as contaminant. To check whether isolated MEs express ABS (as observed *in situ*) (26), aliquots of freshly separated ME were incubated for 10 min with Alb-Au complex in PBS, then fixed and prepared for electron microscopy (9).

**Arterial Endothelial Cells.** Arterial endothelial cells were obtained from rabbit aortae previously flushed with PBS; endothelium was mechanically removed (together with the intima) and freshly used (as negative controls). Adipocytes were isolated from rat epididymal fat pads and cultured after the procedure indicated in (2) and (10) and used as positive controls.

## Preparation of Cell Extracts

After the culture medium was discarded, the cell suspensions or the monolayers (scraped) were washed with PBS, then collected in PBS containing 1% SDS, 0.001 M  $\text{CaCl}_2$ , 0.005 M benzamidine and  $0.25 \times 10^{-3}$  M phenylmethylsulfonyl fluoride (PMSF); homogenization for 1 min with an Ultrasonic Cell Disruptor (Heat Systems-Ultrasonics Inc., Plainview, NY) using the Microtip with the amplitude at setting 6. The homogenate was centrifuged at 13,500 g for 5 min in a Beckman Microfuge B and the supernatant used for electrophoresis or protein determination.

## Preparation of Lung Extracts

Experiments conducted in mouse, rat, and rabbit followed a similar protocol. After general anesthesia and laparotomy, the vasculature was washed

out of blood by perfusion with PBS at  $37^\circ\text{C}$  for 3–5 min at a flow rate of 3 ml/min, using the abdominal aorta as inlet and the punctured abdominal vena cava as outlet. In addition, the lung was perfused via pulmonary artery (catheterized through the right ventricle). In some experiments, the airways were cleared of macrophages by bronchial lavage. The collected lungs were rinsed in PBS containing 1% SDS, 0.001 M  $\text{CaCl}_2$ , 0.005 M benzamidine and  $0.25 \times 10^{-3}$  M PMSF, minced and homogenized for 1 min in an UltraTurax homogenizer at maximum speed. Homogenization was completed with an Ultrasonic Cell Disruptor at setting 6, for 2–3 min. Homogenates were centrifuged for 5 min at 13,500 g and the supernatants used for protein assay and electrophoresis.

## Protein Content

This was determined with the BCA Protein assay reagent from the Pierce Chemical Co. (Rockford, IL) using BSA as standard (24). To determine the amount of ABP on blots, the nitrocellulose strips were stained with Amido Black 10B, cut, the bound stain eluted and the O.D. read at 630 nm.

## Solubilization

To determine the most suitable extraction buffer system, lung fragments were solubilized in various detergents, all at 1% concentration in PBS. The effects of SDS (as denaturing detergent) and *n*-octyl- $\beta$ -D-glucopyranoside (as nonionic detergent) were tested.

## One-dimensional Electrophoresis

Homogenate samples were concentrated by precipitation with TCA at 6–10% final concentration in the presence of 0.015% sodium deoxycholate. After 10–30 min, samples were centrifuged at 13,500 g for 5 min, the supernatant decanted and the pellet resuspended in the electrophoresis solubilization buffer. Concentrated homogenates were supplemented with 2 parts of electrophoresis solubilization buffer.

For sample preparation we have comparatively used various electrophoresis schemes for lung solubilization: (a) nondissociating vs. dissociating buffer systems, (b) nondenaturing vs. denaturing detergents and (c) non-reducing vs. reducing conditions.

The nondissociating buffer system consisted of either PBS, pH 7.4, 0.4 M NaCl, 0.005 M Tris-HCl buffer containing 0.001 M  $\text{MgCl}_2$ , pH 7.4, 0.09 M Tris, 0.08 M  $\text{H}_3\text{PO}_4$  buffer containing 0.003 M  $\text{Na}_2\text{EDTA}$  pH 8.35, or 0.05 M Tris-0.38 M glycine, pH 8.3. Electrophoresis was carried out on precast 2–16% polyacrylamide slab gels under the conditions indicated by the manufacturer (Pharmacia Biotechnology), or on home made 4–10% polyacrylamide slab gels in 0.38 M glycine-0.05 M Tris buffer, pH 8.3. To perform cathodic disc-PAGE system, murine lung was homogenized in 0.35 M  $\beta$ -alanine, 0.14 M acetic acid, pH 4.5. The electrophoresis was done at 125 V on 2–16% precast polyacrylamide slab gels, which were subsequently silver stained (11) or electroblotted (as further described).

The dissociating buffer system mostly used was 0.198 M Tris 0.107 M  $\text{H}_3\text{PO}_4$  buffer, 4.2% SDS, 0.005 M  $\text{Na}_2\text{EDTA}$ , 0.021 M dithiothreitol (DTT) and 0.42 M sucrose pH 6.7. For solubilization of TCA-precipitated samples we also used 0.1 M phosphate buffer, pH 7.0, containing 1% SDS, 1%  $\beta$ -mercaptoethanol and 6 M urea. Electrophoresis was conducted according to (18); the acrylamide concentration of the resolving gel was 5–15% and of the stacking gel 3.75%. The gels (1–3-mm thick;  $13 \times 17\ \text{cm}$ ) were subjected to electrophoresis overnight at 15 mA.

Nondenaturing nonionic detergents such as *n*-octyl- $\beta$ -D-glucopyranoside (25) and denaturing ionic detergents such as SDS with or without urea, were comparatively tested. Since the electrophoretic patterns obtained with these detergents were not significantly different, in most cases we used 1% SDS in PBS or in 0.01 M Hepes, pH 7.4.

Nonreducing vs. reducing conditions were used to test the effect of sulfhydryl-reducing agents on the electrophoretic mobility and the binding activity of ABP. To this intent, 1%  $\beta$ -mercaptoethanol or 0.021 M (DTT) was included in the electrophoresis buffer and before electrophoresis the samples were heated or not for 5 min at  $100^\circ\text{C}$ , in the absence or presence of  $\beta$ -mercaptoethanol.

