

The Human AC133 Hematopoietic Stem Cell Antigen Is also Expressed in Epithelial Cells and Targeted to Plasma Membrane Protrusions*

(Received for publication, November 1, 1999)

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The human AC133 antigen and mouse prominin are structurally related plasma membrane proteins. However, their tissue distribution is distinct, with the AC133 antigen being found on hematopoietic stem and progenitor cells and prominin on various epithelial cells. To determine whether the human AC133 antigen and mouse prominin are orthologues or distinct members of a protein family, we examined the human epithelial cell line Caco-2 for the possible expression of the AC133 antigen. By both immunofluorescence and immunoprecipitation, the AC133 antigen was found to be expressed on the surface of Caco-2 cells. Interestingly, immunoreactivity for the AC133 antigen, but not its mRNA level, was down-regulated upon differentiation of Caco-2 cells. The AC133 antigen was specifically located at the apical rather than basolateral plasma membrane. An apical localization of the AC133 antigen was also observed in various human embryonic epithelia including the neural tube, gut, and kidney. Electron microscopy revealed that, within the apical plasma membrane of Caco-2 cells, the AC133 antigen was confined to microvilli and absent from the planar, intermicrovillar regions. This specific subcellular localization did not depend on an epithelial phenotype, because the AC133 antigen on hematopoietic stem cells, as well as that ectopically expressed in fibroblasts, was selectively found in plasma membrane protrusions. Hence, the human AC133 antigen shows the features characteristic of mouse prominin in epithelial and transfected non-epithelial cells, *i.e.* a selective association with apical microvilli and plasma membrane protrusions, respectively. Conversely, flow cytometry of murine CD34⁺ bone marrow progenitors revealed the

cell surface expression of prominin. Taken together, the data strongly suggest that the AC133 antigen is the human orthologue of prominin.

A characteristic feature of epithelial cells, which is a prerequisite for their function, is the selective association of certain proteins with specific subdomains of the plasma membrane (1–3). An example is the recently identified protein prominin, a 115-kDa five transmembrane domain protein found to be expressed on the apical surface of neuroepithelial cells and several other embryonic epithelia and on brush border membranes of adult kidney proximal tubules (4). Within the apical plasma membrane domain, prominin is selectively associated with microvilli and other related plasma membrane protrusions rather than the planar subdomain of the membrane. Studies with prominin-transfected Madin-Darby canine kidney cells have shown that this selective association is due to a combination of apical sorting and retention in microvilli (5). Remarkably, a plasma membrane protrusion-specific localization of prominin was also observed in prominin-transfected non-epithelial cells (*i.e.* CHO cells),¹ showing that the mechanism underlying the selective association of membrane proteins with plasmalemmal protrusions is conserved between epithelial and non-epithelial cells (4).

These studies were conducted with prominin derived from one species, the mouse. In independent studies, a novel marker of human hematopoietic stem and progenitor cells was identified, characterized, and referred to as AC133 antigen (6, 7). The human AC133 antigen appears to be related to mouse prominin in size (120 kDa), membrane topology (five transmembrane domains, two large extracellular loops), and sequence (8, 9).

However, the tissue distribution reported for the human AC133 antigen (6, 7) is remarkably distinct from that reported for mouse prominin (4). In contrast to the epithelial expression of prominin (4), the AC133 antigen was found to be selectively expressed on human CD34^{bright} progenitors derived from bone marrow, fetal liver, and peripheral blood (6) and on subsets of CD34⁺ leukemia cells, *i.e.* non-epithelial cells, but not in epithelial tissues such as colon and kidney (7). Moreover, the sequence identity between mouse prominin and the human

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^d Supported by the Studienstiftung des Deutschen Volkes.

^h Supported by the Medical Research Council, UK, the Leukemia Research Fund, UK, and EU Biotech and INTAS/RFBR grants.

^j Supported by the National Health and Medical Research Council (NHMRC) of Australia.

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¹ The abbreviations used are: CHO, Chinese hamster ovary; BMNC, bone marrow mononuclear cells; HBSS, Hanks' balanced salt solution; PE, phycoerythrin; sulfo-NHS-LC-biotin, sulfosuccinimidyl-6(biotinamido)-hexanoate biotinylating agent; PBS, phosphate-buffered saline; mAb, monoclonal antibody.

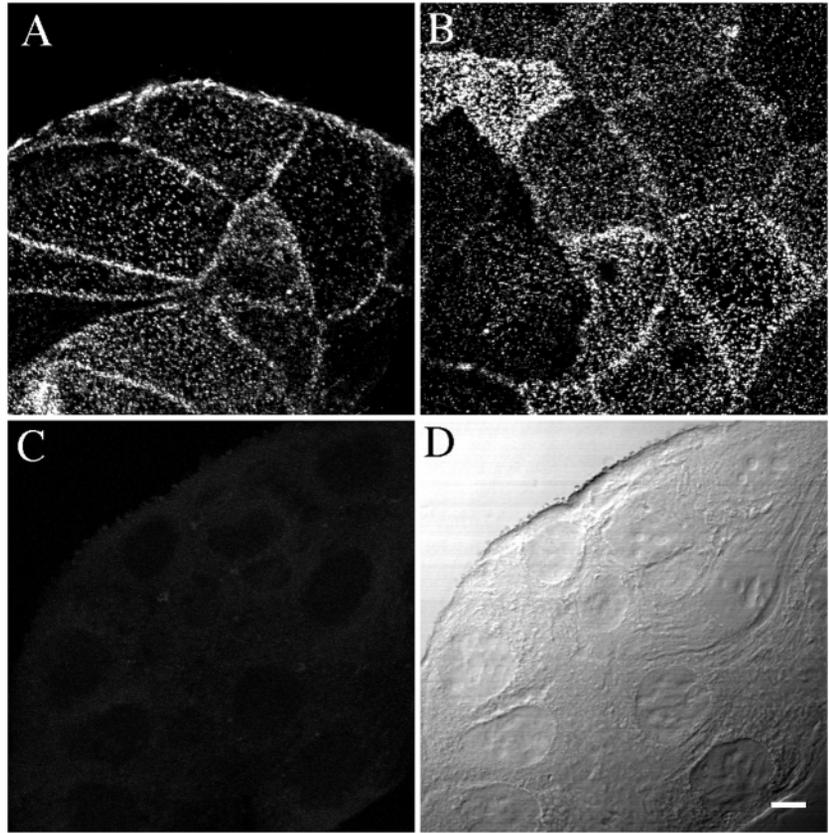


FIG. 1. Cell surface expression of human AC133 antigen in Caco-2 cells. Intact subconfluent Caco-2 cells grown on glass coverslips were incubated at 4 °C with the mAb AC133 (A), mAb AC141 (B), or, as a control, without primary antibody (C), paraformaldehyde-fixed, and then incubated with lissamine rhodamine-conjugated anti-mouse secondary antibody. The samples were viewed with a confocal microscope, and composite pictures of all optical sections are shown. D, Nomarski image of the cells examined in C. Bar in D = 10 μ m. In A and B, note the punctate cell surface staining characteristic of microvilli-associated antigens.

AC133 antigen is relatively low (average 60%), in particular in the three extracellular domains (54%), which comprise more than 70% of the polypeptide.

