Thrombomucin, a Novel Cell Surface Protein that Defines Thrombocytes and Multipotent Hematopoietic Progenitors

Kelly M. McNagny,* Inger Pettersson,* Fabio Rossi,* Ingo Flamme,§ Andrej Shevchenko,‡ Matthias Mann,‡ and Thomas Graf*

*Cell Regulation Program, and ‡Protein and Peptide Program, European Molecular Biology Laboratory, Heidelberg, D-69117 Germany; and §Max-Planck Institut for Physiology and Clinical Research, W.G. Kerckhoff-Institute, Department of Molecular Cell Biology, Bad Nauheim, D-61231 Germany

Abstract. MEP21 is an avian antigen specifically expressed on the surface of Myb-Ets–transformed multipotent hematopoietic precursors (MEPs) and of normal thrombocytes. Using nanoelectrospray tandem mass spectrometry, we have sequenced and subsequently cloned the MEP21 cDNA and named the gene thrombomucin as it encodes a 571–amino acid protein with an extracellular domain typical of the mucin family of proteoglycans. Thrombomucin is distantly related to CD34, the best characterized and most used human hematopoietic stem cell marker. It is also highly homologous in its transmembrane/intracellular domain to podocalyxinlike protein–1, a rabbit cell surface glycoprotein of kidney podocytes.

Single cell analysis of yolk sac cells from 3-d-old chick embryos revealed that thrombomucin is expressed on the surface of both lineage-restricted and multipotent progenitors. In the bone marrow, thrombomucin is also expressed on mono- and multipotent progenitors, showing an overlapping but distinct expression pattern from that of the receptor-type stem cell marker c-kit. These observations strengthen the notion that the Myb-Ets oncprotein can induce the proliferation of thrombomucin-positive hematopoietic progenitors that have retained the capacity to differentiate along multiple lineages. They also suggest that thrombomucin and CD34 form a family of stem cell–specific proteins with possibly overlapping functions in early hematopoietic progenitors.

During embryonic development blood cells arise first in the early yolk sac (primitive hematopoietic cells) and later independently in the vicinity of the dorsal aorta (definitive hematopoietic cells; for reviews see Dzierzak and Medvinsky, 1995; Zon, 1995; Cumano et al., 1996; Dieterlen-Lievre et al., 1996). After the transient production of blood cells in the spleen and fetal liver (mammals), hematopoietic progenitors are produced exclusively in the bone marrow, where their proliferation and maturation is regulated by an intricate set of microenvironmental cues elaborated by stromal cells (Quesenberry, 1992).

The analysis of hematopoiesis has been greatly facilitated by the identification of a variety of cytokines (for review see Callard and Gearing, 1994) and of specific cell surface antigens (for reviews see Spangrude et al., 1991; Uchida et al., 1993) that allow the isolation and expansion of monopotent and multipotent progenitors. In spite of their considerable interest, antigens known to be expressed on the surface of hematopoietic stem cells are still relatively few. They comprise tyrosine kinase receptors such as c-kit (for review see Bernstein et al., 1991) and flk-2 (Matthews et al., 1991), mucins such as CD34 (Simmons et al., 1992), glycosylphosphatidylinositol-linked molecules of unknown function such as Sca-1 and Thy-1 (Uchida et al., 1993; Miles et al., 1997), and the AA4.1 antigen, a specific marker of yolk sac and fetal liver hematopoietic progenitors (Jordan et al., 1990). None of these markers are absolutely specific for hematopoietic stem cells and they must be used in combination with lineage-specific markers to separate monopotent from multipotent progenitors (Uchida et al., 1993).

In previous work we found that the Myb-Ets oncprotein-encoding acute leukemia virus E26 is able to transform primitive hematopoietic progenitors derived from chicken embryo yolk sac. These cells resemble multipotent hematopoietic progenitors since they can be induced to differentiate into either erythrocytes, thrombocytes, myeloblasts, or eosinophils and we have therefore designated them as MEPs1 (Myb-Ets–transformed Progenitors; Graf et al., 1992).

1. Abbreviations used in this paper: cMGF, chicken myelomonocytic growth factor; MEP, Myb-Ets-transformed progenitors; PCLP, podocalyxinlike protein; SCF, stem cell factor.
Using MEPs as a source of antigen for immunizations we have generated a panel of monoclonal antibodies directed against the surface antigens of these progenitors (McNagny et al., 1992). One of these antibodies, named MEP21, was shown to react specifically with an antigen present on MEPs but absent on transformed B and T lymphoid, erythroid, myelomonocytic, and eosinophilic cell lines. Surprisingly the antigen was also found to be expressed on thrombocytes obtained after differentiation induction (through v-Myb inactivation) of MEPs transformed by a temperature mutant of E26 virus (Frampton et al., 1995). Likewise, the MEP21 antigen could be detected on normal chicken thrombocytes, but not on lymphocytes, erythrocytes, eosinophils, neutrophil granulocytes, or macrophages (Graf et al., 1992; McNagny et al., 1992).

