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Laminin binding protein, 34/67 laminin receptor, carries stage-specific embryonic antigen-4 epitope defined by monoclonal antibody Raft.2

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Abstract

We previously produced monoclonal antibodies against the detergent-insoluble microdomain, i.e., the raft microdomain, of the human renal cancer cell line ACHN. Raft.2, one of these monoclonal antibodies, recognizes sialosyl globopentaosylceramide, which has the stage-specific embryonic antigen (SSEA)-4 epitope. Although the mouse embryonal carcinoma (EC) cell line F9 does not express SSEA-4, some F9 cells stained with Raft.2. Western analysis and matrix-assisted laser desorption ionization-time of flight mass spectrometry identified the Raft.2 binding molecule as laminin binding protein (LBP), i.e., 34/67 laminin receptor. Weak acid treatment or digestion with *Clostridium perfringens* sialidase reduced Raft.2 binding to LBP on nitrocellulose sheets and [^{14}C]galactose was incorporated into LBP, indicating LBP to have a sialylated carbohydrate moiety. Subcellular localization analysis by sucrose density-gradient centrifugation and examination by confocal microscopy revealed LBP to be localized on the outer surface of the plasma membrane. An SSEA-4-positive human EC cell line, NCR-G3 cells, also expressed Raft.2-binding LBP.

Keywords: SSEA-4; SialylGb5; Embryonal carcinoma; Laminin binding protein; Laminin receptor; Raft.2; MALDI-TOF MS

EC derived from testicular teratocarcinomas are a subset of germ cell tumors that may contain many embryonic and extra-embryonic tissues, and they represent malignant replicas of normal embryonic cells at specific stages of development. Immunochemical markers, such as SSEA-1, -3, and -4, TRA-1-60, and TRA-1-81, have been utilized to characterize and define the developmental stages of EC lines. For example, early cleavage-stage mouse embryos [1] and the primitive and visceral yolk sac endodermal cells of post-implantation mouse embryos [2] express SSEA-3 and SSEA-4. These carbohydrate antigens are also found on human, but not on murine, EC cells [3]. By contrast, murine EC cells express SSEA-1, while human EC cells do not. Exposure to retinoic acid can prompt EC cells to develop to advanced stages, accompanied by changes in SSEA expressions. Many monoclonal antibodies defining SSEAs were generated in early studies of mammalian development. SSEA-1 is an antigenic epitope defined as a Lewis x (Le^x) carbohydrate structure and is found in both glycosphingolipids and glycoproteins [1,4]. SSEA-3 and -4 defined by MC631 and MC813-70, respectively,

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are located in carbohydrate moieties of globoseries glycosphingolipids [5].

We previously established a monoclonal antibody (Mab) termed Raft.2 by subcutaneously injecting the raft microdomain of a human renal cancer cell line, ACHN, into Balb/c mice and showed that Raft.2 recognizes the carbohydrate structure of sialosyl globopentaosylceramide (sialylGb5), namely GL7, the epitope of SSEA-4 [6]. SSEAs are still among the best markers for characterizing embryonic stem (ES) cells or EC cells and Raft.2 is a potentially useful tool for this purpose.

Although mouse EC F9 cells are known to be SSEA-4 negative, some of these cells stained with Raft.2. In this study, we demonstrated that Raft.2 binds to LBP and that Raft.2-positive LBP is present not only in F9 cells, but also in human EC NCR-G3 cells. We also show LBP to be localized on surface membranes and discuss the significance of LBP carrying SSEA-4. This is the first report, to our knowledge, focusing the SSEA-4 carried by LBP.

Materials and methods

Cell culture and antibodies. The mouse EC cell line F9 and the human renal cancer cell line ACHN were purchased from the American Type Culture Collection. F9 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chem., St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma). ACHN was cultured in Eagle's minimum essential medium (MEM) (Sigma) supplemented with 10% fetal bovine serum and the non-essential amino acid solution (Sigma). The human EC cell line, NCR-G3 [7], was cultured in a 1:1 mixture of DMEM and Ham's F12 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, insulin–transferrin–sodium selenite media (Sigma), and the non-essential amino acid solution. MC-631, Mab for SSEA-3 and MC-813-70, Mab for SSEA–4, to detect Gb5 and sialylGb5, respectively, were purchased from Chemicon International (Temecula, CA). 13C4, Mab for Shiga toxin 1-B subunit (Stx1B), and T-20, a rabbit anti-mouse GTP binding protein β subunit (Gβ) polyclonal antibody, were purchased from the American Type Culture Collection and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Glycolipid analysis. The packed cell pellet (0.5 ml) was extracted with 2 ml chloroform/methanol (C/M) (2:1, v/v) and then with 2 ml of chloroform/isopropanol/water (7:11:2, v/v). Total extracts were combined and evaporated to dryness and then treated with 0.2 N KOH in methanol at 37 °C for 2 h to saponify the phospholipids. After neutralization, methyl esters of fatty acids were removed by mixing with hexane. The extracts were then concentrated to 1/10 volume and dialyzed against water. The retentate was freeze-dried and dissolved in C/M (2:1).