This raises a major question. Is the AC133 antigen the human orthologue of prominin? If so, an explanation would be required as to why the AC133 antigen is apparently not expressed in epithelial tissues such as adult kidney (7), which in the case of mouse does express prominin (4), although mRNA for the AC133 antigen is well detectable (7). In addition, one would then expect the AC133 antigen to be selectively associated with plasma membrane protrusion upon expression in fibroblasts, as previously observed in the case of prominin (4). Conversely, prominin should then be expressed also in murine hematopoietic progenitor cells.

Or, alternatively, are prominin and the AC133 antigen distinct members of a protein family? In the support of this possibility is the occurrence of at least three open reading frames in the *Caenorhabditis elegans* genome related to mouse prominin and the human AC133 antigen (8). In that case, one would not necessarily expect the AC133 antigen to be selectively associated with plasma membrane protrusions.

Answering this question is particularly important in light of a set of recent findings (10) that are as follows. First, a frame-shift mutation in the gene encoding the AC133 antigen, which results in a truncated protein, causes retinal degeneration in humans. Second, a similar truncated version of mouse prominin does not reach the cell surface. Third, consistent with the detection of AC133 immunoreactivity in human retinoblastoma cells (6), in mouse, prominin is found in photoreceptor cells where it is selectively associated with the plasma membrane evaginations at the base of the rod outer segment that are intermediates in disc formation. If the AC133 antigen was the human orthologue of prominin rather than a distinct member of the prominin family, this would suggest that the lack of appearance of this protein in the plasma membrane evagina-

tions of the rod outer segment may lead to impaired disc formation and, eventually, to retinal degeneration.

Here we report several lines of evidence strongly suggesting that the AC133 antigen is the human orthologue of prominin. The evidence includes the expression of the AC133 antigen in certain human epithelial cells. This apparent contradiction of previous observations (6, 7) is very likely explained by our finding that the epitope recognized by monoclonal antibody AC133, which is thought to be a glycosylated structure (7), is down-regulated upon differentiation of epithelial cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Caco-2 cells (kindly donated by Drs. S. Robine and D. Louvard, CNRS-Institut Curie, Paris, France) were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, 1% non-essential amino acids (Life Technologies, Inc.), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin and were cultured in a humidified incubator at 37 °C under 10% CO₂ atmosphere. Cultures were used between passages 8 and 18. CHO cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum and 50 μ g/ml gentamycin at 37 °C under 5% CO₂. For experiments studying polarized cell surface delivery, Caco-2 cells were plated at a density of 4.5×10^5 cells/cm² on permeable membranes (24-mm Transwell™-COL chambers, 0.4- μ m pore size). Media were changed every day, and the experiments were performed 11 days after seeding to allow the development of a tight monolayer.

CHO cells were transfected with the pCR3.1-Uni plasmid containing the AC133 antigen-coding cDNA sequence under the control of the cytomegalovirus promoter (7), using the LipofectAMINE reagent (Life Technologies, Inc.) according to the supplier's instructions. Cells expressing the neomycin resistance gene were then selected by introducing 600 μ g/ml of G418 into the incubation medium. Two weeks later, G418-resistant colonies were pooled and expanded. To enhance the expression of the transgene, cells were incubated for 17 h with 10 mM sodium butyrate (11). All subsequent steps were performed in medium lacking butyrate. Under these conditions 10–30% of neomycin-resistant cells expressed the recombinant AC133 antigen.

Isolation of Human and Mouse Hematopoietic Cells From Bone Marrow—Human bone marrow was aspirated into preservative-free hepa-

rin from the posterior iliac crest of normal adult volunteers according to procedures approved by the ethics committee of the Royal Adelaide Hospital. Bone marrow mononuclear cells (BMNC) were obtained following separation over Ficoll (Lymphoprep, Nygaard) and washed twice at 4 °C in HHF buffer (comprising calcium and magnesium-free Hanks' balanced salt solution (HBSS) supplemented with 5% human AB serum and 5% fetal calf serum). CD34⁺ cells were isolated from BMNC suspensions by means of immunomagnetic beads (Dynal Pty. Ltd). Briefly, BMNC were pelleted by centrifugation, and the pellet was suspended in 100 μ l of CD34 Dynabeads to give a final cell:bead ratio of 4:1. After gentle vortexing for 2 min, the cells were resuspended in 4 ml of HHF and placed on a rotator for a further 30 min at 4 °C to ensure complete capture of CD34⁺ cells. Bead-rosetted CD34⁺ cells were isolated and purified by five sequential rounds of washing with HHF followed by capture on a magnet. The CD34 antibody-coated beads were released from the CD34⁺ cells by the addition of 200 μ l of the CD34 Detachabead reagent (supplied with the kit) followed by incubation in a shaking waterbath for 45 min at 37 °C. After removal of the detached beads using the magnet, the cells were washed three times in HHF, and the aliquots were analyzed for purity using HPCA-2-PE (Becton Dickinson) and for expression of AC133 antigen using AC133-PE conjugates (Amcell). In all experiments, the purity of the CD34⁺ cell preparations obtained in this manner was >98%.

Mouse bone marrow cells were obtained from 8–10-week-old BALB/c mice by flushing dissected femurs, using a syringe with 27G needle, with Hanks' balanced salt solution supplemented with 0.5% bovine serum albumin (Sigma). Red blood cells were lysed by a 5-min incubation with red cell lysing buffer (Sigma), and BMNC were isolated on a Histopaque 1077 gradient (Sigma). Interface cells were harvested and washed twice with HBSS/bovine serum albumin at 4 °C and adjusted to a concentration of 2×10^7 /ml prior to staining for flow cytometric analysis.

Flow Cytometric Analysis—Antibodies were purified from murine ascites or hollow fiber culture harvests (Cellmax QUAD artificial capillary system, Cellco Inc. Germantown, MD) by protein A affinity chromatography and phycoerythrin (PE) conjugates prepared as described previously (6).

10^6 murine BMNC were stained for 30 min at 4 °C with biotinylated anti-mouse CD34 (RAM 34) and, after washing with HBSS/bovine serum albumin, stained with streptavidin-Cy-Chrome (Pharmingen). Cells were washed twice with HBSS/bovine serum albumin before incubation with 150 ng/test of PE-conjugated 13A4 antibody (anti-mouse prominin). Stained cells were analyzed on a FACScan flow cytometer (BDIS, San Jose, CA) with the scatter gates set on the lymphoid population. 50,000 events were acquired and analyzed using Cell Quest software (BDIS).

Northern Blot Analysis—Total RNA from Caco-2 cells was prepared using the acidic phenol/chloroform phase separation method (12). Northern blot analyses were performed using CLONTECH multiple tissue Northern blot membrane and by resolving 20 μ g of total RNA from Caco-2 cells on formaldehyde-agarose gels followed by RNA transfer to nylon membranes and hybridization with the *EcoRI-EcoRI* fragment (nucleotides 756–1195) derived from the human AC133 hematopoietic stem cell antigen cDNA (GenBankTM accession number AF027208). The probe was labeled with [α -³²P]dCTP using the Rediprime kit (Amersham Pharmacia Biotech). Hybridization and washing were performed either at 65 °C using Church buffer as described elsewhere (13) or at 68 °C using the Expresshyb hybridization solution (CLONTECH) according to the manufacturer's instructions. Blots were analyzed using a Fuji phosphoimager.