For several years, we had attempted to sequence MEP21 by conventional protein chemical techniques. However, these attempts were unsuccessful due to the very low amounts of protein that could be purified (silver stained level). Here we report the use of nanoelectrospray mass spectrometry (Wilm and Mann, 1996; Wilm et al., 1996) to sequence the MEP21 protein and clone MEP21-encoding cDNAs and a detailed analysis of the expression of the antigen during ontogeny. The data show that MEP21 is a novel mucinlike protein distantly related to CD34, which is expressed on the surface of mono- and multipotent progenitors of both primitive and definitive origin.

Materials and Methods

Protein Purification and Sequencing

Proteins from ~10^8 HD57 cells were solubilized in 50 ml of lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.5% NP-40) plus protease inhibitors (1 mM PMSF, 20 mM e-aminocaproic acid, 1 mM leupeptin, and 2.5 U/ml tryslodyn) on ice for 30 min. Nuclei were removed by centrifugation at 15,000 g for 30 min at 4°C, and the supernatant was incubated overnight at 4°C with 200 μl of MEP21 antibody-coupled Sepharose beads (2.5 mg of antibody coupled per ml of CNBr-activated Sepharose resin; Pharmacia Diagnostics AB, Uppsala, Sweden). Beads were washed 10 times with 2 ml of lysis buffer containing protease inhibitors, and once with PBS plus PMSF, and bound proteins were eluted in 0.1% trifluoroacetic acid plus PMSF. Eluted fractions were equilibrated to neutral pH by addition of Tris buffer, lyophilized, resuspended in sample buffer, and then resolved on a 10% SDS-PAGE gel.

Staining by Coomassie brilliant blue R-250 did not reveal the location of the band, therefore the gel was restained with silver nitrate according to the protocol of Shevchenko et al. (1996). The silver-stained band was excised and digested in gel with trypsin, as described by Wilm et al. (1996). Half of the peptide mixture obtained after extraction of the gel piece was micropurified on a capillary containing 50 nl of POROS R2 material (PerSeptive Biosystems, Cambridge, MA). After washing with 5 μl of 50% MeOH in 5% formic acid, the peptides were step eluted with 1 μl of 50% MeOH, 1 μl of 5% formic acid, into a nanoelectrospray needle. This needle was transferred to an APIII mass spectrometer (Perkin-Elmer Corp., Toronto, Canada) and sprayed for 30 min. During this time, peptide ions apparent from the mass spectrum were selected and isolated in turn, and then fragmented in the collision chamber of the mass spectrometer. The experiment was repeated with the remaining portion of the digest mixture that had been eserftified in 2 M HCl in MeOH. Amino acid sequences were determined by comparing spectra of esterified and nonesterified peptides, using Apple-ScriptTM (Apple, Cupertino, CA) based scripts developed in our group.

PCR-based Cloning and Library Construction

Degenerate PCR primers were designed based on the sense sequence of the MEP21-1 (5′-GAYCCICGCIGTITYTYGARGAR-3′) and MEP21-2 (5′-GAYCCICGCIGTITYTYGARGAR-3′) peptides. Poly(A)+ RNA was prepared from HD100 total RNA (see below) using oligo(dT)-cellulose (Sambrook et al., 1989), and then converted to single-stranded DNA using a ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. PCR of single-stranded cDNA was performed using the T7 phage arm primer (Pharmacia Diagnostics AB) and either the MEP21-1- or MEP21-2-based primers. Taq polymerase (Pharmacia Diagnostics AB) and buffer conditions were those recommended by the manufacturer. Amplification was performed by 30 cycles of 30-s denaturation at 95°C, 30-s template annealing at 53°C, and 90-s elongation at 72°C, using an “Intelligent Heating Block” (Biometra Inc., Gottingen, Germany). PCR products were cloned into plasmids using a TA cloning kit (Invitrogen, San Diego, CA), transformed into Escherichia coli strain XI-1 blue and two positive clones were characterized by restriction map and sequence analysis.

Construction of the HD100 cDNA phagemid library was described previously (McNagny et al., 1996). The amplified HD100 library was screened three times for MEP21 positive clones. For each screening, one million recombinant phage were plated on XL-1 MRF bacterial host strain and these were screened by hybridization with a 32P-labeled MEP21 PCR fragment probe (Sambrook et al., 1989, and see below). In total, 52 recombinants were identified, plaque purified by two further rescreens, and plasmids were produced by in vivo excision using the protocols recommended by the ZAP-cDNA synthesis kit manufacturer (Stratagene). Inserts were sequenced by the EMBL DNA sequencing service using an automated sequencer (A.L.F. DNA sequencer; Pharmacia Diagnostics AB).

Sequence Comparisons

Sequence comparisons were performed using FASTA and TFASTA searchers of the GenBank/EMBL/DDJB Data Library and Swissprot databases. Homologies were derived using “Pileup” alignments of sequences retrieved from the Swissprot database. The data for MEP21 sequences are available from GenBank/EMBL/DDJB under accession numbers Y13976, Y13977, and Y13978.