Thin layer chromatography (TLC) immunostaining was performed according to a previously described method [8]. Briefly, C/M extracts were separated on plates precoated with Silica gel 60 (HPTLC aluminium sheets, Merck, Darmstadt Germany) using a solvent system consisting of C/M/water containing 0.1% $CaCl_2$ (5:4:1, v/v/v). After drying, the plates were dipped in 0.1% polyisobutylmethacrylate (Aldrich Chem., Milwaukee, WI, USA) in cyclohexane for 1 min and blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The plates were probed with Shiga toxin 1-B subunit (Stx1B) (1 μg/ml in 1% BSA) [9], then Mab 13C4 culture supernatant to detect globotriaosylceramide (Gb3), and with Mab Raft.2 culture supernatant to detect sialylGb5. After three washes with PBS for 5 min each, horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO, A/S, Denmark) at a 1:2000 dilution ratio were used as the second antibody. The antibodies that bound to the plates were visualized with enhanced chemiluminescence reagent SuperSignal (Pierce, Rockford, IL, USA) and detected by LAS-1000 (Fuji Film, Tokyo, Japan).

Flowcytometry. Cells were harvested and incubated with a 1st antibody for 1 h on ice, followed by treatment with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (Jackson Laboratory, West Grove, PA) at a 1:50 dilution ratio and analyzed by flowcytometry (EPICS-XL, Beckman–Coulter).

Western analysis. Cells were homogenized in hypotonic buffer (25 mM NaCl, 0.5 mM $CaCl_2$, 18 mM Tris–HCl buffer, pH 8.0) and cell debris was removed by centrifugation at 200*g* for 5 min at 4 °C. Precipitates were homogenized in the same manner two more times. The combined supernatants were centrifuged at 40K rpm for 30 min at 4 °C in a Beckman 80Ti rotor to obtain crude membrane fractions. The membrane proteins released with 1% Triton X-100 in 25 mM Tris–HCl buffer, pH 7.5, containing 0.15 M NaCl and the cocktail of protease inhibitors, were subjected to 1-dimensional (1-D) or 2-D Western analysis as previously described [10]. In order to remove the sialic acids from the glycoproteins, after 1-D Western blotting, the membrane strips were treated with 25 mM H_2SO_4 at 80 °C for 1 h.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis. The membrane proteins were separated by 2-D polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue (CBB) R-250 (Bio-Rad Lab., Richmond, CA). The CBB-stained protein that corresponded to the position of the Raft.2-reacting spot was excised and digested with trypsin, and the trypsinized peptides were analyzed with an oMALDI-Qq-TOF MS/MS QSTAR Pulser *i* (Applied Biosystems). The mass spectra search was conducted using an NCBI non-reductant database with the MASCOT search algorithm.

Metabolic labeling of F9 cells with [^{14}C]galactose. Subconfluent cells (approx. 1.4×10^6 cells) were cultured in 4 ml of the incubation medium containing 10 μCi D-[1-^{14}C]galactose (57 mCi/mmol, 200 μCi/ml, Amersham Biosciences UK) for 24 h in a 60 mm culture plate. [^{14}C]Galactose-labeled membrane protein was prepared as above and mixed with 50 μg of non-labeled F9 membrane proteins. The F9 membrane protein mixture thus obtained was separated by 2-D PAGE and stained with CBB. Autoradiograms were obtained with BAS 2000.

Sialidase treatment. Proteins on 2-D nitrocellulose sheets were stained with Ponceau 3R Stain Solution (Wako Pure Chem., Osaka, Japan) and the rectangle containing the proteins of interest was excised. The blots were incubated in 50 mM sodium acetate buffer, pH 4.5, containing 0.1% BSA, with or without 100 mU of neuraminidase from *Clostridium perfringens* (Roche Diagnosis GmbH, Mannheim, Germany) at 37 °C overnight.

Subcellular fractionation. Crude membrane fractions obtained as described above were thoroughly suspended in the hypotonic buffer containing 30% sucrose and overlaid on a discontinuous sucrose density gradient of 40%/45%/50%/60% in a 12-ml ultracentrifugation tube, and the suspension was then overlaid with a 20% sucrose layer. The gradient was centrifuged at 25K rpm for 1 h in a Beckman SW40Ti rotor, and after recovery and dilution with PBS, each of the interface layers was sedimented at 40K rpm for 0.5 h in a Beckman 80Ti rotor. Proteins were released from each precipitate with 1% Triton X-100 lysis buffer and subjected to 2-D PAGE.

Staining for fluorescence microscopic observation. EC cells were harvested from cell culture plates and incubated with Raft.2. They were then stained with Alexa Fluor 488-conjugated goat anti-mouse IgM, μ-chain (Molecular probes, Eugene, OR) for 1 h and mounted in Perma Fluor Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA) on a slide glass. The slides were observed with an Olympus LSM-GB200 confocal microscope.