Cell Surface Biotinylation and Immunoprecipitation—All steps of cell surface biotinylation and immunoprecipitation were carried out at 4 °C. Just prior to use, the membrane-impermeable sulfo-NHS-LC-biotin agent (Pierce) was dissolved in Ca/Mg-PBS (PBS containing 1 mM CaCl₂ and 0.5 mM MgCl₂) to a final concentration of 0.2 mM. After repeated washing (three times) with Ca/Mg-PBS, Caco-2 cells (on 100-mm dishes) were incubated for 1 h in 3 ml of the biotin solution. The cells were washed three times with Ca/Mg-PBS and then incubated for 10 min with Ca/Mg-PBS containing 20 mM glycine to quench the residual biotin. Cells were then lysed in ice-cold solubilization buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 5 mM EGTA, 50 mM Tris/HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml leupeptin, 10 μ g/ml aprotinin). Cell lysates obtained after centrifugation (10 min, 10,000 \times g) were subjected to immunoprecipitation. For domain-selective cell surface biotinylation (14), 11-day-old monolayers on duplicate Transwell filters were washed three times with Ca/Mg-PBS and then biotinylated from either the apical (1.5 ml) or basolateral (2.6 ml) chamber compartment. The compartment not receiving the sulfo-NHS-

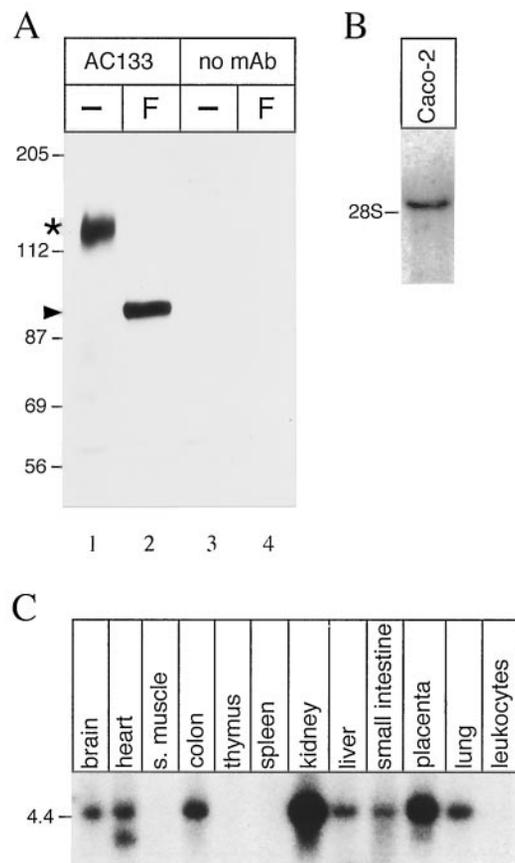


Fig. 2. Characterization of the AC133 antigen in Caco-2 cells and tissue distribution of its mRNA. A, AC133 antigen in Caco-2 cells is a 120-kDa glycoprotein. Confluent Caco-2 cells were incubated at 4 °C with sulfo-NHS-LC-biotin and solubilized, and AC133 antigen was immunoprecipitated using mAb AC133 (AC133), or, as a control, no antibody (no mAb). Solubilized immunoprecipitates were incubated in the absence (–) or presence (F) of PNGase F followed by NeutrAvidin blotting. Asterisk, 120-kDa AC133 antigen; arrowhead, product after N-deglycosylation. B and C, Northern blots of total RNA isolated from Caco-2 cells (B) or poly(A)⁺ RNA (≈ 2 μ g each) from various human tissues (C) and probed with human AC133 antigen cDNA.

LC-biotin was filled with Ca/Mg-PBS. After quenching, cells were scraped into ice-cold solubilization buffer, and cell lysates were prepared as described above.

Cell lysates corresponding to one confluent 100-mm dish or half of one 24-mm filter were diluted 4-fold in immunoprecipitation buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 10 mM EDTA, 50 mM Tris/HCl, pH 7.8, 10 μ g/ml aprotinin, 2 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and monoclonal antibody (mAb) AC133 (10 μ g/ml) was added. Samples were incubated overnight on an end-over-end shaker. Immune complexes were collected with protein A-Sepharose CL-4B beads (Amersham Pharmacia Biotech), pre-adsorbed with rabbit anti-mouse IgG, and used for deglycosylation, SDS-polyacrylamide gel electrophoresis, and NeutrAvidin blotting (see below).

Endoglycosidase Digestions and NeutrAvidin Blotting—Immune complexes containing the cell surface-biotinylated proteins were eluted from protein A-Sepharose beads using glycosidase buffer (1% Triton X-100, 0.1% SDS, 25 mM EDTA, 1% 2-mercaptoethanol, 50 mM sodium phosphate, pH 7.2) at 95 °C. Eluates were incubated for 6 h at room temperature in the absence or presence of 1 unit PNGase F (Roche Molecular Biochemicals). Proteins were then analyzed by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher and Schuell, pore size 0.45 μ m) using standard procedures. After transfer, nitrocellulose membranes were incubated for 2 h in PBS containing 5% low fat milk powder and 0.3% Tween 20 and then washed extensively with PBS. Biotinylated proteins were identified with horseradish peroxidase-conjugated NeutrAvidin (Pierce) (diluted in PBS containing 0.1% Tween 20) followed by incubation with the SuperSignal ULTRA chemiluminescent substrate (Pierce).

Immunofluorescence and Confocal Microscopy—Cell surface immu-

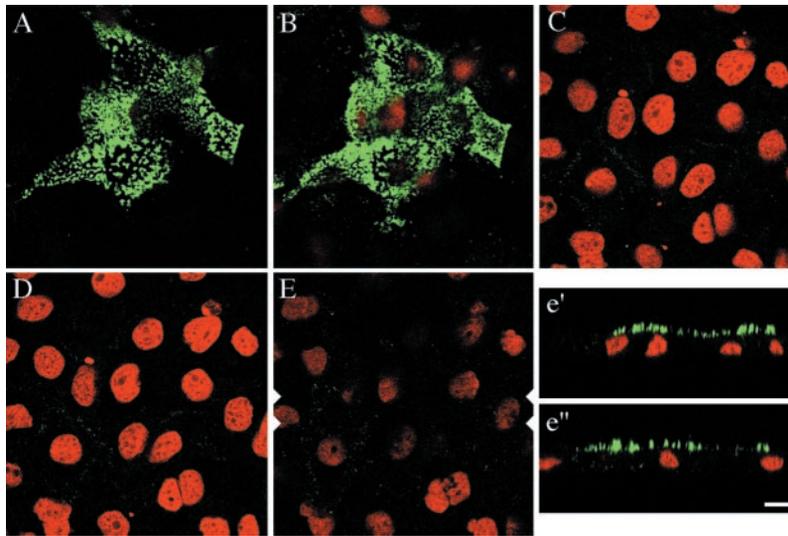


FIG. 3. Apical localization of AC133 antigen in polarized Caco-2 cells. Confluent Caco-2 cells grown on glass coverslips were paraformaldehyde-fixed, saponin-permeabilized, and incubated with mAb AC133 followed by CyTM2-conjugated anti-mouse secondary antibody (green). Nuclei were labeled with propidium iodide (red). *A-E*, a series of single optical xy-plane sections from the apical (*A*) to the basal (*E*) side of the cells. *e'* and *e''*, two single optical xz-plane sections of the AC133 antigen-positive cells shown in *A-E*. The white triangular indentations at the margins of *E* indicate the approximate positions of the xz sections shown in *e'* and *e''*. Bar in *e''* = 10 μ m. Note that in the AC133 antigen-expressing cells, immunoreactivity is confined to the apical surface.

