Endothelial Albumin Binding Proteins Are Membrane-associated Components Exposed on the Cell Surface*

(Received for publication, October 11, 1988)

Nicole Ghineaț, Monica Eșkenasy, Maya Simionescu, and Nicolae Simionescu
From the Institute of Cellular Biology and Pathology, Bucharest 76091, Romania

The heterobifunctional, photocross-linking, thiol-cleavable, cross-linker sulfosuccinimidyl 2-(p-azidosalicylamido)ethyl-1,3’-dithiopropionate (SASD) was radioiodinated and used to determine whether endothelial albumin binding proteins (ABP) recently identified (Ghineaț, N., Fixman, A., Alexandru, D., Popov, D., Hasu, M., Ghitescu, L., Eșkenasy, M., Simionescu, M., and Simionescu, N. (1988) J. Cell Biol. 107, 231-239) are plasma membrane-associated components exposed on the cell surface. Microvascular endothelial cells (MEC) freshly isolated from rat epididymal fat were incubated with 125I-2-(p-azidosalicylamido)ethyl-1,3’-dithiopropionate (ASD)-albumin conjugate which upon photolysis by UV light was cross-linked to the receptor proteins. By cleaving the disulfide linkages of the cross-linker with 5% β-mercaptoethanol and the ligand-receptor interactions with 0.1% sodium dodecyl sulfate, the radioiodinated ASD moiety remained attached to the receptor peptides which were further detected by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. In parallel, samples were examined by ligand blotting with albumin-gold complex. The results showed that in these experimental conditions ABP are represented by two major peptides of 31 and 18 kDa and by two minor bands of 73 and 42 kDa. Densitometric scanning showed that the two major bands constitute more than 70% of the total ABP. The four peptides were not apparent if the samples were not UV-irradiated. The binding of the radioiodinated ligand to ABPs was reduced by ~82% in the presence of excess competitive unlabeled albumin. When MEC were incubated with unlabeled SASD and exposed to UV light, the autoradiographic banding pattern obtained was similar to that of either radioiodinated receptor proteins or MEC not treated with SASD. This indicated that the four albumin binding peptides are distinct proteins of the endothelial cell plasma membrane.

Communication

EXPERIMENTAL PROCEDURES

Materials

Chemicals—The special reagents used were obtained from the following sources: SASD and Iodogen from Pierce Chemical Co.; crystallized bovine serum albumin (BSA), hereafter referred to as albumin, from Miles; Sephadex G-25, Sephadex G-200, and Percoll from Pharmacia, Uppsala, Sweden; collagenase Worthington type I from Biochrom, Berlin, Federal Republic of Germany; culture medium M-199 and Dulbecco’s phosphate-buffered saline, pH 7.2 (PBS), from Gibco; dichloromethane and tetrachloroauric acid from Merck, Darmstadt, F. R. G.; carrier-free Na”1 from the Institute of Atomic Energy, Otwock-Swierk, Poland; myoglobin (horse heart), phenylmethylsulfonyl fluoride, high molecular weight standards, benzamidine hydrochloride, fetal calf serum, endothelial cell growth supplement, and all reagents for SDS-PAGE from Sigma; nitrocellulose membrane filters BA-85, 0.45 μm, from Schleicher & Schuell; Azoix

EXPERIMENTAL PROCEDURES

Materials

Chemicals—The special reagents used were obtained from the following sources: SASD and Iodogen from Pierce Chemical Co.; crystallized bovine serum albumin (BSA), hereafter referred to as albumin, from Miles; Sephadex G-25, Sephadex G-200, and Percoll from Pharmacia, Uppsala, Sweden; collagenase Worthington type I from Biochrom, Berlin, Federal Republic of Germany; culture medium M-199 and Dulbecco’s phosphate-buffered saline, pH 7.2 (PBS), from Gibco; dichloromethane and tetrachloroauric acid from Merck, Darmstadt, F. R. G.; carrier-free Na”1 from the Institute of Atomic Energy, Otwock-Swierk, Poland; myoglobin (horse heart), phenylmethylsulfonyl fluoride, high molecular weight standards, benzamidine hydrochloride, fetal calf serum, endothelial cell growth supplement, and all reagents for SDS-PAGE from Sigma; nitrocellulose membrane filters BA-85, 0.45 μm, from Schleicher & Schuell; Azoix...
Endothelial Albumin Binding Proteins

R-2 x-ray films used for gel autoradiography from Azomureș, Tg. Mures, Romania.