Nucleic Acid Hybridization

For Northern blot analysis, total RNA was prepared by lysis and fractionation in guanidinium/acetate/phenol/chloroform as described by Chromczynski and Sacchi (1987). Approximately 10 μg of each RNA was resolved on a 1% agarose–formaldehyde gel and blotted onto nylon membranes (GeneScreen; Dupont-NEB, Boston, MA) as described by Sambrook et al. (1989). Hybridization of radiolabeled probes and removal of unbound probe was performed in NaHPO4/SDS buffer as described by Church and Gilbert (1991).

All hybridization probes were labeled with [α-32P]dCTP by random hexamer priming as described by Feinberg and Vogelstein (1983). The following cDNA fragments were used as probes: a 1.8-kb PCR fragment of MEP21 (see above), a 0.23-kb Psxl/HindIII fragment specific for MEP21 alternatively spliced and a glyceraldehyde-3-phosphate dehydrogenase–specific probe (Dugaiczyk, 1983).

Immunohistologic Analyses

Whole mount in situ antibody stains and paraffin sections were performed as described by Lopez and Carrasco (1992).

Animals and Cell Culture

Embryos and chicks were produced from fertilized eggs obtained from White Leghorn chickens maintained by Lohmann (Cuxhaven, Germany). Protocols for the isolation, virus infection, and generation of primary transformed cells from embryonic day two, three, and four blastodermes were as described previously (Graf et al., 1992; McNagny and Graf, 1997). Bone marrow was flushed from the tibia and femurs of 5-d-old chickens using PBS and a 5-ml syringe with a 21-gauge needle. Viable leukocytes were isolated by centrifugation at room temperature on “lymphocyte separation medium” (Eurobio, Les Ulis, France) and then converted to single-stranded cells were isolated from the interface, washed three times, stained, and then sorted as described.

The origins of the cell lines used as sources of RNA have been described previously: HD3 erythroblasts (Beug et al., 1982a); HD44 erythroblasts (Metz and Graf, 1991); HD11 macrophages (Beug et al., 1979); HD13 granulocytes (Golay et al., 1988; Kullera et al., 1995); HD75 MEPs (Metz...
and Graf, 1991); HD57 M1 myeloblasts (Graf et al., 1992); HD50 1A1 eosinophils (Kulessa et al., 1995); MSB-1 T cells (Akiyama and Kato, 1974); RP-12 B cells (Siegfried and Olson, 1972); and HD100 MEP/cosinophilic cells (McNagny et al., 1996).

All cells were grown in blastoderm medium (Graf et al., 1992; McNagny et al., 1992; McNagny and Graf, 1997) composed of DME, supplemented with 10% fetal calf serum, 2.5% chicken serum, 0.15% NaHCO3, 56 µg/ml of conalbumin, 80 mM 2-mercaptoethanol, 0.9 µg/ml insulin, and the standard complement of antibiotics at 37°C in 5% CO2. Medium for HD50M and HD11 cells was supplemented with ~10 U/ml of crude chicken myelomonocytic growth factor (cMGF; Leutz et al., 1989). Where indicated, colony assays were performed in the presence of 5% anemic serum as a source of erythropoietin (Radke et al., 1982) and 4 ng/ml chicken SCF (Hayman et al., 1993).

**Colony Assays**

Plasma clot colony assays were performed as described by Cormier and Dieterlen-Liévré (1988). Briefly, sorted cells were resuspended in 1.2 ml of blastoderm medium plus 120 µl of citrated bovine plasma (GIBCO BRL, Gaithersburg, MD) plus 10 µl thrombin (100 IU/ml, Sigma) and 600 µl aliquots from this mixture were seeded in duplicate into a 24-well tissue culture plate (Nunc, Roskilde, Denmark). Cultures were incubated for 3–6 d at 37°C; harvested, and air dried onto microscope slides according to the methods described previously for collagen cultures (Lanotte, 1984). Slides were stained for hemoglobin using diaminobenzidine (see below), counterstained with DiffQuik (May-Gruenwald-Giemsa–like stain; Baxter, Dudingen, Switzerland), and colony types were assessed by morphology. In some experiments, cultures were supplemented with anemic serum, cMGF or stem cell factor (SCF). All colonies obtained from the bone marrow and yolk sac were scored when they contained a minimum of five cells per colony. For liquid culture colony assays, single cells were sorted into individual wells of a 96-well plate containing 150 µl of media. To determine the composition of large, bone marrow–derived blast colonies, these were cultured 10 d in liquid culture, replated in plasma clots and incubated an additional 5 d before DiffQuik staining and morphological analysis.

**Immunofluorescence and Cell Sorting**

For single color, indirect immunofluorescence analysis, 106 cells were stained with MEP21, MEP26, EOS47 (McNagny et al., 1992), MEP17 (anti-VLA-2; McNagny et al., 1992), Brdshaw et al. (1995), MLY5/2 (Kornfeld et al., 1983), JS4 (Schmidt et al., 1986), α5β1 integrin anti-gpIIb/IIIa (Lacoste-Eleaume et al., 1994) or anti–c-kit (Vainio et al., 1996) mAbs, or with normal mouse serum followed by goat anti–mouse FITC-coupled antibodies (Dianova, Hamburg, Germany) as described previously (Graf et al., 1992). For two-color analysis, cells were stained as above, and then with biotinylated anti–c-kit antibodies and streptavidin-phycocerythrin (Dianova). Alternatively, cells were stained with the appropriate mAb followed by goat anti–mouse antibodies coupled to phycoerythrin, and FITC-labeled mAbs to JS4. All flow cytometric analyses were performed using a FACScan® (Becton and Dickinson Co., Mountain View, CA). Cell sorting was performed using FACStar® Plus and FACS® Vantage (Becton and Dickinson Co.) cytometers. Single cell sorts were performed using the Cytocone™ (Becton and Dickinson Co.) cell cloning attachment and wells were checked microscopically for the presence of single cells.