Gels were calibrated using the Pharmacia low molecular weight kit. In some experiments, the standards were iodinated with  $600\ \mu\text{Ci}\ \text{Na}^{125}\text{I}$  in the presence of 60  $\mu\text{g}$  iodogen in 0.1 M borate buffer, pH 8 (7). Molecular weights were determined by the calibration curve methods indicated in (6) and (35). After electrophoresis, the gels were either silver-stained (11) or stained with 0.2% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid. Gel destaining of Coomassie Blue was performed either by passive diffusion of the stain in 25%  $\text{CH}_3\text{OH}$ , 10%  $\text{CH}_3\text{COOH}$  or in a

gel destainer GD-4 (Pharmacia Biotechnology). The stained gels were dried and photographed; the densitometric profiles were traced with a Chromscan apparatus (Joyce, Loebel & Co. Ltd., Gateshead, England).

### ***Isoelectric Focusing***

This assay was conducted on 4% T + 3% C polyacrylamide gels (12 × 15 cm) containing 2% Pharmalyte 3-10 using 0.05 M H<sub>2</sub>SO<sub>4</sub> and 1 M NaOH as electrode solutions. After 30 min prefocusing, the samples (10–20 μl) were applied on the gel surface near the middle of the gel with the aid of paper applicators. The pH gradient was determined using the following pI markers: amyloglucosidase (from *Aspergillus oryzae*) pI 3.55, trypsin inhibitor (from soybean) pI 4.55, α-lactoglobulin A (from bovine milk) pI 5.13, carbonic anhydrase (from bovine erythrocytes) pI 5.85, carbonic anhydrase (from human erythrocytes) pI 6.57, myoglobin (from horse heart) pIs 6.76 and 7.16, L-lactic dehydrogenase (from rabbit muscle) pIs 8.3, 8.4, and 8.5, and trypsinogen (from bovine pancreas) pI 9.3. After 3,000 volthours, the gels were equilibrated for 15 min in Tris-borate buffer, pH 8.3, placed between two nitrocellulose sheets and the electrophoretic transfer of proteins was performed as described below.

### ***Two-dimensional Electrophoresis***

The isoelectric focusing in the first dimension was carried out on 1% agarose IEF gels containing 6% sorbitol, 6% sucrose and 2% Pharmalyte 3-10. The electrode solutions were 0.01 M H<sub>2</sub>SO<sub>4</sub> and 1 M NaOH. 50 μl of rabbit lung homogenate containing 500 μg protein in 0.005 M Tris-HCl buffer, pH 7.4, were applied on the gel surface. After 2,000 volthours, a strip from the agarose gel equilibrated for 30 min in an SDS sample buffer (10% [wt/vol] glycerol, 5% β-mercaptoethanol, 2.3% SDS, 0.0625 M Tris-HCl pH 6.8), was placed on top of a 5–15% polyacrylamide slab gel for electrophoresis in the second dimension. The gel was calibrated with the radioiodinated molecular weight standards. The proteins were transferred to nitrocellulose membrane, preincubated with bovine IgG for 12 h and incubated with Alb-Au for 1 h. The dried nitrocellulose sheet was exposed to X-ray films.

### ***Electroblotting***

The transfer of electrophoretically separated proteins on nitrocellulose membranes was performed in 0.041 M Tris-0.040 M boric acid buffer pH 8.3 for 150 min at 200 mA in an electroblotting chamber (8). The strips were dried and used for either staining with 1% Amido Black 10B in 7.5% acetic acid or for incubation with ligands.

### ***Incubation of Nitrocellulose Strips with Albumin Conjugates***

Before incubation with albumin conjugates, the blots were washed 3 × 10 min with PBS containing 0.001 M CaCl<sub>2</sub>, then incubated with 1–2 mg/ml bovine IgG in PBS for 12 h to prevent nonspecific binding. Unless specified, IgG was present in all incubation steps. The strips were further incubated for 1 h at 22°C with one of the following ligands: (a) Alb-Au solution in PBS, at A 1.0 cm/515 nm = 1.0; (b) [<sup>125</sup>I]monomeric Alb in PBS, followed by autoradiography; (c) [<sup>125</sup>I]polymeric Alb in PBS, with subsequent autoradiography.

For a better visualization of Alb-Au binding to the ABP bands, alternatively the silver enhancement procedure recommended by Janssen Life Science Products (Beerse, Belgium) was applied.

### ***Characterization of ABP-binding Properties***

Binding assays were carried out on small segments of the nitrocellulose strips containing the ABP peptides, using a procedure adapted from (19). After electrotransfer, two strips from the lateral edges of the nitrocellulose sheets were cut out, incubated with either 2 mg/ml bovine IgG or 2% polyvinylpyrrolidone (PVP) in PBS, and stained with Alb-Au in the presence of the quencher. After aligning these strips with the remaining nitrocellulose sheet, small segments of equivalent area (0.5 cm × 0.5 cm) that correspond to the 31- or the 18-kD peptides, were excised and used for the following binding assays.

**Saturability.** Nitrocellulose segments obtained as described above were incubated with 2 mg/ml bovine IgG in PBS for 12 h at room temperature. Subsequently, the segments were incubated for 60 min with increasing concentrations (from 9 nM to 273 nM) of [<sup>125</sup>I]Alb, washed 3 × 30 min with 2 mg/ml bovine IgG in PBS, and counted. The nonsaturable nonspecific

ligand binding was determined by conducting the incubations in the presence of a 100-fold excess of unlabeled albumin. The saturable binding was estimated by measuring the difference in detected ABP-bound radioactivity in the presence and absence of excess unlabeled albumin. The specific binding was calculated by subtracting nonspecific binding from total binding. The data for specific binding were then used for constructing double-reciprocal plot to estimate the dissociation constant (*K<sub>d</sub>*).