Monomeric albumin was prepared from commercial BSA by gel filtration on Sepadex G-200.

Bovine immunoglobulin G, albumin-free, was kindly prepared by Dr. Szegli (Institute Dr. I. Cantacuzino, Bucharest, Romania). All other reagents were of the highest grade.

Abnormal-Gold Complex—Au was prepared as described previously (2).

125I-Albumin—Chromatographically purified monomeric albumin was radioiodinated by using Iodogen (10 μg/160 μg protein) in the presence of 200 μCi of Na125I (10).

Microvascular Endothelial Cells—MEC were freshly isolated from rat epididymal fat pads according to Refs. 11 and 12; these cells express albumin binding sites (8). The cell suspension obtained was devoid of adipocytes and may contain up to a maximum of 15% pericytes as contaminants; in most preparations, the pericytes represented less than 10% of the cell population. We assumed that even if pericytes contain ABP, their potential contribution to the detected ABP should be minor. The endothelial origin of ABP is supported by the previous evidence of albumin-specific binding to MEC both in situ (1-4, 7) and in culture (6). Cells were maintained for 10-12 h in culture medium M-199 supplemented with 15% fetal calf serum, 2 mg/ml histamine, 100 μg/ml heparin, 20 μg/ml endothelial cell growth supplement, 100 units/ml penicillin, and 100 μg/ml streptomycin. At the start of the experiments, the culture medium was discarded and the cells extensively washed in PBS.

Methods

Since SASD contains a photolabile azido group, before photolysis all experiments were carried out in a dark room under red light using vessels covered with aluminum foil.

Preparations of Radiolabeled Putative ABP

Radioiodination of SASD—the protocol followed was that described in Ref. 9 with slight modifications. Borosilicate glass tubes were first coated with 400 pg of Iodogen (for each tube). SASD was dissolved by emptying the reaction tube. The solution was used directly for the cells, which were washed with PBS (3 min × 3). MEC (3 mg of protein) were suspended in 1 ml of 20 mM borate buffer, pH 8.0, and incubated with 2 mM SASD in the same buffer as above for 30 min at 22°C. The unbound SASD was removed by washing cells in PBS (5 min × 2). The cells were then irradiated with UV light from a 100-W xenon lamp (as above) for 70 s on ice, washed twice with PBS, and homogenized in PBS containing 5% SDS, 5 mM benzamidine, and 0.25 mM phenylmethylsulfonyl fluoride. After centrifugation at 13,500 × g for 5 min, the supernatant was used for protein assay and SDS-PAGE (without β-mercaptoethanol). The resolved proteins were then electrophoresed to nitrocellulose membranes and incubated with albumin-gold complex. Controls consisted in using protein extracts from freshly isolated MEC not treated with SASD.

Identification of ABP

SDS-PAGE and Autoradiography—Homogenate samples were concentrated by precipitation on ice, with trichloroacetic acid at 10% final concentration, in the presence of 0.015% sodium deoxycholate.

Alcohol precipitation was performed at 15°C. Samples were centrifuged at 15,500 × g for 10 min at 4°C, and the precipitate was dissolved in solubilization buffer (198 mM Tris, 107 mM H2PO4, pH 6.8, containing 5% SDS, 5% β-mercaptoethanol and 420 mM sucrose, β-Mercaptoethanol was omitted in experiments requiring nonreducing conditions. Aliquots of 5 μl were counted in a Beckman model 4000 γ counter. Electrophoresis was conducted according to Ref. 15, the acrylamide concentration being 5-15% in the resolving gel and 3.75% in the stacking gel. Gels were calibrated using a Sigma high molecular weight kit supplemented with myoglobin (horse heart), and the molecular weights were calculated by the calibration curve method (16). The gels (13 × 17 cm and 1.4 mm thick) were electrophoresed overnight at 5 mA and then stained with 0.2% Coomassie Brilliant Blue R-250 in 45% methanol, 10% acetic acid. Autoradiograms of the dried gels were made on Azoix R-2x2-s ray film.