**Cytological Stainings**

Peroxidase staining was performed by a procedure that allows the detection of peroxidase activity (an exclusive marker of chicken eosinophil granules; Brune and Spitznagel, 1973) in cells suspended in culture medium (Graf et al., 1992). Acid and neutral benzidin stains for hemoglobin detection were performed as described previously (Beug et al., 1982a).

**Results**

**MEP21 Is a 150-kD Protein Expressed by Thrombocytes**

To determine whether MEP21 antigen is coexpressed by thrombocytes with known thrombocyte markers, and

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Figure 1. Expression of MEP21 protein by peripheral blood thrombocytes. (A and B) Immunofluorescence analysis of peripheral blood leukocytes from 5-wk-old chicks. (A) Cells were stained with a mouse mAb to α5β1 integrin followed by a phycoerythrin-conjugated anti–mouse antibody and an FITC-coupled MEP21 antibody. (B) Another aliquot of the above cells was stained with MEP21 antibody followed by a phycoerythrin-conjugated anti–mouse antibody. MEP21+ and MEP21− fractions (gates R1 and R2, respectively) were sorted by flow cytometry and gave populations of >98% purity. (C)DiffQuik stained cells from R1 and R2 fractions. (D) Western blot analysis of MEP21 expression of hematopoietic cell lines and sorted peripheral blood cells. Designations of cells are indicated on the top of each lane.
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11C3, directed against the integrin αIIIβ1, which is a hallmark of thrombocytes (Barclay et al., 1993; Lacoste-Eleaume et al., 1994). As can be seen from the FACS© plot in Fig. 1 A, >90% of the cells that stained with the 11C3 antibody also reacted strongly with MEP21. The remaining 10% of the cells expressed low levels of MEP21 and no MEP21-positive cells could be detected that did not also stain with 11C3. Next, the leukocytes were stained with MEP21 antibody and sorted by FACS®. The plot in Fig. 1 B shows that ~51% of the cells stained positive with MEP21 antibodies while 49% were negative. Analysis of the sorted fractions by cytocentrifugation and staining with DiffQuik (Fig. 1 C) revealed that the MEP21-positive fraction consisted almost entirely of thrombocytes (98% of 309 cells counted), while the negative fraction contained no thrombocytes at all (335 cells counted). Finally, both fractions were subjected to Western blotting using the MEP21 antibody. As shown in Fig. 1 D, the MEP21-positive but not the MEP21-negative fraction expressed a protein identical in size to that seen in lysates from two different MEP cell lines. In addition, erythrocytes as well as myeloid and eosinophil cell lines, were negative in this assay.

Cloning of the cDNA-encoding MEP21 Protein Reveals a Mucinlike Structure

To clone MEP21-encoding cDNA, MEP21 protein was detergent solubilized from the MEP cell line HD57 and purified by a combination of immunoaffinity chromatography and preparative gel electrophoresis. The protein amount was estimated to be ~0.5 pmol based on subsequent mass spectrometric analysis. Peptides were micropurified into a nanoelectrospray needle and the sequences of seven tryptic peptides of MEP21 were determined as explained in Mann and Wilm (1995) and Wilm et al. (1996) (Fig. 2). Several of the peptides overlapped, leading to a total of 44 unique amino acid residues (underlined in Fig. 3). Database searches of these sequences revealed no significant homology. The sequences of peptides MEP21-1 and MEP21-2 were used to prepare degenerate oligonucleotides to PCR amplify and to clone MEP21-specific cDNAs from an HD100 λZAPII bacteriophage library (McNagny et al., 1996). One such PCR product was sequenced and found to contain the MEP21-2 peptide sequence, and this was subsequently used to isolate 54-phage cDNA clones. These clones fell into three categories according to differences in their coding sequence (Fig. 3 A).