**Competition.** ABP-containing nitrocellulose segments were incubated for 1 h with 9.4 nM of [<sup>125</sup>I]Alb in the presence of increasing concentrations of unlabeled albumin (from 15 × 10<sup>-8</sup> to 44 × 10<sup>-6</sup> M). After 3 30-min washes with 2 mg/ml bovine IgG in PBS, the remaining radioactivity bound to either the 31- or 18-kD was counted.

**Binding Kinetics.** The pH sensitivity of albumin binding to the ABP peptides was assessed by incubating the nitrocellulose strips with [<sup>125</sup>I]Alb in 2% PVP in 0.1 M phosphate buffer at pH ranging from 5.0 to 9.0.

The time-dependence of Alb binding to the ABP peptides was determined by incubating nitrocellulose strips with [<sup>125</sup>I]Alb in 2% PVP in PBS for periods of 20, 40, 60, and 80 min. After 3 30-min washes in PBS, the retained radioactivity was counted.

**Binding Specificity.** In addition to competition experiments, binding specificity was tested by incubating electroblotting strips of lung extracts with either IgG-Au or PEG-Au at A 1.0 cm/515 nm = 1.0.

**Effect of High Ionic Strength.** To determine whether the [<sup>125</sup>I]Alb or Alb-Au binding to ABP is purely due to a nonspecific electrostatic interaction, electroblotting strips were incubated with either radioiodinated albumin or Alb-Au in PBS containing 2 mg/ml IgG in the presence of 0.45 to 2 M NaCl (final concentration). In other experiments, NaCl was applied after the ligand was bound to the ABP bands.

**Effect of Heparin.** Electroblots incubation with Alb-Au was carried out in the presence of 4 mg/ml heparin (activity = 500 UI/ml).

### ***Detection of ABP Exposure on Endothelial Cell Surface***

The membrane proteins exposed on the cell surface of MEs were radioiodinated using Na<sup>[125]I</sup> and iodogen as oxidizing reagent. The radiolabeled cells were then processed for electrophoresis followed by incubation with Alb-Au and autoradiography.

**Radioiodination.** The procedure used was adapted from (7) and (33). Suspensions of MEs maintained in serum-free culture medium for 24 h at 4°C were pelleted by 10 min centrifugation at 170 g, washed twice by resuspension in PBS, and harvested by centrifugation as above. The pellet was suspended in 1 ml of 0.1 M borate buffer pH 8.0 containing 530 μCi of carrier-free Na<sup>[125]I</sup>. The mixture was transferred to test tubes coated with 120 μg iodogen and the reaction was carried out for 20 min at 4°C. Since the oxidizing agent (iodogen) was immobilized on the wall of the test tube, the iodination could occur only at the surface of the cells. The reaction was stopped by transferring the suspension to centrifuge tubes containing 0.1 M KI in PBS (vol/vol). Cells were pelleted for 5 min at 200 g and resuspended in 1 ml PBS. Proteins were extracted and precipitated overnight at 4°C in 0.015% sodium deoxycholate and 10% TCA containing 0.1 M KI. The precipitated proteins were pelleted by 10 min centrifugation at 13,500 g and washed twice to be cleared from unreacted radioiodide.

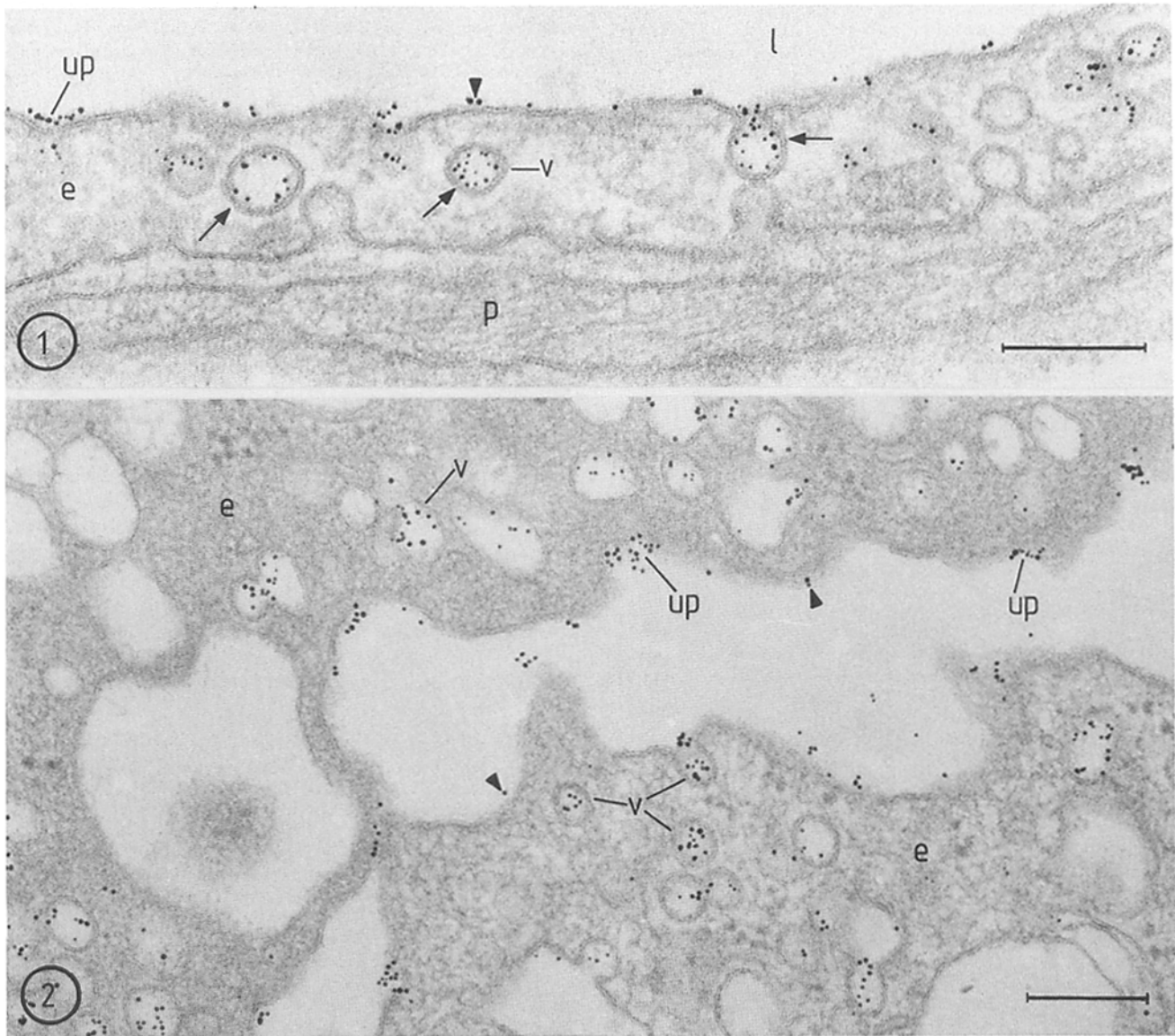
**Electrophoresis, Blotting, and Autoradiography.** The labeled proteins were solubilized in 0.1 M phosphate buffer, pH 7, containing 1% SDS, 1% β-mercaptoethanol and 6 M urea and were run on 5–15% SDS-PAGE as above. The gels were either used for electroblotting or stained with Coomassie Blue R-250, air-dried and exposed to X-ray films.