Scanning-Photocoupling with Albumin-Gold—The transfer of electrophoretically separated proteins to nitrocellulose membrane was performed in 41 mM Tris, 40 mM boroate buffer, pH 8.3, for 3 h at 200 mA in an electroblotting chamber (17). The blots were washed with PBS (10 min × 3) containing 1 mM CaCl2 and, to prevent nonspecific binding, were preincubated with 2 mg/ml bovine lIgG in PBS for 12 h at 22°C. The strips were then incubated with A-Au in PBS (concentration corresponding to Al250 g = 1.0). Visualization of A-Au complex binding to the ABP bands was enhanced by the silver method recommended by Janssen Life Science Products (Beerse, Belgium).

RESULTS AND DISCUSSION

Cross-linking of monomeric albumin to its putative endothelial receptor proteins (ABP) was achieved by using SASD,
a heterobifunctional, iodinatable, photoactivatable, and cleavable reagent. SASD has one chemically and one photochemically reactive group connected by a thiol-cleavable linkage.

Fig. 1 illustrates the sequence of steps involved in the present study. SASD was first radioiodinated in the 2-(p-azidosalicylamido) moiety using Na$^{125}$I and Iodogen (Step 1). The radioiodinated SASD was attached to monomeric albumin (BSA) by acylation for 60 min at room temperature, which resulted in the loss of the sulfosuccinimidyl moiety. The radioiodinated 2-(p-azidosalicylamido)-1,3'-dithiopropionate remained coupled to albumin ($^{125}$I-ASD-A) (Step 2). This albumin derivative was separated from free $^{125}$I and unreacted $^{125}$I-SASD by chromatocentrifugation and dialysis against PBS. Aliquots of $^{125}$I-ASD-A (50 μg of BSA; specific radioactivity 0.7 μCi/μg protein) were incubated with MEC (3 mg of protein) (Step 3). About 15 ± 1.2 μg of BSA was bound to 3 mg of MEC protein. In the presence of a 300-fold excess of competing unlabeled albumin, this value decreased to 2.3 ± 0.6 μg of BSA/3 mg total MEC protein ($n = 3$).

After extensive washing with PBS, the $^{125}$I-ASD-A was allowed to bind to the albumin receptor proteins (ABP) to which it was cross-linked by photolysis with UV light for 70 s at 0 °C (Step 4). After washing with PBS, the disulfide linkages of the cross-linker were cleaved with 5% β-mercaptoethanol, and albumin-receptor interactions were dissociated with 0.1% SDS. As a result, the radioiodinated ASD moiety remained attached to the receptor peptides (ABP) (Steps 5 and 6). After additional washings with PBS, the labeled ABP were extracted, subjected to electrophoresis and visualized by autoradiography.

Fig. 2A shows the banding pattern obtained with MEC subjected to the protocol depicted in Fig. 1. Four bands appeared (lane b): two major bands at 31 and 18 kDa, and two minor bands at 73 and 56 kDa. Densitometry indicated the following approximate relative contributions of each peptide to the total radioactivity associated with the ABP: 7, 21, 39, and 33% for the 73, 56, 31, and 18-kDa peptides, respectively. The two major bands (31 and 18 kDa) therefore constitute more than 70% of the total ABP. The four bands did not appear if MEC were not exposed to UV light (lane c). As already mentioned, the binding of the radiolabeled ligand to ABP was reduced in the presence of 300-fold excess of unlabeled BSA (by ~82%), suggesting that the binding is susceptible to competitive inhibition.

Fig. 2B illustrates the detection of ABP by ligand blotting...
Endothelial Albumin Binding Proteins

with the A-Au complex followed by silver enhancement. When MEC were incubated with unlabeled SASD and exposed to UV light (lane d), the banding pattern obtained was the same as that observed with untreated MEC (lane e). The polypeptides revealed were the same as in the experiments with radioiodinated receptor (lane b). Assuming that SASD can covalently link adjacent membrane proteins, these findings suggest that the four albumin binding peptides may be distinct entities rather than parts of an oligomer. However, stronger evidence is needed to elucidate convincingly the in vivo interaction between the ABP peptides.

