Molecular Cloning and Characterization of EndoGlyx-1, an EMILIN-like Multisubunit Glycoprotein of Vascular Endothelium

Received for publication, July 2, 2001, and in revised form, September 13, 2001
Published, JBC Papers in Press, September 14, 2001 DOI 10.1074/jbc.M106152200

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EndoGlyx-1, the antigen identified with the monoclonal antibody H572, is a pan-endothelial human cell surface glycoprotein complex composed of four different disulfide-bonded protein species with an apparent molecular mass of approximately 500 kDa. Here, we report the purification and peptide analysis of two EndoGlyx-1 subunits, p125 and p140, and the identification of a common, full-length cDNA with an open reading frame of 2847 base pairs. The EndoGlyx-1 cDNA encodes a protein of 949 amino acids with a predicted molecular mass of 105 kDa, found as an entry for an unnamed protein with unknown function in public data bases. A short sequence tag matching the cDNA of this gene was independently discovered by serial analysis of gene expression profiling as a pan-endothelial marker, PEM87. Bioinformatic evaluation classifies EndoGlyx-1 as an EMILIN-like protein composed of a signal sequence, an N-terminal EMI domain, and a C-terminal C1q-like domain, separated from each other by a central coiled-coil-rich region. Biochemical and carbohydrate analysis revealed that p125, p140, and the two additional EndoGlyx-1 subunits, p110 and p200, are exposed on the cell surface. The three smaller subunits show a similar pattern of N-linked and O-linked carbohydrates, as shown by enzyme digestion. Because the two globular domains of EndoGlyx-1 p125/p140 show structural features shared by EMILIN-1 and Multimerin, two oligomerizing glycoproteins implicated in cell-matrix adhesion and hemostasis, it will be of interest to explore similar functions for EndoGlyx-1 in human vascular endothelium and adult tissues as well as human cancer tissues, H572 immunostaining was found exclusively on blood vessel endothelium. Notably, these included capillaries, veins, arterioles, and muscular arteries, but interestingly no immunoreactivity was observed in the sinusoidal endothelial cells of the spleen and liver. In neoplastic tissues, H572 immunostaining was consistently found in tumor capillaries, including “hot spots” of neoangiogenesis in certain tumors (2). The endothelial staining pattern revealed a uniform cell surface and cytoplasmic distribution of the antigen, in some cases with an accentuated staining at the abluminal side of the endothelial cell layer. All nonendothelial cell types in normal and tumor tissues were unreactive with mAb H572. The expression of the antigen on cultured human tumor cell lines and normal cells in vitro was also studied in detail. Thus, a host of cultured transformed cells of mesenchymal, neuroectodermal, and epithelial derivation as well as normal lymphocytes, hematopoetic cells, and platelets were found to be H572 antigen-negative. Cells of endothelial origin, such as human umbilical vein endothelial cells (HUVEC), microvascular endothelial cells, and the immortalized endothelial hybrid cell line EA.hy926 express high levels of the antigen in tissue culture. Stimulation of HUVECs with a variety of regulatory peptides and mediators that are known to induce expression of endothelial activation antigens found in inflammatory lesions (3, 4) did not result in an altered expression of the H572 antigen.

Radioimmunoprecipitation studies with mAb H572 identified the target antigen on cultured HUVECs as a high molecular mass glycoprotein complex of ~500 kDa composed of at least four different disulfide-bonded protein subunits migrating at distinct molecular sizes in reducing SDS gels. Direct identification of the epitope-carrying subunit(s) was unsuccessful because of the fact that the epitope was destroyed by the immobilization procedure. To reflect the biochemical characteristics and the endothelium-specific expression of the H572 antigen, the molecule was designated EndoGlyx-1.

In the absence of any information on the EndoGlyx-1 molecular structure, no clues were available regarding its potential function in endothelial cells. Considering the strong interest in mechanisms that control assembly of endothelial cells into vascular structures and regulate the biological function of established vessels, the present study was designed to characterize the EndoGlyx-1 gene. The results should provide essential information and molecular tools to investigate EndoGlyx-1 function in suitable model systems and to investigate the mode of EndoGlyx-1 regulation in the vascular bed.

