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Cloning and Characterization of Mouse Vascular Adhesion Protein-1 Reveals a Novel Molecule with Enzymatic Activity

Petri Bono, Marko Salmi, David J. Smith, and Sirpa Jalkanen

Human vascular adhesion protein-1 (VAP-1) is a sialylated endothelial cell adhesion molecule mediating the initial L-selectin-independent interactions between lymphocytes and endothelial cells in man. In this work we cloned and characterized mouse VAP-1 (mVAP-1) and produced an anti-mVAP-1 mAb against a recombinant mVAP-1 fusion protein. The isolated cDNA encodes a novel 84.5-kDa mouse molecule. The anti-mVAP-1 mAb stained high endothelial venules in peripheral lymph nodes, and smooth muscle cells and lamina propria vessels in gut. During immunoblotting, this anti-mVAP-1 mAb recognized a 110/220-kDa Ag, suggesting that mVAP-1 is a dimer. Since mVAP-1 has significant sequence identity to members of a family of enzymes called the copper-containing amine oxidases, we showed that mVAP-1 possesses monoamine oxidase activity. Thus, mVAP-1 is the first mouse membrane-bound amine oxidase identified at the molecular level. Based on the 83% identity between the isolated cDNA and human VAP-1 cDNA, the expression pattern, the molecular mass, and the enzyme activity against monoamines, the cloned molecule represents a mouse homologue of human VAP-1. Cloning of mVAP-1 provides a valuable tool for in vivo studies of the significance of VAP-1 for lymphocyte-endothelial cell interactions and of the possible relationship between leukocyte adhesion and amine oxidase activity. The Journal of Immunology, 1998, 160: 5563–5571.

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Sequence data have been submitted to the EMBL/GenBank databases under accession number AF054831.

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Abbreviations used in this paper: HEV, high endothelial venule; VAP-1, vascular adhesion protein-1; hVAP-1, human vascular adhesion protein-1; PLN, peripheral lymph node; PNAd, peripheral lymph node addressin; mVAP-1, mouse vascular adhesion protein-1; GST, glutathione S-transferase; FAD, flavin adenine dinucleotide; BSAO, bovine serum amine oxidase; SSAO, semicarbazide-sensitive amine oxidase.
Materials and Methods

Isolation of mVAP-1 cDNA

Total RNA from BALB/c mouse kidney was prepared using an UltraSpec kit (Biotech, Houston, TX), and a mVAP-1 PCR fragment was amplified using partially degenerate oligonucleotide primers PB3 and PB5 (KEBO Laboratory, Espoo, Finland); PB3 TTTGGTTGAATGGYGCAACAY (designed from the hVAP-1 protein sequence FCFVFEQY; residues 329-335 of the predicted hVAP-1 protein sequence) and PB5 GTTRGGAATRT CYTCWGCRTG (designed from the hVAP-1 protein sequence HAE-DIPP; residues 687-693, respectively). The reaction conditions in PCR were 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, repeated for 30 cycles. The amplified 800-bp fragment was then blunt end cloned into pUC18 vector using a SureClone kit (Pharmacia, Uppsala, Sweden). The cloned DNA fragment was sequenced using a Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland, OH) and the sequencing service facilities of the Department of Medical Genetics, University of Turku (Turku, Finland). Sequence analysis and database comparisons were performed using the Wisconsin Package version 8.1-UNIX of the Computer Group (Madison, WI).

Approximately 1 x 10^11 recombinant λ phage from a mouse heart 5' STRETCH PLUS CDNA library in the Agt10 cloning vector (Clontech Laboratories, Palo Alto, CA) were screened with the reverse transcriptase-PCR-amplified mVAP-1 PCR fragment from mouse kidney mRNA. The fragment was labeled using an Amersham Multiprime DNA labeling kit and [32P]dCTP (about 3000 Ci/mmol (Amersham International, Aylesbury, U.K.). Approximately 100,000 plaques/plate were transferred to Hybond-N nylon filters (Amersham, and duplicate filters were hybridized at 65°C in 5 x SSC, 5 x Denhardt's reagent, 0.5% SDS, and 0.5 mg/ml denatured, sheared salmon sperm DNA. The filters were washed twice for 30 min each time at 65°C in 0.1 x SSC and 0.1% SDS and autoradiographed with intensifying screens (Biomax MS film, Eastman Kodak, Rochester, NY). One positive clone was identified, which was subjected to secondary screening, from which a single plaque was purified, and the insert of this clone was subcloned into EcoRI-digested pUC18 for sequencing. Plaque hybridization, λ phage purification, Escherichia coli transformation, and plasmid purifications were all performed according to standard molecular biology protocols (12).