Detection of albumin binding proteins was carried out by blot incubation with Alb-Au, as described above. The blots were then washed with PBS for 5 min, dried between filter papers and exposed to X-ray films at -70°C for different periods of time according to the level of radioactivity.

## ***Results***

### ***Detection of ABS on Isolated Capillary Endothelium***

We have previously shown that Alb-Au perfused in situ binds specifically to the plasmalemmal vesicles of capillary endothelium of the lung, heart, muscle and adipose tissue (9, 26, 27). Fig. 1 shows an example of in situ Alb-Au binding to capillary endothelium of adipose tissue. The occurrence of specific and restrictively located ABS made these endo-



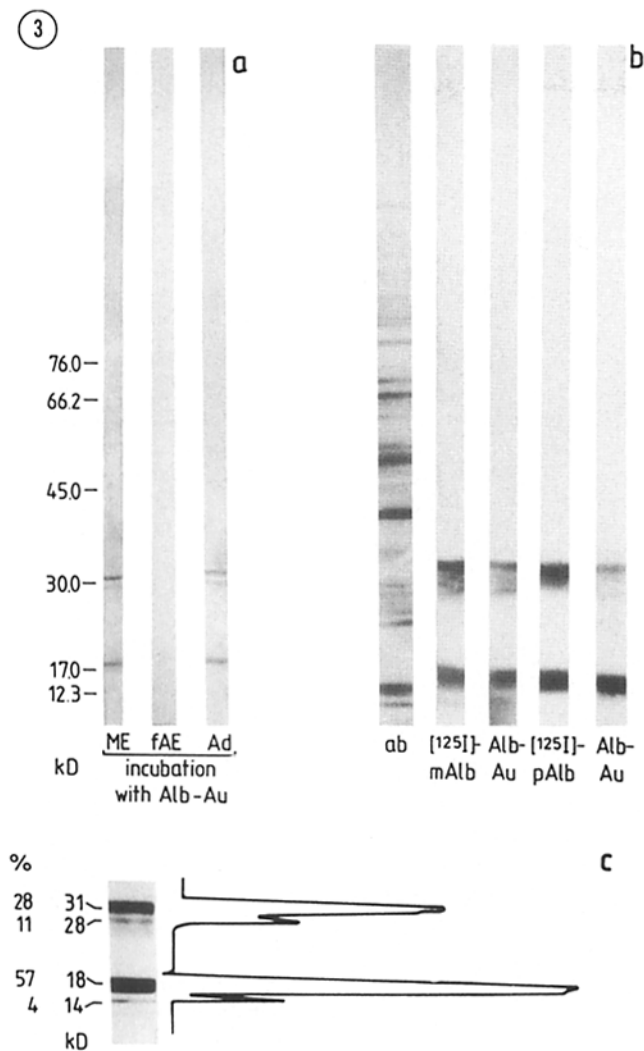
**Figures 1 and 2.** (Figure 1) Capillary endothelium of adipose tissue after 3 min in situ perfusion of Alb-Au: tracer particles occur only occasionally on plasma membrane (*arrowhead*) but bind avidly in one or two rows (*arrows*) on uncoated pits (*up*) and virtually all plasmalemmal vesicles (*v*) apparently associated with the luminal front. (*l*) Lumen; (*p*) pericyte; (*e*) endothelial cell. (Figure 2) Microvascular endothelial cells freshly isolated from rat epididymal fat and incubated for 3 min with Alb-Au: numerous plasmalemmal vesicles (*v*) are labeled by tracer particles, predominantly in an adsorptive pattern. Some particles are bound to uncoated pits (*up*) and rarely appear on plasma membrane proper (*arrowhead*). (*e*) Endothelial cell. Bars, 0.2  $\mu$ m.

thelia the main choice as starting material for the identification of putative ABPs. MEs freshly isolated from rat epididymal fat appeared mostly as small sheets of cells; they were used either in suspension or as primary culture. When suspensions of ME were incubated with Alb-Au for 10 min, a large number of plasmalemmal vesicles, especially those associated with the former luminal front, were characteristically labeled in an adsorptive pattern by the tracer particles which were not removed by extensive washing with PBS (Fig. 2). As in situ, binding appeared to be restricted to capillary endothelium (small vascular tubes <6- $\mu$ m wide) and was negligible in endothelia of larger vessels containing Weibel-Palade bodies (presumably arterioles and venules). This indicated that isolated and freshly cultured capillary en-

dothelia largely maintain their ABS. In cultured MEs the main contaminants were pericytes which could represent up to 15% of the cell population; no adipocytes were detected in these preparations.