EXPERIMENTAL PROCEDURES
Cells and Antibodies—HUVECs were purchased (BioWhittaker, Walkersville) and cultured on collagen I-coated cell culture plates
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Vascular Expression of EndoGlyx-1—To compare vascular expression of EndoGlyx-1 with VE-cadherin, a protein complex constituting an antigen specific for the intercellular junctions of endothelium, serial frozen sections obtained from normal human tissues were tested with mAb H572 specific for human VE-cadherin. Acetone-fixed frozen sections were stained with the avidin-biotin immunoperoxidase method. The sections were counterstained with Harris hematoxylin. The bar represents 50 μm.

Fig. 1. Immunohistochemical staining of EndoGlyx-1 on human blood vessels. Serial sections of normal breast tissue were stained with mAb H572 for EndoGlyx-1 or with mAb TEA-1 for VE-Cadherin. Acetone-fixed frozen sections were stained by the avidin-biotin immunoperoxidase method. The sections were counterstained with Harris hematoxylin. The bar represents 50 μm.

Transfection—Transient transfection of HeLa-S3 cells plated in 60-mm tissue culture dishes (Becton Dickinson) was carried out with the transfection reagent FuGENE 6 (Roche Diagnostics) and expression vector pMH (Roche Diagnostics), containing the complete EndoGlyx-1 p125 protein coding sequence or as empty vector. The cells were analyzed for expression 48 h after transfection by immunoprecipitation of metabolically labeled cells with mAb H572 or control IgG, essentially as described (7).

Northern Blot Analysis—The SV total RNA isolation System (Promega) was used for total RNA isolation according to the manufacturer’s instructions. Oligo(dt)-cellulose (Life Technologies, Inc.) was taken for poly(A)+ RNA isolation as described (9). For Northern blots, 1.5 μg of poly(A)+ RNA were electrophoresed on a 0.8% (w/v) agarose gel containing 20 μT NP-40, 5 μM sodium acetate, pH 6.6, and 1.1% formaldehyde. The RNA was blotted to 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) on a Hybond N+ membrane (Amerham Pharmacia Biotech) for 16–20 h and UV-cross-linked. To detect EndoGlyx-1 by endo-Glyx-1 p140 mRNA, two polymerase chain reaction fragments, a 201-bp 5′ probe comprising positions +174 to +375 or a 290-bp central probe comprising positions +1124 to +1414 of EndoGlyx-1 cDNA, were used. Immunoprecipitation Assays and Carbohydrate Analysis—Immunoprecipitation assays were performed as described (9). For cell surface expression analysis, HUVECs (5 × 105 cells) were washed three times with cold phosphate-buffered saline on a cell culture plate and incubated with biotinylation buffer (200 mM HEPES, pH 7.45, 5 mM KCl, 130 mM NaCl, 0.8 mM MgCl2, 1 mM CaCl2, 0.5 mg/ml EZ link Sulfo-NHS-Biotin; Pierce) for 1 h at 4 °C. After removal of the reagent, the cells were washed three times with wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2) and lysed in 1% Triton X-100 lysis buffer for 1 h. The lysates were centrifuged for 15 min at 15,000 × g, and the supernatant (1% SDS, 100 mM NaH2PO4) was separated by SDS-PAGE and transferred to nitrocellulose (10). The nitrocellulose membranes were subsequently incubated, after blocking, with ExtrAvidin-peroxidase conjugates diluted 1:5,000 (Sigma) for 1 h at room temperature. Signals were detected after incubation with ECL reagent and exposure to Hyperfilm ECL (Amerham Pharmacia Biotech).