Northern blot analysis

A mouse multiple tissue Northern blot was obtained from Clontech Laboratories (Palo Alto, CA) and was hybridized according to the manufacturer's instructions at 65°C using the PCR-amplified 800-bp mVAP-1 PCR fragment as a probe.

Abs and immunohistochemistry

An anti-mVAP-1 mAb was produced by immunizing rats with recombinant mVAP-1. In brief, soluble mVAP-1 fusion protein was produced by amplifying the 1168-bp C-terminal part of human mVAP-1 cDNA (pMVPGST8; GTC AGG ATC CGG GAG GGC CAG GAT (designed from the mVAP-1 protein sequence REGQDA; residues 726-731 of the predicted protein sequence) and MVAPGST9; TCC CGA ATT CAG TCT CTT TGA GCA AAC C (designed from the protein sequence GFAYRD; residues 759-765, respectively). Reaction conditions in the amplification were 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 30 cycles. The amplified fragment was digested with BamHI and EcoRI and ligated into BamHI- and EcoRI-digested pGEX-3X vector (glutathione S-transferase (GST) gene fusion vector, Pharmacon). A portion of the fusion protein was run on SDS-PAGE gel and stained with Coomassie blue to confirm the purity and solubility of the resulting GST-mVAP-1 fusion protein. Finally, the fusion protein was mixed with IFA and used to immunize specific pathogen-free Sprague-Dawley rats. Three s.c. injections into the footpads at 1-wk intervals, the rats were sacrificed, and the popliteal lymph nodes were collected. The isolated lymphocytes were then fused with nonsecreting Sp2/0 myeloma cells using standard procedures. The resulting hybridoma supernatants were tested by immunoperoxidase staining of acetone-fixed cryostat sections obtained from specific pathogen-free BALB/c mice gut. The sections were incubated with primary Abs (100 µl of hybridoma supernatant from mVAP-1 fusions or 100 µl of supernatant from a hybridoma producing a negative control mAb, Hermes-1, which is a rat mAb against human CD44) (13) for 120 min, washed twice in PBS, and incubated for 30 min with peroxidase-conjugated goat anti-rat IgG (Dako, Glostrup, Denmark) containing 5% normal mouse serum. The reaction was developed for 5 min using 3,3'-diaminobenzidine hydrochloride in PBS containing 0.03% hydrogen peroxide as a chromogen. After the stainings, the sections were counterstained with hematoxylin. One hybridoma supernatant (TK10–79) positively stained the sections and was subcloned twice by limiting dilution before use in additional experiments.

Transfections and cell culture

For transfections, the mVAP-1 cDNA fragment covering 1 to 3101 bp was isolated from pUC18 after EcoRI and XhoI digestions and subcloned into the pcDNA3 expression vector (Invitrogen, San Diego, CA). CHO cells were used as hosts in transfections to express the mVAP-1 protein, and they were grown in α-MEM (Life Technologies, Paisley, U.K.) plus CHO nucleosides supplemented with 20% FCS, 2 mM glutamine (Biological Industries, Beit Haemek, Israel), 128 U/ml penicillin, and 128 µg/ml streptomycin. Electroporation was performed using a Bio-Rad Gene Pulser apparatus (Richmond, CA; 0.3 kV, 960 µF, 0.4-cm cuvette in RPMI plus 1 mM sodium pyruvate, 2 mM l-glutamine without serum, and 20 µg of expression plasmid). These stably transfected cells were selected by culturing them in the presence of 0.5 mg/ml genetin (Life Technologies, Grand Island, NY).