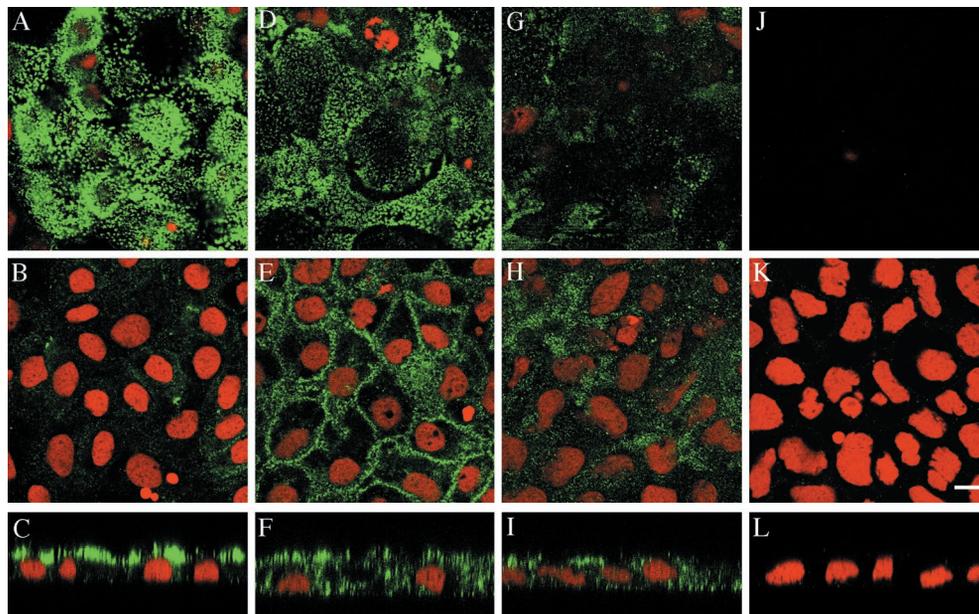


FIG. 4. Variable cellular expression and subcellular localization of the AC133 antigen in Caco-2 cells. 16-day-old post-confluent Caco-2 cells grown on glass coverslips were treated and stained as in Fig. 3. Four sets of three panels each, *A-C*, *D-F*, *G-I*, and *J-L*, show distinct areas of the same cell monolayer. Single optical xy-plane sections at the apical surface of the cells (*top panels*), at the level of the nuclei (*middle panels*), and single optical xz-plane sections (*bottom panels*) are shown. AC133 antigen are pseudocolored in green and nuclei are in red. Bar in *K* = 10 μ m.

no fluorescence was carried out as described previously (5). Cells grown on glass coverslips were washed with Ca/Mg-PBS, first at room temperature and then on ice, and surface-labeled for 30 min at 4 °C by the addition of the mAb AC133 (10 μ g/ml) or mAb AC141 (10 μ g/ml) or double surface-labeled by an additional incubation for 10 min with fluorescein isothiocyanate-conjugated wheat germ agglutinin (Sigma). Both antibodies and wheat germ agglutinin were diluted in ice-cold immunofluorescence buffer (Ca/Mg-PBS containing 0.2% gelatin). Unbound antibodies or free wheat germ agglutinin were removed by five washes with ice-cold immunofluorescence buffer. Fixative (3% paraformaldehyde (Sigma) in PBS) was added to the cells on ice, and the coverslips were placed at room temperature for 30 min. The fixative was removed by three washes with Ca/Mg-PBS, and the residual formaldehyde was quenched for 30 min with 0.1 M glycine in PBS. Cells were then incubated for 30 min at room temperature with lissamine rhodamine-conjugated goat anti-mouse IgG (H + L) (Dianova). Coverslips were rinsed sequentially with immunofluorescence buffer, PBS, and distilled

water and mounted in Mowiol 4.88 (Calbiochem).

In experiments with permeabilized cells, cells grown on glass coverslips were washed with PBS and fixed with 3% paraformaldehyde in PBS for 30 min at room temperature. Coverslips were then rinsed with and incubated for 10 min in PBS containing 50 mM ammonium chloride. Cells were permeabilized and blocked with 0.2% saponin/0.2% gelatin in PBS (blocking solution) for 30 min. Cells were then incubated sequentially with mAb AC133 (10 μ g/ml), 1 mg/ml RNase A for 10 min at room temperature (to digest cytoplasmic mRNA), and CyTM2-conjugated goat anti-mouse IgG (H + L) (Dianova), all in blocking solution. Nuclei were labeled with propidium iodide (0.3 μ g/ml) during the incubation of secondary antibody. Coverslips were rinsed and mounted as described above. Cells were observed with a Leica TCS^{4D} confocal laser scanning microscope. The confocal microscope settings were such that the photomultipliers were within their linear range. The images shown were prepared from the confocal data files using Adobe photoshop software.

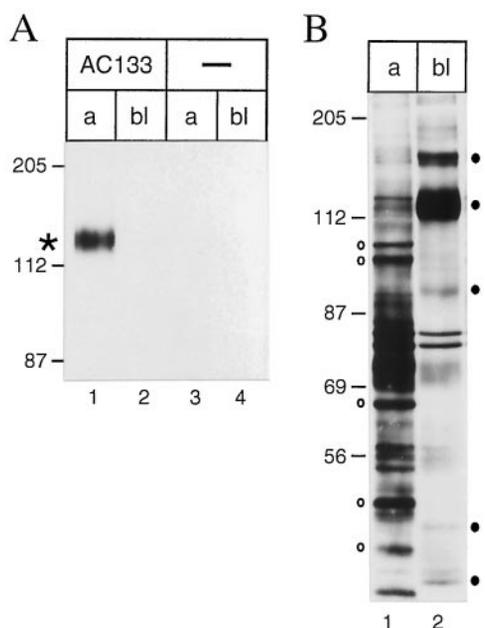


FIG. 5. Steady-state distribution of the AC133 antigen between the apical and basolateral plasma membrane of Caco-2 cells. Transwell filter-grown Caco-2 cells were surface-biotinylated at 4 °C from either the apical (a) or basolateral (bl) side using sulfo-NHS-LC-biotin. A, cell lysates were subjected to immunoprecipitation using either mAb AC133 (AC133) or, as a control, no antibody (—), and the immunoprecipitates were analyzed by NeutrAvidin blotting. Asterisk, 120-kDa AC133 antigen. B, cell lysates were directly analyzed by NeutrAvidin blotting. Open circles, apical plasma membrane-specific proteins; filled circles, basolateral plasma membrane-specific proteins.

Immunocytochemistry of Human Embryonic Tissues—A human embryo at 32 days of development was obtained immediately after voluntary termination of pregnancy induced with the RU 486 antiprogesterone compound. Informed consent for the use of the embryo in research was obtained from the patient, and the embryo was collected according to the guidelines and with the approval of both national and institutional ethics committees.