For glycosylation studies, EndoGlyx-1 was immunoprecipitated from cell surface biotinylated HUVECs as described before, using Affigel 10-bound mAb H572 (4.9 mg/ml). After washing four times with IP wash buffer, the immunoprecipitates were split into four equal aliquots, resuspended in 40 μl of reaction buffer (50 mM sodium phosphate, pH 7.2) and incubated for 16 h at 37°C with 1 unit of sialidase from Flavobacterium meningosepticum or (b) with 10 units of sialidase from Arthrobacter ureafaciens, 0.5 units of O-glycosidase from Diplococcus pneumoniae, or (c) with 1 unit of N-glycosidase F, 10 units of sialidase and 0.5 units of O-glycosidase (Roche Diagnostics) or (d) in the absence of enzyme (mock treatment). Subsequent washing, elution in Laemmli sample buffer, and immunoblotting followed by ECL detection were performed as described before.

Data Base Mining and Sequence Analysis—Data base mining and sequence analyses were performed as essentially outlined in Ref. 7 and are described in great detail at the supplementary World Wide Web page mendel.imp.univie.ac.at/SEQUENCES/endoglyx1/.

RESULTS
Protein Purification of Human EndoGlyx-1 and Identification of the cDNA—The H572 antigen-positive cell line EA.hy926, obtained by fusion of a human umbilical vein endothelial cell with the transformed tumor cell line A549 (12), was selected as a source for EndoGlyx-1 purification. Protein was purified from detergent extracts of 3 × 10^6 EA.hy926 cells. Matrix-bound material was eluted with Laemmli sample buffer and electrophoresed under reducing conditions on a 6% polyacrylamide gel. The expected p110, p125, p140, and p200 EndoGlyx-1 species are marked by arrows. A trypptic peptide pattern was obtained for the p125 and p140 protein (filled arrows) resulting in identification of a common cDNA sequence; the positions of the molecular mass markers (in kDa) are indicated to the left.

**Fig. 2. EndoGlyx-1 protein purification.** Silver-stained gel showing proteins isolated with mAb H572-Affi-Gel 10 matrix (H572) or a negative control matrix with mouse IgG (mlG) from detergent extract of 3 × 10^6 EA.hy926 cells. Matrix-bound material was eluted with Laemmli sample buffer and electrophoresed under reducing conditions on a 6% polyacrylamide gel. The expected p110, p125, p140, and p200 EndoGlyx-1 species are marked by arrows. A trypptic peptide pattern was obtained for the p125 and p140 protein (filled arrows) resulting in identification of a common cDNA sequence; the positions of the molecular mass markers (in kDa) are indicated to the left.

Protein Purification of Human EndoGlyx-1 and Identification of the cDNA—The H572 antigen-positive cell line EA.hy926, obtained by fusion of a human umbilical vein endothelial cell with the transformed tumor cell line A549 (12), was selected as a source for EndoGlyx-1 purification. Protein was purified from detergent extracts of 3 × 10^6 EA.hy926 cells using mAb H572 covalently coupled to Affi-Gel 10 as an immunoaffinity matrix. To analyze the quality of the preparation, an aliquot of the eluted material was separated on a reducing SDS-polyacrylamide gel, and four major protein bands of 110 kDa (p110 subunit), 125 kDa (p125 subunit), 140 kDa (p140 subunit), and 200 kDa (p200 subunit) were visualized by silver staining (Fig. 2, arrows). This is in agreement with the EndoGlyx-1 subunit pattern described previously for human umbilical vein endothelial cell cultures (1). The remaining eluate was separated under reducing conditions on a quantitative SDS-PAGE and yielded 0.1–0.5 pmol of the four specific protein species as determined by colloidal Coomassie staining. Gel fragments containing the EndoGlyx-1 subunits were subjected to a trypsin digest, the eluted peptides were separated by HPLC and analyzed by matrix-assisted laser desorption ionization-time of flight. Subsequent peptide-mass fingerprint analysis gave rise to the molecular masses of nine peptides derived from the p125 subunit (Fig. 3, dashed lines) and fourteen peptides from the p140 subunit (Fig. 3, underlined) matching with the same entry in the public data base of a full-length cDNA (EMBL data base accession number AF088916) and 34% sequence identity with the protein precursor of human multimerin (SwissProt data base, Q13201), respectively (Fig. 4B).