#### Detection of ABPs on Electroblothing Strips

**Cell Extracts.** In Fig. 3 extracts of (MEs), arterial endothelium freshly isolated from the rabbit aorta (fAE), as well as isolated adipocytes (Ads) were analyzed by SDS-PAGE. After transfer on nitrocellulose membrane, the blots were incubated with Alb-Au. The ME lane revealed two pairs of bands which strongly bound Alb-Au and became clearly visible due to their red color. As shown by Fig. 3, the poly-



**Figure 3.** (a) Detection of ABPs by ligand blotting of SDS-gel extracts subjected to electrophoresis extracts of microvascular endothelial cells (MEs), freshly isolated rabbit aortic endothelium (fAE) and adipocytes (Ad). After strips incubation with Alb-Au, two sets of albumin-binding band with an apparent molecular mass of 31 and 18 kD appeared in ME and Ad lanes, but not in the fAE track. (b) Lung extract electrotransferred. The first strip stained with Amido black 10B revealed the broad spectrum of proteins transferred. Two strips were incubated with radioiodinated monomeric or polymeric albumin and autoradiographed (lanes marked  $[^{125}\text{I}]m\text{Alb}$  and  $[^{125}\text{I}]p\text{Alb}$ , respectively), and two other similar strips were overlaid with albumin-gold complex (lanes marked *Alb-Au*). Note the superposition of the two peptides revealed by autoradiography with those labeled by Alb-Au, as well as between the bands obtained with monomeric and polymeric Alb. (The blots were purposely overloaded to render more visible the ABP bands and the nonspecific binding, if existent.) (c) Densitometric scanning followed by the determination of the relative contribution (in percentage) of each peptide to the total detected ABPs.

peptides binding Alb-Au consisted of a doublet composed of a major band with an apparent  $M_r$  of 30,000 to 31,000 (hereafter named the 31-kD peptide) and a variable minor band with  $M_r$  28,000. A lower molecular weight doublet was usually represented by a band corresponding to a  $M_r$  of 17,000 to 18,000 (hereafter named the 18-kD peptide) and a variable

minor band with an apparent molecular weight of 16,000. An additional faint band may show up at  $M_r$  14,400 (the 14-kD peptide). The minor variable bands may represent proteolytic degradation products of ABP. Since *in situ* ABS occur restrictively on capillary endothelium, it is likely that the ABP detected in the ME homogenates originate primarily or solely from capillary endothelial cells. With this procedure and using the same amount of protein applied on the SDS-gel, no detectable ABPs were revealed in the extract of aortic endothelial cells; such bands were present, however, in the extracts of adipocytes (Fig. 3). The latter occurrence is in agreement with previous reports on the existence of albumin receptors on the Ad plasma membrane (2, 20).

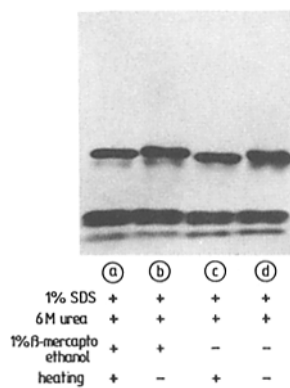
**Lung Extract.** In the heart or lung extracts, prepared as described, Alb-Au-binding peptides similar to those found in MEs were detected. Since in the heart homogenates, ABPs may originate from myocytes, for our experiments we used lung extracts only (as an alternative to MEs of adipose tissue) (15, 16, 23). The ABP peptides were labeled both by radioiodinated monomeric albumin ( $[^{125}\text{I}]m\text{Alb}$ ) or polymeric albumin ( $[^{125}\text{I}]p\text{Alb}$ ) with a pattern superposable to that resulted upon Alb-Au incubation (Fig. 3 b). The removal of macrophages by bronchial lavage did not significantly change the electrophoretic pattern and ligand affinity of the electrotransferred ABP peptides. Since *in situ* the bronchial and alveolar epithelium did not show any special affinity for the Alb-Au complex infused intratracheally (Simionescu, M., manuscript in preparation), it was assumed that in the lung extract the detected ABP originate mainly from capillary endothelia.

Densitometric profiles of extracts of murine lung blots incubated with Alb-Au gave an approximation of the fractional contribution of the four ABP peptides. The major component appears to be the 18-kD peptide that amounts to  $\sim 57\%$ ; the 31-kD peptide accounts for  $\sim 27\%$ , while the 28- and the 14-kD bands represent only 10% and 4%, respectively (Fig. 3 c).

#### **ABP Electrophoretic Pattern as a Function of Solubilization Conditions**

**Nondissociative vs. Dissociative Conditions.** Lung fragments which were homogenized in buffer systems devoid of detergent, upon electrophoresis, transfer on nitrocellulose, and incubation with Alb-Au, gave a band at the top of the resolving gel. This was taken as an indication that ABP material was present in the crude homogenate but was not dissociated from the membrane, and as such, it penetrated minimally the polyacrylamide gel. When similar lung homogenates were centrifuged for 1 h at 114,000 g in a Beckman SW 50.1 rotor and the supernatant processed as above, no Alb-Au-reacting bands were visualized on the electroblotting strips; the ABP remained associated with the membrane material spun down in the pellet. It was concluded that in the mild conditions used for extraction (low to high ionic strength at neutral or slightly alkaline pH), ABP cannot be obtained in soluble lipid-free form, presumably due to strong hydrophobic interaction with other membrane components.