Type 1 clones, found 39 times, contain a 5' untranslated region of 52 bp, followed by an open-reading frame of 1,713 bp encoding 571 amino acids, which includes three out of four unique sequence stretches found by mass spectrometric sequencing. The 3' end consists of an untranslated region of 1,347 bp (Fig. 3 A). In the extracellular domain, the predicted protein contains a putative amino terminal signal peptide and a 342-amino acid serine-, threonine-, and proline-rich domain with five potential N-glycosylation sites and numerous O-glycosylation sites. This is followed by a stretch of 108 amino acids containing four cysteines that could form two globular domains, by a hydrophobic sequence of 24 amino acids that encodes a putative transmembrane region, and by a cytoplasmic region.

whether in these cells it exhibits a similar molecular weight as in MEPs (150 kD; McNagny et al., 1992), peripheral blood leukocytes were prepared and analyzed. They were first stained with a combination of the mAbs MEP21 and

\[ \text{Figure 2. Sequencing of MEP21 by nanoelectrospray mass spectrometry.} \]

(A) Mass spectrum of the unseparated peptide mixture obtained after in-gel tryptic digestion of the protein band. (B) Peptide ion T1 (A) was isolated and fragmented in the collision chamber of the mass spectrometer, leading to the spectrum shown. Fragmentation of tryptic peptides predominantly produces nested sets of fragments containing the peptide COOH terminus (\(Y^a\), \(Y^b\), etc. [Roepstorff and Fohlmann, 1984]), which allow assignment of the sequence by their mass differences. (C) Tandem mass spectrum of the same peptide as in B after esterification of the whole peptide mixture. Esterification results in 14-D mass shifts for the ions containing COOH terminus plus an additional shift of 14 D for each Asp and Glu residue as indicated by filled circles. Comparison of the tandem mass spectra of native and esterified peptide, B and C, allowed unambiguous assignment of the peptide sequence. Note that the isobaric amino acids Leu and Ile could not be distinguished and are designated by the letter L. The following peptide sequences were determined: T1, [AS] NEAFFEVFCSGR; T2, [AS] NEAFFEVFCSSGR; T3, WAVHVLVHR; T4, VLDPAAVFEELK; T5, VLDPAAVFEELKEK; T6, ALLFLNR.
containing a consensus protein kinase C and CKII phosphorylation site each. The observed difference between the size of the predicted protein (54 kD) and the apparent molecular weight of MEP21 antigen (150 kD) suggests that MEP21 is highly glycosylated. Together these features suggest that the \textit{mep21} gene encodes a member of the mucin family of glycoproteins and we therefore now call the MEP21 protein “thrombomucin”.

A second type of clone, found 10 times, encodes an additional 18 amino acids in the cytoplasmic domain and includes the sequenced peptide MEP21-3 (Fig. 3, \textit{A} and \textit{C}). This peptide was found at relatively low amounts in the preparation used for sequence analysis, suggesting that the protein encoded by this cDNA is less abundantly expressed than type 1. Half of the type 2 clones terminated immediately after the first 62 nucleotides of the 3' untranslated region of type 1 clones in an A-rich region, suggesting that they originated because of internal priming of A-rich sequences.

Type 3 clones, found three times, are identical to type 1 and 2 clones but contain a novel cytoplasmic and 3' untranslated region beginning at the point where the deletion in type 1 clones begins (Fig. 3, \textit{A} and \textit{C}). Unlike the other clones, type 3 clones contain a consensus polyadenylation signal.

A comparative protein sequence analysis with those present in the Swissprot and TFASTA data bases revealed no significant similarity between the extracellular domain of MEP21 and any previous entries. As shown in Fig. 3 \textit{B}, the cytoplasmic and transmembrane domain of type 1 clones, however, showed 96% similarity and 86% identity to the cytoplasmic domain encoded by the rabbit podocalyxinlike protein (PCLP)-1 gene (Kershaw et al., 1995). Kyte-Doolittle hydrophobicity plots also revealed strong similarity in this domain as well as a general similarity in the extracellular domain (data not shown). The PCLP-1 gene is thought to encode the major sialoglycoprotein of kidney podocytes and of vascular endothelia (Kershaw et al., 1995).

\textbf{Endogenous and Exogenous Expression of MEP21/Thrombomucin Confirms the Identity of Cloned cDNAs}

To verify that the cDNAs cloned indeed correspond to the mRNAs expressed in MEP21-positive cells, Northern blots were performed. As shown in Fig. 4 \textit{A}, RNA from a variety of chicken hematopoietic cell lines revealed a major transcript of 6 kb in the antigen-positive MEP cell lines HD100 and HD57 but not in several antigen-negative cell lines belonging to the B and T lymphoid as well as to the myelomonocytic, eosinophilic, and erythroid lineages. To demonstrate that the cloned cDNAs encode a protein of the expected size and immunoreactivity, a type 1 cDNA was subcloned into the pSFCV viral expression vector and
The homology detected between thrombomucin and PCLP-1/podocalyxin prompted us to examine the reactivities of the MEP21 antibody in nonhematopoietic tissues. For this purpose we prepared fixed tissue sections from 5-d-old chicks and examined them by immunohistochemistry. As illustrated in Fig. 5, the antigen was found to be expressed on the luminal face of vascular endothelia in a variety of tissues including kidney, lung, intestine, spleen, thymus, and brain. In the kidney, by far the most prominent expression was observed on the podocytes (cells that form the filtration apparatus in Bowman’s capsule). The antigen is not expressed by cells comprising the thick- and thin-walled proximal and distal tubules, nor is it expressed by cells of the collecting ducts. In the lung, thrombomucin is expressed at high levels by capillary networks surrounding the air sacs and by major vessels, but not by bronchial-associated epithelia. In intestine, MEP21 antibody demarcated the luminal face of capillaries in the lamina propria of the villi. Isolated vessels in the smooth muscle layer were also stained, while brush borders of epithelial cells and intra-epithelial lymphocytes scored negative. In the spleen (and thymus, not shown) blood vessels were positive as were occasional cells in the red pulp of the spleen, probably corresponding to thrombocytes.
Thrombomucin Is Expressed on the Surface of Extra- and Intraembryonic Hematopoietic Cells