Immunoblotting

The mVAP-1 CHO transfectants were scraped from cell culture flasks and lysed with lysis buffer (150 mM NaCl, 10 mM Tris base (pH 7.2), 1.5 mM MgCl2, 1% Nonidet P-40, 1% protamine, and 1 mM PMSF). After 2 h at 7°C, the lysates were centrifuged (13,000 rpm, 30 min, 7°C), and the supernatants were collected. Noninfamed mouse gut samples from BALB/c mice were dissected free from the lamina propria. The smooth muscle wall was minced into small pieces, lysed 2 h in the lysis buffer at 7°C, and centrifuged, and the supernatants were collected. Twenty-five micrograms of the gut smooth muscle lysate supernatant or CHO cell lysate supernatant was mixed with an equal volume of Laemmli’s sample buffer for electrophoresis under nonreducing conditions. Before loading on a 5 to 12.5% SDS-PAGE gel, the samples were boiled for 5 min at 95°C. The resolved proteins were transferred onto nitrocellulose sheets (Hybond-ECL, Amersham) by a Hoefer electrophoblotter (San Francisco, CA). Nitrocellulose strips were stained using an enhanced chemiluminescence detection kit (Amersham) according to the manufacturer’s recommendations. Briefly, blocking was performed with PBS containing 10% nonfat milk powder and 0.3% Tween-20 for 1 h, and a 1/20 dilution of the hybridoma TK10–79 supernatant was used as a primary Ab. The mAb Hermes-1 was used as a negative control Ab. Peroxidase-conjugated goat anti-rat Ig containing 5% normal mouse serum was used in the second stage of staining.

Amine oxidase assays

The mVAP-1 transfected and mock (pcDNA3 vector) transfected CHO cells (typically 10–15 x 10^6) were scraped into 10 ml of PBS and centrifuged at 4°C. The cell pellet was washed twice with PBS and finally suspended in 1 ml of PBS before sonication on ice at medium power (Braun sonicator, Melsungen, Germany). Sonicated lysates were used in the assay immediately or after storage at −20°C. All enzyme reactions were performed with or without heating the cell lysates for 5 min at 95°C before addition of the substrate. The enzyme activity assay protocol was modified from the method described by D’Agostino (14). Briefly, in benzylamine assay, 100 µl of the cell lysate was mixed with 300 µl of 100 mM NaPO4 and 100 µl of the substrate, and in the putrescine assay, 100 µl of the cell lysate was mixed with 1.7 ml of 100 mM NaPO4, 200 µl of 400 mM acetaldehyde, and 100 µl of the substrate. [14C]benzylamine hydrochloride (0.2 µCi; Amersham) in 100 mM NaPO4 containing 0.8 mM unlabeled benzylamine (Sigma, St. Louis, MO) or 0.1 µCi of [14C]putrescine dihydrochloride (Amersham) plus 0.04 mM unlabeled putrescine (Sigma) were used as substrate in these reactions. One milligram of porcine kidney diamine oxidase (Sigma) was used as a positive control for diamine oxidase activity, and 0.5 mg of bovine plasma monamine oxidase was used as a control source for monamine oxidase activity. The reactions were incubated at 37°C for 60 min before double extraction of the labeled aldehyde reaction products in 700 µl of toluene-based scintillation mixture containing toluene and 0.35 g/l diphenyloxazole. The extractions were measured for 14C by a liquid scintillation counter (15). Hydroxylamine hydrochloride (5 µM; Sigma) and semicarbazide hydrochloride (0.1 mM; Sigma) were used as specific inhibitors (16) and incubated for 10 min with 100 µl of the cell lysate before addition of the substrate. Total protein concentration measurements were performed using the Bradford method with bovine γ-globulin as a standard. The results are presented as picomoles of substrate used per minute per milligram of protein.
Results

Isolation of a mVAP-1 cDNA

Based on the hVAP-1 sequence, several degenerate oligonucleotide primers were designed to be used in reverse transcriptase-PCR experiments with mRNA isolated from different mouse tissues. A single 800-bp fragment of the expected size was amplified from mouse kidney mRNA with the degenerate oligonucleotides PB3 and PB5 as primers, and this specific fragment was cloned for sequencing. The sequence revealed that the amplified 800-bp fragment had 80% amino acid identity to hVAP-1, indicating that the amplified fragment could represent mVAP-1. PCR screening of different mouse libraries showed that a mouse heart cDNA library was positive with the same primer pair, so this library was selected for screening with the PCR-amplified mVAP-1 fragment as a probe to search for the full-length mVAP-1 cDNA. One phage with a large 4.4-kb insert was isolated, cloned, and sequenced, and the preliminary sequence revealed that the isolated cDNA contained the whole 800-bp PCR fragment sequence. Thus, the whole isolated insert was sequenced in two directions. The insert had an ATG starting codon 350 bp down from the 5’ end of the cDNA and was followed by one continuous open reading frame of 2298 bp (Fig. 1). A 5’ untranslated region of 349 bp and a 3’ untranslated region of 1830 bp following the TGA stop codon were present in the isolated clone. At the 3’ end of the clone, a polyadenylation signal and a poly(A) tail were found, suggesting that the isolated clone also included the whole 3’ end of the cDNA encoding mVAP-1.