In the cathodic electrophoresis system followed by electroblotting, the absence of detectable amounts of Alb-Au-binding peptides suggested that ABPs have a low solubility in aqueous solutions, their solubilization requiring detergents.



**Figure 4.** Rabbit lung extract subjected to electrophoresis on 5–15% polyacrylamide gel under reducing and nonreducing conditions. After electroblotting, the nitrocellulose strips were incubated with Alb-Au. The conditions used for sample solubilization are indicated in the lower part of the figure. When the SDS-urea-β-mercaptoethanol mixture was heated before electrophoresis (lane *a*), the mobility of the 31-kD peptide was slightly faster than in lanes *b* and *d*. Same increase in

the mobility of this peptide was obtained by only heating the sample without addition of β-mercaptoethanol (lane *c*). When the SDS-urea solution was supplemented with β-mercaptoethanol alone (lane *b*), the apparent molecular weight and binding activity of the ABP peptides was similar to those of the control (standard procedure) (lane *d*).

**Nondenaturing vs. Denaturing Conditions.** When lung fragments were solubilized in denaturing (SDS) or nonionic detergents (n-octyl-β-D-glucopyranoside), the electrophoretic patterns obtained were similar. Accordingly, either 1% SDS (in most cases) or 1% n-octyl-β-D-glucopyranoside in PBS or 0.01 M Hepes pH 7.4 were alternatively used in the extraction buffers. Heating of the samples for 5 min at 100°C resulted in a slight increase in the electrophoretic mobility of the 31-kD polypeptide (Fig. 4) without significantly affecting the ABP-binding properties. This effect occurred only when urea was added to the SDS gel. The presence of urea in the SDS-electrophoretic buffer without heating did not significantly change ABP mobility or its binding of albumin. These observations were taken as a suggestion that SDS-denatured ABPs maintain to a large extent their binding affinity for albumin.

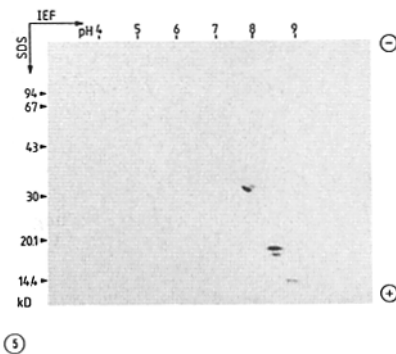
**Nonreducing vs. Reducing Conditions.** Addition of sulfhydryl reducing agents such as β-mercaptoethanol or DTT did not induce significant changes in the ABP electrophoretic mobility or binding activity (Fig. 4). This indicated that disulfide bonds, if existent in the ABP molecules, are in very low number and are not required for the expression of the specific binding properties.

### Isoelectric Focusing

After the electrophoretic transfer of proteins to nitrocellulose membranes and incubation with Alb-Au, five bands were stained by the ligand. Three bands appeared labeled on the anodic sheet: two soluble ABP bands correspond to pIs of 8.05 and 8.15 and a third band (insoluble ABP) remained in start. On the cathodic nitrocellulose membranes, two soluble ABP bands of pIs 8.55 and 8.75, respectively, were stained by Alb-Au.

### Two-dimensional Electrophoresis

By applying the ligand blotting with Alb-Au to two-dimensional isoelectric focusing/SDS polyacrylamide gels it was found that the 31-kD peptides correspond to the isoelectric points of 8.05 and 8.15, the 18-kD peptides have an isoelectric point of 8.55 and the 14-kD peptide a pI of 8.75. No other

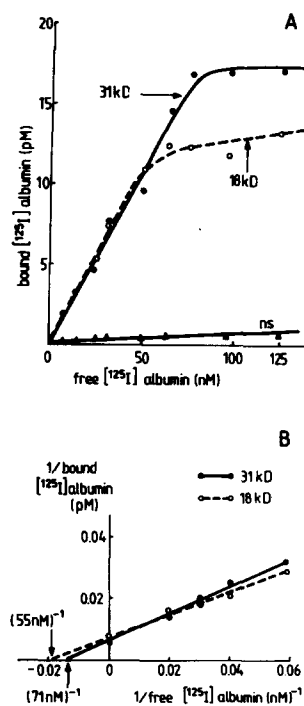


**Figure 5.** Two-dimensional electrophoresis and ligand blotting of ABPs. Rabbit lung homogenate (500 μg protein) was subjected to two-dimensional electrophoresis, electrophoretic transfer and incubation with Alb-Au. The isoelectric points of ABPs visualized by the ligand binding are: pI 8.05 for the 31 kD, pI 8.15 for the 33 kD, pI 8.55 for the 17 kD and 8.75 for the 14-kD peptides. Molecular mass standards are indicated.

peptides resolved by this technique showed affinity for Alb-Au (Fig. 5).