By using a detergent concentration higher (5%) than in our previously reported experiments (1% SDS) (8), in addition to the two major peptides (31 and 18 kDa) revealed in those conditions, two other minor peptides (73 and 56 kDa) were also extracted from MEC plasma membranes. However, when the SDS concentration was raised to 10%, no additional bands appeared. Densitometry of lanes d and e in Fig. 2B showed that the two minor bands extracted at high detergent concentration contribute less than 30% of the total mass of detected ABP. Cultured MEC are reported to express a single 60-kDa glycoprotein that interacts weakly but specifically with albumin (18).

These observations suggest that under the experimental conditions used in this study, ABP (represented by two major and two minor polypeptides) appear to be distinct membrane components exposed on the endothelial cell surface. Their exact interactions remain to be elucidated. The findings also suggest that two minor ABP may occur either in small concentration or have a lower affinity for albumin than the 31- and 18-kDa peptides, or both. Kinetic studies to define the binding properties of each ABP and the detection of their location to different microdomains of the endothelial plasma membrane are currently being conducted.

Acknowledgments—We are grateful to A. Radu for his help with the photoactivation procedure and to V. Crician and M. Toader (electrophoresis), F. Georgescu and D. Taflan (radioisotope experiments), E. Stefan (photography), C. Neacsu (graphics), and D. Neacsu (word processing) for the excellent technical assistance provided.

REFERENCES
1. Simionescu, N., and Simionescu, M. (1984) International Symposium on Membrane Biogenesis and Recycling, September 1-3, Kanazawa, Japan, pp. VI-1
2. Ghiteascu, L., Fixman, A., Simionescu, M., and Simionescu, N. (1986) J. Cell Biol. 102, 1304-1311
3. Simionescu, M., Ghiteascu, L., Fixman, A., and Simionescu N. (1987) News Physiol. Sci. 2, 97-100
4. Ghiteascu, L., Galis, Z., Simionescu, M., and Simionescu, N. (1988) J. Submicrosc. Cytol. Pathol. 22, 358-365
5. Milici, A. J., Watrous, N. E., Stukenbrok, H., and Palade, G. E. (1987) J. Cell Biol. 105, 2603-2612
6. Schnitzer, J. E., Carley, W. W., and Palade, G. E. (1988) Am. J. Physiol. 254, H425-437
7. Simionescu, N., and Simionescu, M. (1987) in Microcirculation: An Update (Tsauhunga, I. M., Asano, M., Mishima, Y., and Oda, M., eds) Vol. I, pp. 87-93, Excerpta Medica, Amsterdam
8. Chinea, N., Fixman, A., Alexandru, D., Popov, D., Hasu, M., Ghiteascu, L., Eskenasy, M., Simionescu, M., and Simionescu, N. (1988) J. Cell Biol. 107, 231-239
9. Shephard, E. G., De Beer, F. C., von Holt, C., and Hapgood, J. P. (1988) Anal. Biochem. 168, 306-313
10. Frazer, P. J., and Speck, J. C. (1974) Biochem. Biophys. Res. Commun. 80, 849-857
11. Carson, M. P., and Haudenschild, C. C. (1966) In Vitro Cell Dev. Biol. 22, 344-354
12. Waggoner, R. C., Robinson, C. S., Cross, P. J., and Devenny, J. J. (1983) Microvasc. Res. 25, 387-396
13. Sorensen, P., Farber, N. M., and Krystal, G. (1986) J. Biol. Chem. 261, 9094-9097
14. Sheffield, J. B., Graff, D., and Li, H. P. (1987) Anal. Biochem. 168, 49-54
15. Maizel, J. V., Jr. (1971) Methods Virol 4, 179-246
16. Weber, K., and Osborn, M. (1967) J. Biol. Chem. 244, 4406-4412
17. Gerashoni, J. M., and Palade, G. E. (1982) Anal. Biochem. 124, 396-405
18. Schnitzer, J. E., Carley, W. W., and Palade, G. E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6773-6777