Human embryonic tissue, fixed in 4% paraformaldehyde (v/v) in PBS for 1 h at 4 °C, was rinsed in PBS for several hours and then twice in 15% sucrose in PBS for at least 24 h. The tissue was then embedded in 15% sucrose, 7.5% gelatin in PBS, and frozen at -70 °C. Five-micron-thick frozen sections were thawed, hydrated in PBS, and endogenous peroxidases were inhibited for 20 min in PBS containing 0.2% hydrogen peroxide. Sections were then washed with 0.25% Triton X-100 in PBS and the mAb AC133 (20 µg/ml) was added overnight at 4 °C. After washing with PBS-Triton X-100, incubation was performed for 1 h at room temperature with biotinylated rabbit anti-mouse antibody (DAKO) and subsequently with peroxidase-coupled streptavidin (DAKO). Peroxidase activity was revealed with 0.025% (v/v) 3,3'-diaminobenzidine (Sigma) in PBS containing 0.015% hydrogen peroxide. Slides were counterstained with Harris' hematoxylin, and mounted in XAM neutral medium (BDH).

Immunogold Electron Microscopy of Caco-2 Cells and AC133-transfected CHO Cells—Caco-2 cells or CHO cells stably transfected with the human AC133 gene cultured on collagen-coated 60-mm Petri dishes (Collagen R, Serva) were washed with 200 mM HEPES-NaOH, pH 7.4, and fixed with 4% paraformaldehyde (Caco-2 cells) or 8% paraformaldehyde (CHO cells) in HEPES buffer for 4 h at 4 °C. CHO cells were embedded in 10% gelatin in PBS. Ultrathin cryosections were prepared as described previously (5). Immunogold labeling of cryosections using either mAb AC133 (0.5–1.5 mg/ml) or mAb AC141 (0.15–2.3 mg/ml) followed by goat anti-mouse antibody coupled to 15-nm gold particles (Aurion) was performed according to the manufacturer's instruction (Aurion) with minor modifications.

Immunogold Electron Microscopy of Human CD34⁺ Hematopoietic Progenitor Cells—Human CD34⁺ hematopoietic progenitor cells were incubated for 45 min at 4 °C with saturating concentrations of AC133 antibody and with an equivalent concentration of an IgG1 isotype-matched nonbinding control antibody, 1B5 (generously supplied by Dr. Graham Mayerhofer, Hanson Center for Cancer Research). After two

washes with HHF buffer, the cells were incubated with goat anti-mouse IgG coupled to colloidal gold particles of average diameter of 10 nm (GAM-Au10, Janssen Pharmaceutica) for 1 h at 4 °C with gentle rocking, washed three times with HHF buffer, and then fixed in a 3% (v/v) solution of EM-grade glutaraldehyde (Agar Aids) in 150 mM cacodylate buffer (pH 7.3). Additional aliquots of CD34⁺ cells were stained in parallel using an identical protocol with mAb DREG-56 (anti-CD62L) and with mAb 7E10 (an antibody prepared in P. J. Simmons' laboratory, which identifies a class 3 epitope on the CD34 molecule (15)). Immunolabeled cells were then washed three times in cacodylate buffer, post-fixed for 1 h in 1% osmium tetroxide (Johnson Matthey Chemical Ltd.) in the same buffer, and then dehydrated by exposure to a graded series of ethanol. Following dehydration in 100% ethanol, the cells were transferred to propylene oxide (BDH) prior to infiltration with epon-araldite resin overnight at room temperature. The resin was polymerized for 8 h at 80 °C after which ultrathin sections were cut and examined without counterstaining in a transmission electron microscope (Jeol) operating at 80 kV. The qualitative and quantitative statements about the distribution of the various antigens between protrusions and planar regions of the plasma membrane are based on the examination of 80–100 cells and the counting of at least 250 gold particles for each antibody.

RESULTS

The AC133 Antigen Is Endogenously Expressed in the Human Intestine-derived Epithelial Cell Line Caco-2—Both previously described (6) mouse mAb against the human AC133 hematopoietic stem cell antigen, mAb AC133 (Fig. 1A) and mAb AC141 (Fig. 1B) were found to stain, on cell surface immunofluorescence, Caco-2 cells, a human intestine-derived epithelial cell line (16). The staining showed a punctate pattern, which is characteristic of a microvilli-associated antigen. Caco-2 cells did not show any staining when primary antibodies were omitted (Fig. 1C).

The antigen recognized by mAbs AC133 and AC141 on the surface of Caco-2 cells was characterized. Both mAb AC133 (7) and mAb AC141² recognize a glycosylated structure. We therefore labeled the surface of Caco-2 cells with the membrane-impermeant sulfo-NHS-LC-biotin and subjected the antigen immunoprecipitated with mAb AC133 to incubation with and without PNGase F, followed by SDS-polyacrylamide gel electrophoresis and blotting with horseradish peroxidase-conjugated NeutrAvidin (Fig. 2A). Without PNGase F treatment, a band with an apparent molecular mass of ≈120 kDa was detected (Fig. 2A, lane 1). Deglycosylation yielded a 95-kDa band (Fig. 2A, lane 2). These apparent molecular masses are those expected for the AC133 antigen (7). The same results were obtained using mAb AC141 instead of mAb AC133 for the immunoprecipitation (data not shown). Furthermore, native and deglycosylated protein immunoprecipitated from Caco-2 cells with mAb AC133 exhibited the same molecular mass as authentic human AC133 antigen obtained from CHO cells transfected with the AC133 antigen cDNA (data not shown).

Northern blot analysis of Caco-2 cell RNA, using an *EcoRI* fragment derived from the human AC133 antigen cDNA as probe, revealed the presence of a ≈5.0-kilobase mRNA (Fig. 2B), as expected for the AC133 antigen (7). Taken together, the data of Fig. 1 and Fig. 2, A and B, show that the AC133 antigen is endogenously expressed in the epithelial cell line Caco-2.

We examined the presence of the AC133 antigen mRNA in various human tissues. AC133 antigen mRNA was detected in brain, heart, kidney, liver, lung, and placenta, as reported previously (7), as well as in the colon and small intestine (Fig. 2C). Consistent with the presence of AC133 antigen mRNA in the human colon, six expressed sequence tags derived from this source were identified (GenBankTM accession numbers AI469575, AI273443, AI308936, D25789, AA622198, and AA577606). It should be noted that the presence of the AC133

² D. W. Buck, unpublished data.

antigen mRNA in adult colon is in contrast to the lack of AC133 immunoreactivity in that tissue (see Table II within Ref. 7).

Polarized Distribution of the AC133 Antigen at the Plasma Membrane of Caco-2 Cells—Consistent with their epithelial origin, Caco-2 cells exhibit an apical-basal polarity with distinct, apical *versus* basolateral, plasma membrane domains (17, 18). To determine the localization of AC133 antigen with regard to the apical *versus* basolateral plasma membrane domain of these cells, indirect immunofluorescence using mAb AC133 followed by confocal laser scanning microscopy was performed on Caco-2 cells grown on glass coverslips for 16 days after reaching confluence (Fig. 3). To facilitate the analysis, nuclei were labeled with propidium iodide (*red*). A series of single optical xy-plane sections from the apical (Fig. 3A) to the basolateral (Fig. 3E) side revealed that AC133 immunostaining (*green*) was predominantly located at the apical domain. Two single optical xz-plane sections through the AC133 antigen-positive cells shown in *panels A–E* confirmed the apical localization of the AC133 antigen (Fig. 3, *e'* and *e''*).