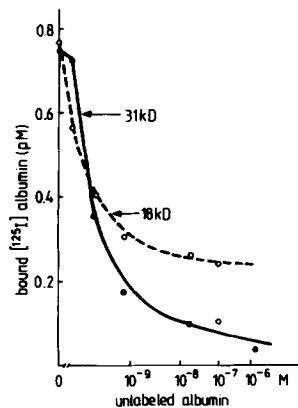
### Binding Characteristics of the ABP Peptides

**Saturability.** The binding of radioiodinated albumin to the electrophoretically resolved and transferred ABP peptides, was saturated at  $80 \times 10^{-9}$  M concentration of labeled ligand. The profiles of the saturation curves were similar for the two sets of ABP peptides. At all concentrations used, the



**Figure 6.** Saturation of [<sup>125</sup>I]-albumin binding to the ABP peptides subjected to electrophoresis and transferred on nitrocellulose sheets. (A) Concentration dependency of [<sup>125</sup>I]-albumin binding; (B) Double reciprocal plot constructed with the data in A. Murine lung homogenate was solubilized and aliquots containing 1.4 mg protein were applied to a preparative SDS-polyacrylamide gel (12.3-cm wide well). After electrophoresis and transfer to nitrocellulose sheets, 0.5 cm × 0.5 cm slabs corresponding to the 31- and 18-kD (detected with Alb-Au on the marginal strips) were excised. Slabs were incubated with 2 ml of increasing concentrations of [<sup>125</sup>I]albumin (0.14 μCi/μg protein) for 1 h at room temperature. The diagram in A shows the values of the saturable binding corrected for background by subtracting the nonsaturable binding (*ns*) measured in the presence of a 100-fold excess of unlabeled albumin from the total binding.





**Figure 7.** Competition for [<sup>125</sup>I]-albumin binding to the ABP peptides by unlabeled albumin. Nitrocellulose strips containing 31 and 18 kD were obtained as described in the legend to Fig. 6. Strips were incubated in PBS containing 9.4 nM [<sup>125</sup>I]Alb, 2 mg/ml bovine IgG and the indicated concentrations of unlabeled albumin. After 1 h at room temperature, the medium was removed and the strips washed three times with PBS containing IgG, and the remained radioactivity was counted.

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values of the nonsaturable nonspecific binding were low (less than 4% of the total binding) and were ascribed to a straight line (Fig. 6 A). Analysis of the binding values in Fig. 6 A by double reciprocal plots gave, from the abscissa intercepts, an apparent equilibrium dissociation constant  $K_d$  of  $55 \times 10^{-9}$  M and  $71 \times 10^{-9}$  M for the 18-kD and 31-kD peptides, respectively (Fig. 6 B).

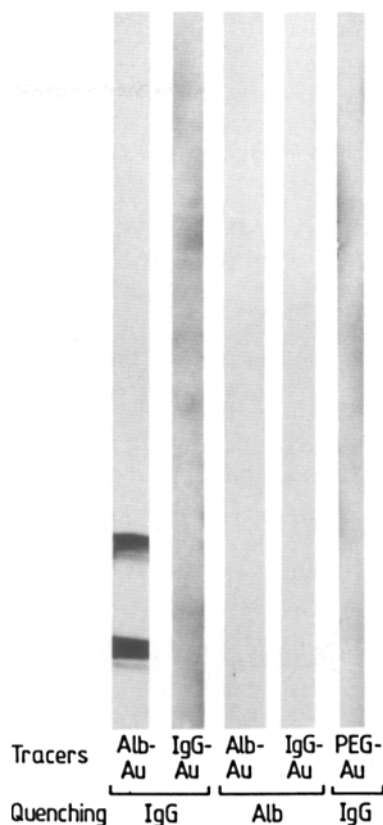
**Competition.** When the electroblots exposed to the same concentration of radioiodinated albumin (9.4 nM) were competed for 60 min with increasing concentrations of unlabeled albumin, the [<sup>125</sup>I]Alb binding was inhibited by 95% by concentrations of unlabeled albumin as low as  $44 \times 10^{-6}$  M (Fig. 7).

**Binding Kinetics.** The data obtained showed that the electrophoretically resolved and transferred ABP peptides bound radioiodinated albumin in a pH-dependent manner. The highest binding values occurred at pH 5.0 (close to the pI of albumin). At pH 8.0, close to the pI of ABP peptides, the [<sup>125</sup>I]Alb binding reached half the values recorded at pH 5.0. In our experiments the incubations were performed at pH 7 within the range of the physiological pH in plasma where 64% of the highest binding occurred. When the strips were incubated with the same concentration of [<sup>125</sup>I]Alb, but for different time intervals, the binding reached a steady-state plateau at 1 h (that was used as standard incubation period in our experiments).

**Specificity.** On the nitrocellulose strips no protein was detected if IgG-gold or PEG-gold were used as ligands or when the blots were quenched with albumin before the ABP detection with Alb-Au, or control incubation with IgG-Au (Fig. 8). The experiments with radioiodinated monomeric or polymeric albumin showed that the ABP peptides bound each of these ligands as well as the Alb-Au complex (Fig. 3 b).

**Effects of High Ionic Strength and Heparin.** The [<sup>125</sup>I]-Alb binding to the ABP peptides could not be either prevented or displaced by NaCl in concentration up to 2 M when it was either added in the incubation medium concomitantly with the ligand or was applied after the radiolabeled Alb was bound to ABP. Similar results were obtained when Alb-Au was used as ligand.

Heparin at a concentration of 4 mg/ml: 500 UI/ml did not impair the Alb-Au [A 1.0 cm/515 nm = 1.0] binding to the ABP bands after a 1-h incubation.



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**Figure 8.** Tests for assessing the specificity of Alb-Au binding to the ABPs. Electroblotting strips of murine lung extracts (which were subjected to electrophoresis) upon quenching with Alb or bovine IgG were incubated with Alb-Au, IgG-Au and PEG-Au. Unlike Alb-Au, neither IgG- nor PEG-gold complexes bind to any transferred protein. When quenching was performed with albumin, this fully competed the binding of Alb-Au to the ABP bands.

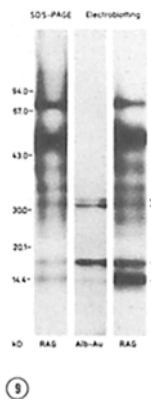
These findings showed that Alb/ABP interaction are not of a pure electrostatic nature.