**Domain Structure of EndoGlyx-1 p125/p140—**Bioinformatic analysis identifies EndoGlyx-1 p125/p140 as a typical precursor sequence for a eukaryotic protein resulting evolutionarily from exon shuffling (Fig. 4A). First, there is a putative N-terminal sequence of 23 amino acids similar to a signal leader peptide for translocation into the lumen of the endoplasmatic reticulum. Next there are two major globular segments, an N-terminal EMI domain and a C-terminal C1q-like domain spaced by a central coiled-coil rich region. The EMI domain comprises the amino acids 55–130 including 7 cysteine residues (C1–C7), which potentially form two subdomains (C1–C4 and C5–C7) as determined by sequence similarity searches in data bases by PSI-BLAST tools (13). This is followed by a coiled-coil region comprising more than half of the polypeptide (547 amino acids), composed of five subdomains with a strong prediction (amino acids 323–346, 374–481, 532–553, 554–594, and 687–713) and two subdomains with a weak prediction (amino acids 166–188 and 290–313) for coiled-coil formation. These are interrupted by noncoiled regions, allowing structural flexibility. A short cluster (amino acids 779–806) rich in positively charged amino acids separates the coiled-coil region from the C1q-like domain-containing consensus sequences, common in proteins known to interact with glycosaminoglycans such as heparin and heparan sulfate (14). There are two well conserved regions in the C1q domain: an aromatic motif (amino acids 844–873) as defined by the Prosite C1q pattern is located within the first part of the domain (amino acids 827–873), the other conserved region is near the extreme C terminus (amino acids 912–947). A C-terminal C1q domain is a common feature of the C1q-family of proteins such as the A, B, and C chains of the complement C1q protein (15, 16), the type VIII and X collagens (17, 18), the recently identified and broadly expressed extracellular matrix glycoprotein EMILIN (19), and a large protein of homomultimeric structure called multimerin (20) found in the α-granules of platelets and Waibel-Palade bodies of endothelium.

**EndoGlyx-1 Is a High Molecular Mass Complex of Disulfide-linked Protein Subunits—**To determine the EndoGlyx-1 subunit composition, detergent extracts of metabolically labeled human umbilical vein endothelial cells were purified with mAb H572, and eluted material was separated either by nonreduc-
ing or reducing polyacrylamide gel electrophoresis. EndoGlyx-1 was identified by mAb H572 under nonreducing conditions as a single protein species with a molecular mass of ~500 kDa that barely entered the separating gel (Fig. 6A). Reduction previous to SDS gel electrophoresis resulted in detection of three distinct EndoGlyx-1 species, p125, p140, and p200, indicating a high molecular mass complex cross-linked through intermolecular disulfide bridges (Fig. 6B). To test this, the 500-kDa EndoGlyx-1 protein and a respective control were cut from the nonreduced polyacrylamide gel and re-electrophoresed under reducing conditions on a second gel (Fig. 6, A and B, asterisks), resulting in a protein pattern that appeared to be identical to Fig. 3.

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The sequence of full-length cDNA was identified as an entry for an unnamed protein in the public data base using a matrix-assisted laser disorption ionization-time of flight-based peptide mass fingerprint analysis resulting in molecular mass detection of 9 tryptic peptides derived from the p125 protein species (dashed lines) and 14 tryptic fragments derived from the p140 protein species (underlined amino acids). The putative N-terminal signal sequence is boxed, and the potential polyadenylation signal is double underlined. The 11 predicted N-linked glycosylation sites are marked by circles.
that of the directly reduced H572 precipitates. This indicates that EndoGlyx-1 is composed of at least three disulfide-bonded protein subunits: p125, p140, and p200 (Fig. 6, arrows). In contrast, no major reduction-dependent shift in electrophoretic mobility was observed for proteins in TEA-1 immunoprecipitates, which is in agreement with the reported noncovalent association of VE-cadherin with catenins (22).