The staining for the AC133 antigen was not uniform across the entire postconfluent cell monolayer (Fig. 3), in contrast to the observations on subconfluent cultures where almost all cells were stained (Fig. 1A). Approximately 20% of the postconfluent Caco-2 cells showed immunofluorescence staining at the apical membrane (Fig. 3, *A–E* and Fig. 4, *A–C*), whereas $\approx 80\%$ of the cells did not show any prominent immunofluorescence staining (Fig. 4, *J–L*). Mosaic expression of the AC133 antigen in postconfluent Caco-2 cells is reminiscent of that reported for several brush-border hydrolases (19, 20) and transporters (21). The intensity of immunostaining for the AC133 antigen at the apical membrane was variable (compare Fig. 4, *A, D*, and *G*). In addition, some Caco-2 cells showed staining for the AC133 antigen also in intracellular compartments (Fig. 4H) and on the lateral domain of the cells (see ring-like staining in Fig. 4E). The latter observation suggested that the AC133 antigen in these cells was located either at the lateral plasma membrane and/or in vesicles closely associated with this membrane.

To quantitate the cell surface distribution of the AC133 antigen in Caco-2 cells, domain-selective cell surface biotinylation was performed. 11-day-old postconfluence Caco-2 cell monolayers grown on Transwell filters were biotinylated with sulfo-NHS-LC-biotin agent on either the apical or basolateral membrane. The AC133 antigen was immunoprecipitated from cell extracts, and cell surface-labeled AC133 antigen was then quantified by probing the immunoprecipitates with horseradish peroxidase-conjugated avidin (Fig. 5A). Biotinylated AC133 antigen was recovered from the apical (Fig. 5A, *lane 1*) but not basolateral (Fig. 5A, *lane 2*), plasma membrane. The absence of biotinylated AC133 antigen at the basolateral cell surface suggested that either the lateral staining for the AC133 antigen detected by light microscopy in some cells (Fig. 4E) occurred in an intracellular membrane compartment closely associated with the basolateral membrane, or the amount of AC133 antigen in the lateral plasma membrane was below the level of biochemical detection.

To rule out the possibility that the biotinylation agent did not have access to the basolateral membrane due to steric hindrance, we analyzed the protein composition of the apical and basolateral membranes after domain-selective cell surface biotinylation. Visualization of biotinylated proteins with horseradish peroxidase-conjugated NeutraAvidin revealed that both membrane domains contain a distinct set of cell surface proteins (Fig. 5B, *lanes 1* and *2*), which indicated that the biotinylation agent reached both the apical and basolateral surface of Caco-2 cells grown on permeable supports. These data also

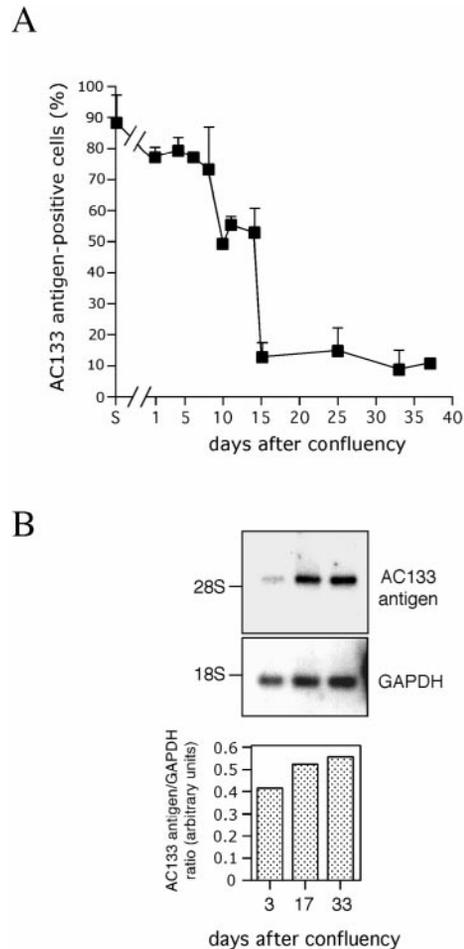


FIG. 6. Expression of the AC133 antigen and its mRNA during differentiation of Caco-2 cells. *A*, down-regulation of AC133 immunoreactivity. Intact Caco-2 cells, grown on glass coverslips for the indicated periods of time, were surface double-labeled at 4 °C with the mAb AC133 and fluorescein isothiocyanate-conjugated wheat germ agglutinin, paraformaldehyde-fixed, and incubated with lissamine rhodamine-conjugated anti-mouse secondary antibody. AC133 antigen-positive cells are expressed as the percentage of wheat germ agglutinin-labeled cells. Values represent the mean of two to four independent coverslips; *bars* indicate S.D. or the variation of the individual values from the mean. More than 400 cells were counted for each time point. *S*, quantification of cells in subconfluent state. *B*, Northern blot of total RNA ($\approx 20 \mu\text{g}$) isolated from Caco-2 cells, grown for the indicated periods of time, and probed with human AC133 antigen cDNA followed by re-probing with a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA. The bottom panel shows the ratio of the two RNA signals quantified by densitometric scanning.

document the integrity of the cell monolayer used in our assays.

Down-regulation of the AC133 Epitope upon Differentiation of Caco-2 Cells—Enterocytic differentiation of Caco-2 cells is a growth-related process, which starts about 7 days after the cells reach confluence (17, 18) and is complete within 20–30 days (20). To further analyze the mosaic expression of the AC133 antigen in Caco-2 cells, we investigated in greater detail its expression pattern in relation to the time cells spent in a subconfluent/confluent state in culture. AC133 antigen expression was monitored by indirect cell surface immunofluorescence using either mAb AC133 (Fig. 6A) or mAb AC141 (which gave the same results as mAb AC133 (data not shown)). As mentioned above, virtually all cells in subconfluent culture were labeled (Fig. 6A). After confluence, AC133 immunoreactivity persisted for about a week in 70–80% of the cells (Fig. 6A). Interestingly, upon differentiation of Caco-2 cells, the AC133 immunoreactivity decreased rapidly, and at 15 days

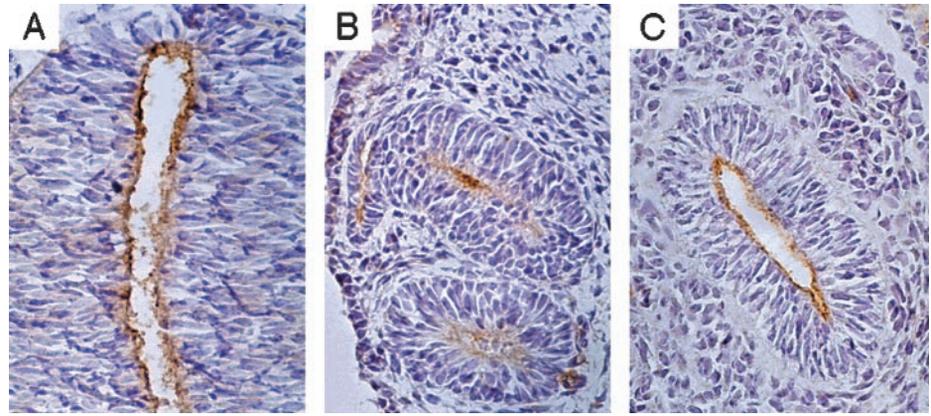


FIG. 7. Immunoperoxidase localization of the AC133 antigen in human embryonic tissue. Transverse sections of 32-day-old embryonic tissue stained with mAb AC133. *A*, neural tube; *B*, mesonephros; *C*, gut. In all panels, AC133 immunoreactivity is confined to the apical, luminal side of the epithelia. Panels are the same magnification.

post-confluence and thereafter only 10–20% of the cells were stained by the mAb AC133 (Fig. 6A).