### **ABPs Are Probably Membrane-Associated Components Exposed on Endothelial Cell-Surface**

The results obtained from MEs whose surface proteins were iodinated and the emerging electroblots exposed to X-ray films and incubated with Alb-Au showed that proteins with the same molecular weight as ABPs are detected on the cell surface of MEs. The 14-kD peptide often displayed the strongest radiolabeling suggesting a content in tyrosine residues higher than the other two sets of ABP peptides (Fig. 9).

### **Discussion**

The existence of an albumin receptor that mediates the uptake of free fatty acids has been reported for the heart myocytes (15, 17, 24), adipocytes (2, 20) and hepatocytes (20, 37, 38). The specificity of the latter has been recently challenged (1, 30, 31, 36, 38). Attempts to identify and isolate the pro-



**Figure 9.** Microvascular endothelial cells isolated from rat epididymal fat were surface radioiodinated, subjected to electrophoresis on 5–15% polyacrylamide gels, and proteins transferred on nitrocellulose membranes. Some strips were autoradiographed (*left lane*), while others were first incubated with Alb-Au (*middle lane*), which revealed the two sets of ABP bands. Subsequently same strips showed in the middle lane were exposed to X-ray films (*right lane*). Note that proteins with the same molecular weight as ABPs are radioiodinated.

tein(s) responsible for the specific cellular uptake by the liver or kidney of the native or chemically modified serum albumin have been only partially successful (12, 13, 22). A hepatitis B surface antigen polypeptide of 31,000 D which binds human and chimpanzee glutaraldehyde-polymerized albumin has been reported (17).

In our experiments, the detection of ABPs by the ligand blotting relied on the ability of this technique to reveal such proteins in crude extracts of cells and tissues when no anti-receptor antibody was yet available. Ligand blotting was shown to be as sensitive as immunoblotting for the identification of receptors such as low density lipoprotein receptor (5).

The visualization of ABPs by ligand blotting was facilitated by several characteristics of these proteins: (a) they retain their binding capacity after SDS-gel electrophoresis and electroblotting on nitrocellulose paper even in the presence of sulfhydryl reducing agent and after heating, (b) they interact strongly with either monomeric or polymeric albumin in radioiodinated or gold-conjugated form; and (c) quenching with IgG or PVP permits extensive washings required for the removal of nonspecifically bound material.

In our ligand blotting survey, ABP were found to be well expressed in the microvascular endothelial cells of the adipose tissue as well as in the lung (presumably due to its ABS-positive capillary endothelium). Because of the ubiquitous role of albumin as carrier for free fatty acids and other molecules, the occurrence of ABPs (in various density and affinity) in other cells has to be expected, but this remains to be investigated. In our preparation conditions, ABP were not detected in extracts of rabbit aortic endothelium. The preliminary observation reported in (27) on cultured bovine arterial endothelium and rat pericytes remained to be confirmed (or infirmed) by using higher protein concentrations applied on gels and transferred on nitrocellulose sheets. The present observations suggest, but not yet proved, that ABPs may represent the specific binding sites for albumin expressed in situ. Experiments to test their possible identity are currently conducted. In SDS-PAGE, the two pairs of ABP polypeptides, 31 and 18 kD, have virtually the same apparent molecular weight and binding affinity in all cells and tissues expressing these proteins. In the case of adipose tissue, lung and heart, this uniformity may be explained by the presence in these organs of ABS-positive capillary endothelia (9, 26, 27).

Since in the intact isolated microvascular endothelial cells ABPs were readily iodinated, one can assume that they are

membrane-associated proteins with the albumin binding sites exposed on their ectodomains.

Final demonstration will be possible when antibodies against the ABP peptides will become available.

As revealed by the densitometric profiles of the electrophoretic bands (not shown), ABPs bind with similar affinity monomeric and polymeric albumin regardless whether the latter was obtained by intermolecular self-aggregation or by adsorption on gold particles. From these results, it may be assumed that—at least in the conditions described—the ABP interaction with its cognate ligand is not significantly dependent on its mono-, or polymeric character or on the presence of the gold tag.

As detected by ligand blotting, the specificity of ABP was indicated by the demonstration that: (a) other gold-conjugated ligands such as IgG-Au or PEG-Au do not bind to the ABP peptides; (b) binding is not of purely electrostatic nature since neither high ionic strength nor heparin are able to displace the ligand; and (c) binding is specifically competed by increasing concentration of free albumin (and not by IgG) in a dose-dependent manner. However, an inquiry of a large number of plasma proteins and cells remains to elucidate how restricted is the ligand specificity and tissue specificity of the ABPs.

In the conditions of the electroblotting technique applied to receptor-ligand interactions (20), the albumin binding to the ABP peptides, subjected to electrophoresis, reaches saturation at  $\sim 80$  nM concentration. A 50% inhibition of ligand binding is achieved by 5.5  $\mu\text{g/ml}$  of competing albumin, suggesting a strong interaction with ABPs ( $K = \sim 60 \times 10^{-9}$  M). We do not know yet how these values compare with those of the ABPs in situ; the latter are under current investigation.

As suggested by the electrophoretic patterns, the ABP cationic peptides may represent dimer or oligomer of nonidentical subunits, noncovalently associated and containing a relatively reduced number (if any) of intramolecular disulfide bonds. The integrity of the latter seems not to be required for the binding activity.

The excellent technical assistance of V. Craciun, I. Andreescu, M. Toader, D. Profir (electrophoresis); F. Georgescu, and D. Taflan (radioassay); I. Manolescu (cell culture), E. Stefan and G. V. Ionescu (photographical work); C. Neacsu and M. Schean (graphics); and D. Neacsu (word processing) is gratefully acknowledged.

The work was supported by the Ministry of Education, Romania, and by the National Institutes of Health (NIH) grant HL-26343.

Received for publication 22 October 1987, and in revised form 7 March 1988.

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