Transient transfection studies with the EndoGlyx-1-negative cell line HeLa-S3 were used to confirm that the putative cDNA indeed specifies the antigen defined by mAb H572. Unlike the parental or mock transfected cells, which did not react with mAb H572 (data not shown), H572 antigen was immunoprecipitated from detergent lysates of metabolically labeled transfectants generated with an expression vector containing full-length EndoGlyx-1 p125/p140 cDNA, revealing under nonreducing conditions a high molecular mass species of H11011 kDa, which gave rise after reduction to a major protein band of 125 kDa and to two less prominent species of about 110 and 200 kDa (Fig. 6C, arrowheads). The lack of the p140 species in the transfectants could be due to alternate splicing or differential protein processing. Thus, recombinant EndoGlyx-1 assembles into a disulfide-bonded complex with subunits similar in size to those found in the endogenous EndoGlyx-1 of HUVECs and EA.hy926.

Vascular Surface and Cell Surface Expression of EndoGlyx-1—To investigate whether the EndoGlyx-1 antigen is expressed on the luminal side of intact blood vessel endothelium, mAb H572 or a corresponding isotype-matched control antibody was infused intraluminally into the umbilical cord vein. To detect bound antibody, frozen sections generated after antibody perfusion were incubated with a biotinylated horse anti-mouse IgG followed by the avidin-biotin complex and diaminobenzidine as substrate for the reaction. Our results show a strong immunoreactivity of mAb H572 with the endothelial lining (Fig. 7A), whereas no staining was detected for the isotype control (data not shown). This indicates that at least a sizable fraction of EndoGlyx-1 is exposed on the luminal surface of human blood vessels.

To discriminate between intra- and extracellular distribution of the distinct EndoGlyx-1 subunits, lysates of cell surface biotinylated human umbilical vein endothelial cells were gen-
erated and tested by immunoprecipitation with mAb H572 immobilized to Affi-Gel 10. The bound antigen was eluted, separated by reducing SDS-PAGE, and transferred to nitrocellulose, and the subsequent avidin-peroxidase conjugate-based ECL reaction gave rise to the detection of four biotinylated proteins. The loss in signal intensity after N-glycosidase F treatment could be due to a removal of biotin label immobilized to N-bound sugars.

**DISCUSSION**

We have cloned the gene coding for the human EndoGlyx-1 subunits p125 and p140 based on two lines of evidence. First, 17 peptide sequences found in the p125 and/or p140 proteins are present in the putative EndoGlyx-1 cDNA. Second, transfection of this cDNA into antigen-negative HeLa-S3 cells results in expression of a protein recognized by cognate mAb H572. The apparent molecular size of transfected recombinant EndoGlyx-1 protein under reducing and nonreducing conditions is in good agreement with the observed size of the endogenous antigen.

The present study is not the only evidence linking EndoGlyx-1 expression to the endothelium. Rather, an independent line of investigation, aimed at dissecting comprehensive gene expression profiles for blood vessels in cancer versus normal tissues with the SAGE method, has implicated an EndoGlyx-1-specific tag sequence (23). This study identified a SAGE tag sequence designated PEM87 among 93 tagged transcripts elevated at least 20-fold in normal and tumor endothelium compared with other nonendothelial cell types. The PEM87 tag matches the 3′-untranslated region of a full-length cDNA transcript coding for an unnamed protein in human
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placenta and three oriented expressed sequence tags deposited in the public data base. We show here that PEM87 is the EndoGlyx-1 p125/140 gene. Taking together the EndoGlyx-1 expression data generated with mAb H572 (1) and the RNA expression data derived from SAGE, a consistent picture of EndoGlyx-1 expression in endothelium emerges.