To determine whether down-regulation of AC133 immunoreactivity is associated with a change in mRNA expression, we examined transcripts by Northern blot analysis on days 3, 17, and 33 post-confluence (Fig. 6B). Remarkably, a small increase, rather than a decrease, in the relative level of the AC133 antigen mRNA over time was detected (Fig. 6B).

Expression of the AC133 Antigen in Human Embryonic Tissues—Prominin immunoreactivity has previously been detected in various murine embryonic epithelia (4). Given that the AC133 antigen is expressed in a human epithelial cell line, Caco-2, we examined the expression of the AC133 antigen in human embryonic epithelia. 32-day-old human embryonic tissues were stained with mAb AC133 (Fig. 7). Interestingly, AC133 immunoreactivity was detected on the apical, but not basolateral, side of the neural tube (Fig. 7A). AC133 immunoreactivity was also observed on the apical side of the mesonephros (Fig. 7B) and gut (Fig. 7C). Hence, the tissue expression of the AC133 antigen in the human embryo is similar to that of prominin in the mouse embryo (4).

The AC133 Antigen Is Associated with Apical Microvilli in Epithelial Cells and with Plasma Membrane Protrusions in Non-epithelial Cells—The remarkable feature of mouse prominin, *i.e.* its specific localization in microvillar membranes of epithelial cells (4, 5) and related plasma membrane protrusions of non-epithelial cells (4), prompted us to investigate the ultrastructural localization of the AC133 antigen in Caco-2 cells and, upon transfection, in CHO cells (Fig. 8). Immunogold EM revealed that AC133 immunoreactivity was specifically associated with microvilli at the apical surface of Caco-2 cells and lacking from the small intermicrovillar regions of the plasma membrane (Fig. 8, *A* and *B*). The preferential localization of the AC133 antigen to plasma membrane protrusions was also observed in AC133 antigen-transfected fibroblasts (Fig. 8, *C* and *D*). In this case, gold particles were conspicuously concentrated on microvilli-like protrusions, whereas the large neighboring planar areas of the plasma membrane were labeled to a much lesser extent, if at all.

The AC133 Antigen Is Enriched in Plasma Membrane Protrusions of Human CD34⁺ Hematopoietic Progenitor Cells—To investigate the ultrastructural localization of the AC133 antigen in hematopoietic progenitor cells, CD34⁺ cells were isolated from human bone marrow, subjected to cell surface immunogold labeling, and analyzed by EM (Fig. 9). Remarkably, cell surface AC133 immunoreactivity was preferentially associated with plasma membrane protrusions (Fig. 9A, *arrows*), where it appeared to be concentrated at the tip (Fig. 9B). Specifically, about half of the gold particles were associated with protrusions, which however constituted only a minor proportion of the plasma membrane. Control experiments per-

formed with mAb 7E10 (anti-CD34) revealed that the CD34 antigen was randomly distributed over the surface of CD34⁺ cells (Fig. 9C), with a minor fraction ($\approx 5\%$) of the gold particles being associated with protrusions. In contrast, cell surface staining with mAb DREG 56 (anti-CD62L) showed, as anticipated, the preferential association of L-selectin (CD62L) with plasma membrane protrusions (Fig. 9D, *arrows*), with about half of the gold particles being associated with these structures, as was the case for the AC133 antigen.

Prominin Is Found on Murine Hematopoietic Progenitor Cells—The data presented above show that the human AC133 antigen exhibits the same key features as previously reported for mouse prominin. This in turn raises the possibility that prominin is expressed by murine hematopoietic progenitor cells. To examine this possibility, we analyzed by flow cytometry the expression of prominin in mouse bone marrow CD34⁺ cells (22–24) (Fig. 10). Double cell surface staining of the lymphoid-gated cell population with mAb anti-CD34 and mAb 13A4 (anti-mouse prominin) (4) revealed that 36% ($n = 3$) of the CD34⁺ cells (which represented 2.85% of nucleated cells in bone marrow) express prominin. This expression level was comparable to that reported for the AC133 antigen in human bone marrow cells (6, 25).

DISCUSSION

The present data indicate that the human AC133 antigen shows a very similar, if not identical, cellular distribution and subcellular localization as previously reported for mouse prominin (4, 5). The human AC133 antigen, previously found in flow cytometry to be restricted to the CD34^{bright} population of progenitor cells from adult and fetal bone marrow, fetal liver, cord, or peripheral blood (6, 25), and to subsets of CD34⁺ leukemias (7), is like prominin (4) expressed in epithelial cells. Conversely, mouse prominin is also expressed on a subset of CD34⁺ progenitors isolated from murine bone marrow, consistent with the expression of the AC133 antigen on human hematopoietic stem cells (6, 25). This finding suggests that prominin could be of use as a marker of murine hematopoietic progenitors, and therefore its value in studies of murine hematopoiesis may be considerable. Further studies will be needed to characterize the specific subpopulation of mouse bone marrow cells expressing prominin.

As previously reported for mouse prominin in epithelial cells (4, 5), the human AC133 antigen is mainly confined to the apical plasma membrane, where it appears to be concentrated in microvilli. This microvillar localization is independent of the epithelial phenotype of the cell, as shown by the ectopic expression of the AC133 antigen in fibroblasts and its physiological expression in hematopoietic progenitor cells. Considering also the sequence homology between the human AC133 antigen and mouse prominin (8, 9), the present results strongly suggest that