To study the expression of thrombomucin during ontogeny, whole mounts were prepared from 4-d-old embryos and analyzed by immunoperoxidase staining with MEP21 antibodies. Thrombomucin expression was detected on virtually all embryonic and extraembryonic blood vessels (Fig. 6A). Sections of such embryos also showed strong antigen expression by cells of a variety of tissues including the developing neural tube, aorta, glomerulus, liver, heart, and coelomic cavity (Fig. 6B). In each of these tissues, the antigen appeared to be restricted to cells comprising the lumenal surfaces of tissues or boundary elements between tissues such as in the liver capsule, aorta, mesonephros, coelomic cavity, and the central canal of the neural tube. As can be seen from the inset, we also observed expression of thrombomucin by isolated cells in the ventral wall of the dorsal aorta, in the precise region where the earliest intraembryonic hematopoietic cells develop (Cormier and Dieterlen-Lievre, 1988).

MEP21/thrombomucin Is Expressed on Hematopoietic Precursors in Early Avian Embryos

To determine whether hematopoietic progenitors express the protein, cell suspensions were prepared from the yolk sac of 3-d-old chick embryos and analyzed by FACS® for the expression of thrombomucin as well as for a variety of other cell surface antigens. While a large percentage of the cells from both stages were stained with MEP21 and JS4 antibodies (this antibody detects a late erythroid-specific marker; Schmidt et al., 1986) they were negative for most other hematopoietic markers, including c-kit (a progenitor-specific receptor tyrosine kinase; for review see Callard and Gearing, 1994), MEP26 (an MEP antigen that is also expressed on thrombocytes and early erythroid cells; Graf et al., 1992; McNagny et al., 1992), the integrin αIβ3, EOS47 (an eosinophil-specific transferrin; McNagny et al., 1992, 1996), and MYL51/2 (a myeloid-specific antigen; Kornfeld et al., 1983). MEP21 and JS4 double staining revealed two distinct subpopulations of MEP21-expressing cells: MEP21hi only, and JS4lo/MEP21lo double-positive cells (Fig. 7A). To determine the colony-forming potential of cells from the two subpopulations (R1 and R2), they were FACS® sorted (>95% purity) and seeded into plasma clot cultures. The resulting colonies were then prepared 5 d later for DiffQuik staining and cytological evaluation. As shown in Fig. 7B, the JS4lo/MEP21lo population gave rise exclusively to late erythroid colonies (4–10 eryth-
rocytes per colony), whereas all other hematopoietic precursors (early erythroid, thrombocytic, mixed erythroid/thrombocytic, myelomonocytic, and eosinophilic) were enriched in the MEP21hi-only fraction. Double-negative cells yielded no colonies, suggesting that essentially all colony-forming units from these yolk sac preparations express thrombomucin.

MEP21-only cells also yielded mixed erythroid/thrombocytic/myelomonocytic colonies. However, the low frequency of these precursors compared to the monopotent precursors (<1 mixed colony in 100) made it difficult to rule out the possibility that they actually represented artifacts that arose due to an overlapping of monopotent colonies. To clarify this issue, a total of 2,112 MEP21-positive cells were sorted as single cells into 96-well plates, cultured in blastoderm medium, and evaluated without staining after 6 d. Of the cells seeded, 22% formed colonies that could be classified relatively easily because of their small size and the distinct morphology of nucleated erythrocytes, thrombocytes, macrophages, and eosinophils. As illustrated in Fig. 8 A, the frequencies of the various colony types observed were comparable to those determined in the plasma clot assay (Fig. 7). Four of the cells (that is ∼1 in 500 cells seeded), yielded mixed erythrocyte, thrombocyte, and macrophage colonies, proving that thrombomucin is indeed expressed on the surface of multipotent progenitors. These colonies were significantly larger than the JS4hi/MEP21lo-derived ones, consisting of >100 cells (Fig. 8 B).

**Thrombomucin Is Expressed by Mono- and Multipotent Progenitors in the Bone Marrow with a Distribution Distinct from that of c-kit**

To determine whether thrombomucin is also expressed by hematopoietic progenitors later in ontogeny, bone marrow from 1-wk-old chicks was stained with MEP21 antibodies and analyzed by FACS®. Approximately 7% of the cells were stained and their sorting resulted in an enriched population of both thrombocytes and larger cells with a “blast”-like morphology (McNagny et al., 1992; data not shown). As hematopoietic precursors from mammalian bone marrow express c-kit (Ogawa et al., 1993) and respond to kit ligand (stem cell factor or SCF) we performed a two-color immunofluorescence analysis of chick bone marrow using MEP21 and c-kit antibodies (no antibodies to avian CD34 are available). As shown in Fig. 9 A, this analysis revealed four distinct subpopulations: 13.1% c-kit single-positive cells (R1); 5.8% c-kit/MEP21 double-positive cells (R2); 3.3% MEP21 single-positive cells (with a small proportion of c-kitlo cells; R3); and 87.8% double-negative cells (R4).