EndoGlyx-1 represents a new member of the unique group of EMILIN-like proteins. This group is shared by two Elastin microfibril interface-located proteins, designated as EMILIN-1 and EMILIN-2, and multimerin, a massive, soluble protein found to be expressed in platelets, megakaryocytes, and vascular endothelium (24). Unlike EndoGlyx-1, EMILINs are expressed in connective tissues of blood vessels, skin, heart, lung, kidney, and cornea (25–27). EndoGlyx-1 p125/p140 diverges in domain architecture from EMILIN-1 and multimerin by a short cluster of charged amino acids located in the transition between the coiled-coil and the C1q-like domains. EMILIN-1 shows two leucine zipper motifs followed by a collagenous domain instead. This region is dominated by basic amino acids (10 of 27 residues) arranged in a sequence similar to consensus motifs found in heparin binding proteins, such as von Willebrand factor (28), which are known to be important for ionic interactions with glucosaminoglycans, such as heparin and heparan sulfate (14). Because glucosaminoglycans provide antithrombotic activities to endothelium through activation of the coagulation protease inhibitor ATIII, it will be interesting to test whether EndoGlyx-1 is capable of recruiting glucosaminoglycans or related structures to the endothelial cell surface.

The molecular organization of EMILINs and multimerin is highly characteristic; both form homotrimers and larger homomultimers via interchain disulfide bonds, giving rise in the case of EMILINs to high molecular aggregates deposited as a fine network in the extracellular matrix (29), whereas multimerin oligomers assembled from a precursor protein, prepromultimerin, are stored in secretory granules of platelets and endothelium (24). Exploiting the yeast two-hybrid system with EMILIN-1 indicates that trimerization is initiated by the C1q-like domain followed by a subsequent quaternary assembly mediated by intermolecular disulfide bridges, which was further supported by the fact that a deletion mutant of EMILIN-1, lacking the C1q-like domain, was incapable in multimer formation (21). Our analysis shows that the C-terminal portion of EndoGlyx-1 p125/p140 consists of an C1q-like domain homologous to EMILIN-1 and multimerin, with 26 and 32% amino acid sequence identity, respectively, which is in accordance with the oligomerization of the different EndoGlyx-1 subunits mediated by intermolecular disulfide bridges.

Analysis of tryptic peptides revealed that the p125 and p140 species originate from the same EndoGlyx-1 mRNA precursor. A likely explanation for the difference in molecular mass of the p125 and p140 subunits is proteolytic processing, as has been shown for platelet multimerin, which, expressed as a 170-kDa glycosylated precursor protein form, is proteolytically converted into a stable 155-kDa protein subunit (30, 31). The size difference of the p125 and p140 polypeptide backbone is further supported by enzymatic removal of N- and O-linked carbohy-
drates, which resulted in an almost identical reduction in the molecular mass of both species excluding the possibility of a differentially glycosylated common core protein.

Because sequence analysis of EndoGlyx-1 p125/p140 cDNA revealed no potential motif for membrane localization, cell surface exposure on endothelium is putatively mediated through association with a plasma membrane-bound and cell surface exposed molecular species. In this context, the as yet unidentified and cell surface exposed subunits p110 and p200 of EndoGlyx-1 are potential candidates for membrane targeting of the complex. However, how the four identified covalently linked subunits contribute to the molecular composition of the individual EndoGlyx-1 complexes awaits elucidation.

In conclusion, the surprisingly restricted tissue distribution and unique domain architecture of EndoGlyx-1 implies a potentially important role in vasculogenesis, angiogenesis, or hemostasis. Future investigations focusing on the biological function will benefit from availability of mAb H572 and recombinant EndoGlyx-1 protein.

Acknowledgments—The expert technical assistance of Claudia Eiberle, Anita Fischbach, Petra Lahm, Hans-Peter Rodi, Karin Ruehe, Anit ach, P., E., R., F., and L., and Reisch Ele, A., Old, L., and J., and Rettig, W. J. (1994) Lab. Invest. 71, 566–573
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