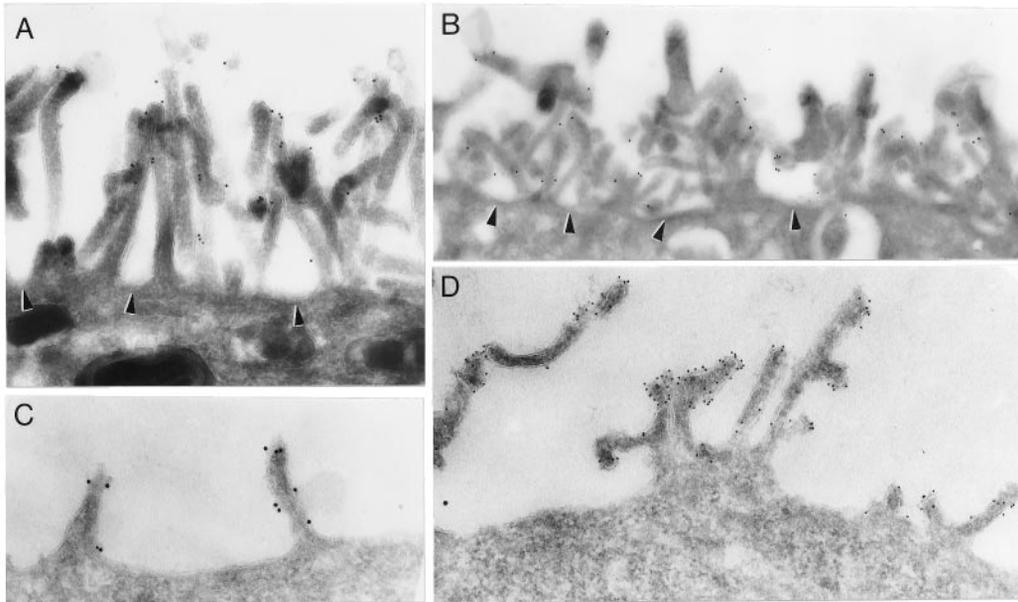


FIG. 8. Immunogold electron microscopy of the AC133 antigen on the apical plasma membrane of Caco-2 cells and on the plasma membrane of AC133 antigen-transfected CHO cells. Ultrathin cryosections of paraformaldehyde-fixed Caco-2 cells (A and B) or AC133 antigen-transfected CHO cells (C and D) were stained with mAb AC133 (A, C, D) or mAb AC141 (B) followed by goat anti-mouse IgG coupled to 15-nm gold particles. Arrowheads, intermicrovillar regions of the plasma membrane. Magnifications: A, $\times 15,800$; B, $\times 24,200$; C, $\times 34,200$; D, $\times 25,300$.

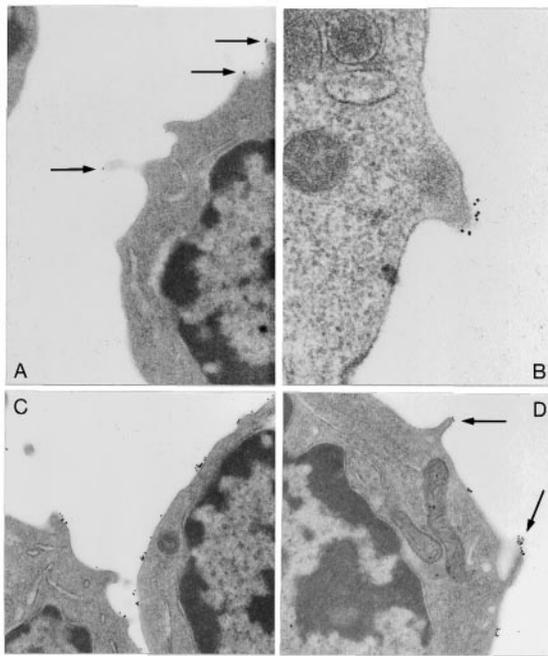


FIG. 9. Immunogold electron microscopy of the AC133 antigen on the cell surface of human $CD34^+$ hematopoietic cells. $CD34^+$ hematopoietic progenitor cells, isolated from human adult bone marrow, were incubated at $4^\circ C$ with mAb AC133 (A and B), mAb anti- $CD34$ (C), or mAb anti- $CD62L$ (D) followed by goat anti-mouse IgG coupled to 10-nm gold particles. Immunolabeled cells were fixed with glutaraldehyde and post-fixed with osmium tetroxide, and ultrathin plastic sections were examined without counterstaining. Arrows indicate AC133 antigen (A) and L-selectin (D) immunoreactivity at the tip of plasma membrane protrusions. Magnifications: A, C, and D, $\times 2500$; B, $\times 5000$. Note that the AC133 antibody selectively binds to plasma membrane protrusions of the cell surface (A and B).

the AC133 antigen is the human orthologue of prominin rather than a distinct member of the prominin family. Following a suggestion of the HUGO Nomenclature Committee, we propose to refer to the AC133 antigen as "prominin (mouse)-like 1"

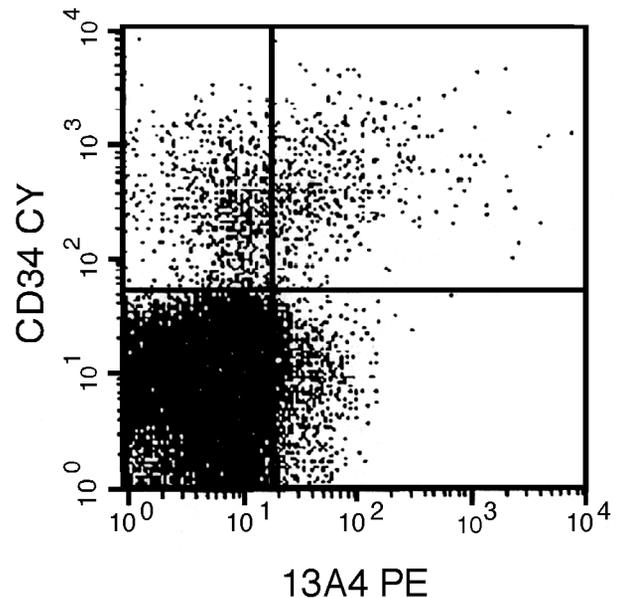


FIG. 10. Cell surface expression of prominin in mouse bone marrow $CD34^+$ cells. Mouse bone marrow cells were double-labeled with biotinylated mAb anti- $CD34$ followed by streptavidin-Cy-Chrome ($CD34$ CY) and mAb 13A4 conjugated to phycoerythrin (13A4 PE). Labeled cells were analyzed by flow cytometry. The upper right quadrant shows cells stained for both $CD34$ and prominin.

(PROML1) until completion of the sequencing of the human genome; should the latter fail to reveal the existence of a human open reading frame more closely related to mouse prominin than the human AC133 antigen, the latter should be called prominin like its mouse orthologue.

The presence of mRNA for the AC133 antigen/PROML1 in adult colon is in contrast to the lack of AC133 immunoreactivity in this tissue reported previously (7). This discrepancy may be explained by the fact that mAb AC133 binds to a glycosylation-dependent epitope (7) and that processing of asparagine-linked oligosaccharides has been reported to change upon differenti-

ation of HT-29 cells (26), a model cell line to study enterocytic differentiation (18). Our results with Caco-2 cells, showing a decrease of AC133 immunoreactivity, but not of the mRNA level for the AC133 antigen/PROML1, upon differentiation are consistent with differential glycosylation depending on the state of enterocytic differentiation. Perhaps the more widespread presence of AC133 immunoreactivity in embryonic as compared with adult human epithelia reflects, at least in part, differential glycosylation rather than a variation in protein expression of the AC133 antigen/PROML1. Further investigation of this issue will require the generation of antibodies directed against the AC133 antigen/PROML1 polypeptide.

The likely orthologue relationship between the human AC133 antigen/PROML1 and mouse prominin has important implications for studies addressing the as yet unknown function of this protein. In particular, insight into the molecular basis of prominin's preference for plasma membrane protrusions, derived from murine models, can now be used to understand human disease caused by mutation of the AC133 antigen/PROML1 gene, as exemplified by that causing retinal degeneration (10).

Acknowledgments—S. M. W. thanks Professor Sir David J. Weatherall for his support.

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