These four subpopulations were sorted (the purity of each fraction was >95%) and seeded in plasma clot cultures in three different media: (a) SCF plus anemic serum (containing erythropoietin), plus cMGF; (b) SCF plus anemic serum; (c) SCF plus cMGF. 6 d later the cultures were harvested and stained with DiffQuik for morphological analysis. As shown in Fig. 9 B, double-positive cells (R2) were found to be highly enriched in colony forming units (CFU) (one CFU per two cells plated), followed by c-kit–only cells (R1, one CFU per five cells plated) and MEP21-
only cells (R3, one CFU per 10 cells plated). Unsorted cells yielded an average of one CFU per 17 cells plated, while double-negative cells gave essentially no colonies (data not shown) and were not characterized further. Little variations were observed between the different culture conditions except that the formation of myeloid colonies was found to be dependent on an activity present in the anemic serum.

Phenotypic analysis of the resulting colonies (Fig. 9 B) revealed that a combination of MEP21 and c-kit staining allows the separation of committed monopotent from multipotent precursors. Thus, macrophage and/or granulocyte precursors were present in the c-kit–only fraction, but absent from all fractions expressing thrombomucin. Conversely, thrombocytic precursors were present exclusively in the thrombomucin-only fraction, but not in the c-kit–only fraction. Thrombomucin was also expressed by erythroid precursors but, in contrast to the expression by thrombocytic cells, its expression decreases as a function of erythroid maturation. Thus, thrombomucin-only cells gave rise to mixed thrombocytic/erythroid colonies and early erythroid colonies, including burst forming units, whereas MEP21 low or negative fractions yielded predominantly late erythroid colonies (5–20 cells per colony).

Approximately 1 in 200 double-positive cells gave rise to colonies containing blasts. To determine whether these colonies correspond to multipotent progenitors, a total of 960 cells from each fraction shown in Fig. 9 A were sorted singly into 96-well plates, cultured for 10 d in the presence of SCF, anemic serum, and cMGF, and the wells scored microscopically for the presence of large, blastlike colo-
nies. As the colonies derived from bone marrow were >50-fold larger than those from embryos they could only be classified after cytological staining. Three colonies containing >5,000 immature-looking cells developed from the MEP21/c-kit double-positive fraction (Fig. 10 A), while no such colonies were detected in the other fractions. To analyze the differentiation potential of these blast colonies, they were replated in plasma clot under the same culture conditions, incubated for an additional 5 d, and stained with DiffQuik. Myeloid, erythroid, and thrombocytic colonies were obtained with one of the replated blast colonies (Fig. 10 B), while the other two yielded myeloid and thrombocytic cells (Fig. 10 C).

Discussion

In this paper we describe the cloning of thrombomucin, an antigen identified by the mAb MEP21 directed against the surface of E26 leukemia virus-transformed multipotent hematopoietic progenitors and of normal thrombocytes.

Figure 10. Micrographs of primary and secondary colonies obtained from bone marrow–derived progenitors. (A) Primary blast-type colony, 10 d after seeding of MEP21-positive cells (fraction R2) in medium containing SCF, anemic serum, and erythropoietin. (B and C) Secondary plasma clot colonies obtained after seeding two different blast colonies into plasma clot for 5 d and staining with DiffQuik. M, macrophages; E, erythrocytes; T, thrombocytes.
We now report that it is also expressed on the surface of both mono- and multipotent progenitors from the bone marrow (Fig. 11 B). These findings support the notion that the Myb-Ets oncoprotein of the E26 avian leukemia virus induces the proliferation of hematopoietic cells that resemble normal multipotent progenitors both functionally and phenotypically.

Here we used a recently developed method, nanoelectrospray mass spectrometry, for the sequencing of MEP21 at silver-stained levels. Despite the low amount, all amino acids were sequenced correctly as evidenced by the agreement with the sequence deduced from the cDNA. Interestingly, the expression of a splicing variant could also be directly verified at the protein level.

The most striking feature of the thrombomucin sequence is its Ser-Thr-Pro–rich extracellular domain that is typical of cell membrane–associated mucins, and which is known to be subject to extensive O-linked glycosylation, sialylation, and sulfation (for review see Hilkens et al., 1992). In agreement with this notion, thrombomucin has a calculated mol wt of 54 kD and an apparent mol wt of 150 kD. However, unlike many other mucins such as episialin, this domain does not contain discernable sequence repeats (Hilkens et al., 1992; Gendler and Spicer, 1995). The identification of differentially spliced transcripts, including one that essentially lacks the cytoplasmic domain, suggests that the protein has several functions. That these variant cDNA forms are indeed translated into protein is suggested by the isolation of a peptide specific for type 2 cDNA as well as the detection of two proteins with slightly different apparent molecular weights (Fig. 1). The cytoplasmic and transmembrane domains of thrombomucin exhibit ~90% sequence similarity to the protein encoded by the rabbit PCLP-1 cDNA (Kershaw et al., 1995). PCLP-1, in turn, probably corresponds to the kidney sialoglycoprotein, podocalyxin, an antigen defined by mAb, which is expressed on the surface of rat and human podocytes (Kerjaschki et al., 1984; Kershaw et al., 1995).

Despite the lack of sequence homology between thrombomucin and PCLP-1, in the extracellular domain both proteins contain a Ser-Thr-Pro–rich domain. In addition, their almost-identical calculated and apparent molecular weights (Kershaw et al., 1995), and the presence of thrombomucin on podocytes as well as on a subset of vascular endothelial cells (Horvat et al., 1986) raises the possibility that these proteins represent homologs from different species. It will be interesting to determine whether PCLP-1/podocalyxin is expressed on hematopoietic cells.

Thrombomucin also bears similarities to CD34, another highly glycosylated mucinlike cell surface protein (Simmons et al., 1992). This includes an intracellular domain of about 75 amino acids containing consensus protein kinase C and CKII phosphorylation sites and the generation of both full-size and cytoplastically truncated forms of the molecule (Nakamura et al., 1993). In addition, we have shown that like CD34 (Fina et al., 1990), thrombomucin is expressed on embryonic and adult hematopoietic progenitors and vascular endothelial cells. However (at least in mammals, and in contrast to thrombomucin), CD34 is expressed on committed granulocyte and macrophage precursors and has not been reported to be a marker of platelets (Olweus et al., 1996). This, along with the lack of any clear sequence identity between murine CD34 and the murine thrombomucin homolog (McNagny, K.M., unpublished observations), argues that the two proteins are distinct. It is therefore possible that the very mild hematopoietic defect observed in CD34-ablated mice (Cheng et al., 1996) could be due to compensation by other mucins expressed by early progenitors such as thrombomucin.

During the development of chickens, thrombomucin seems to be one of the earliest markers of hematopoietic cells. Thus, it is expressed in the precirculation yolk sac and on intraembryonic hematopoietic cells before c-kit is expressed. In mice, precirculation yolk sac cells express c-kit, but, as in chickens, these cells are unresponsive to SCF (Ogawa et al., 1993; Bernex et al., 1996). This suggests that in both species c-kit is dispensable for progenitor cell development. It is possible that the small numbers of thrombomucin-positive multipotent progenitors observed in 3-d-old yolk sac are derived from definitive progenitors since at this stage the first definitive blood cells develop and the circulation is established (Dieterlen-Lievre et al., 1996). In adult bone marrow, c-kit and thrombomucin can be detected on the surface of multipotent hematopoietic progenitors as well as on erythroid precursors, but thrombomucin is present in addition on thrombocyte precursors and c-kit on myeloid precursors (Fig. 11 B). Thus, the combination of these two markers provides a simple method for separation of different types of precursors.

We were initially surprised to find that normal and transformed multipotent progenitors share an antigen with mature thrombocytes. However, it is now clear that the thrombocyte integrin αthβ3 is also expressed on both MEPs (Frampton et al., 1995) and normal mouse GEMM cells (Tronik-Le Roux et al., 1995). Likewise, the thrombopoietin receptor is coexpressed by committed mammalian thrombocytes and multi-lineage precursors (Ku et al., 1996). Thrombomucin may therefore be one of a family of molecules shared between thrombocytes and multipotent progenitors.

The distribution of thrombomucin on embryonic progenitors is, in general, similar to that of definitive bone marrow progenitors except that embryonic but not adult myeloid progenitors express the antigen. This might reflect the fact that embryonic myeloid progenitors differentiate more rapidly (in about 3 d versus 10 d for the bone marrow) and have a much more limited division potential than definitive ones (McNagny, K.M., and T. Graf, unpublished observations). Thus, multipotent progenitors might not have sufficient time to clear thrombomucin from their surface as they differentiate along the myeloid lineage. This notion is supported by the observation that yolk sac- derived myeloblasts transformed by the E26 virus express no MEP21/thrombomucin.

What could the function of thrombomucin be? Mucins have been shown to play both positive and negative roles in adhesion processes. For example, specific oligosaccharide side groups of CD34 can serve as ligands for selectins (for review see Kansas, 1996) while ectopic expression of episialin in adherent cell lines has shown that mucins can act as anti-adhesion molecules (Hilkens et al., 1992; Gendler and Spicer, 1995). An anti-adhesive function has also been discussed for thrombomucin’s potential homolog, podocalyxin. Podocalyxin maintains the glomerular filtra-
tion slits of podocytes in an open configuration via its strong negative charge (Seiler et al., 1975). By analogy, it is possible that thrombomucin prevents thrombocytes from inappropriately adhering to vessels walls. Another possibility is that, similarly to CD34, thrombomucin may prevent the differentiation of hematopoietic cells. These possibilities are now being explored by forced expression of thrombomucin in myeloid and erythroid cell lines and by ablating its expression in mice using a mouse thrombomucin homolog that we have recently isolated.

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