The mouse VAP-1 protein and its homology studies

The open reading frame encoded a 765-amino acid protein with a predicted molecular size of 84.5 kDa (Fig. 1). The mVAP-1 protein has six potential N-glycosylation sites and six potential O-glycosylation sites (according to the O-glycosylation site prediction server, NetOglyc@cbs.dtu.dk (17)). The predicted amino acid sequence revealed that the identity of mVAP-1 to hVAP-1 is 83%, distributed evenly throughout the molecules. A search of the most recent releases from SwissProt and GenEMBL sequence databanks revealed that mVAP-1 has no significant identity to any other adhesion molecule or mouse protein, but it has, like hVAP-1, significant identity to a family of enzymes called copper-containing amine oxidases.

Amine oxidases are enzymes classified into two main classes on the basis of the chemical nature of their cofactors (16, 18): the flavin adenine dinucleotide (FAD)-containing amine oxidases and the copper-containing amine oxidases. The FAD-containing amine oxidases include both the A and B forms of the widely studied outer mitochondrial membrane monoamine oxidases, which possess activity not only against primary amines but also against secondary and tertiary amines (19). In addition to the differences in their cofactors, the copper-containing amine oxidases are distinct from the FAD-containing amine oxidases with regard to their location inside the cell and their substrate specificity. A multiple alignment of mVAP-1, hVAP-1, and five other cloned mammalian members of the copper-containing amine oxidases is shown in Figure 2. The identity of mVAP-1 to these molecules varied between 42 and 95%. The highest identity (95%) was found to the recently published partial cDNA sequence of a rat membrane-bound amine oxidase (20), which together with hVAP-1 comprises the first identified transmembrane protein members of this family. Figure 2 shows also that the hydrophobic amino acids present in the rat (residues 6–26) and human (residues 5–27) membrane-spanning regions are 100% identical between mouse and rat molecules and highly conserved between mouse and human VAP-1. In contrast, the putative transmembrane sequence in the mouse has no homology to other members of the copper-containing amine oxidase family (for example bovine serum amine oxidase (BSAO), which are secreted proteins. This indicates that mVAP-1 probably has a transmembrane domain close to the N-terminus of the molecule and that it belongs to the membrane-bound glycoprotein group of the copper-containing amine oxidase family.

Expression of mVAP-1

In Northern blot analysis of mRNA from BALB/c mice using the 800-bp mVAP-1 PCR fragment as a probe, a single 4.4-kb band was found to hybridize from different tissues (Fig. 3). No other mRNA species were detected even after prolonged autoradiography (data not shown). The expression levels varied; they were strong in heart, lung, skeletal muscle, kidney, and testis. In liver, brain, and spleen only low amounts of mVAP-1 mRNA were detected in longer exposures. The expression pattern of mVAP-1 mRNA is very similar to that of hVAP-1, except that in mouse brain a weak mRNA message was detected during the prolonged exposure, whereas human brain did not express any detectable hVAP-1 mRNA in Northern blot analysis (data not shown).

The mVAP-1 possesses enzyme activity against monoamines

The fact that mVAP-1 is the first cloned mouse molecule with significant identity to proteins belonging to the amine oxidase family (16) led us to test whether mVAP-1 possesses enzyme activity against different amines. In enzyme assays, mVAP-1-transfected CHO cell lysates were assayed with benzylamine (a monoamine) or putrescine (a diamine) as a substrate. In these experiments mVAP-1 showed a clear activity against benzylamine but not against putrescine (Table I), and the activity could be destroyed by heating the cell lysates for 5 min at 95°C before addition of the substrate. Mock transfected CHO cell lysates did not show activity against any substrates. Porcine kidney diaminodioxide (for diamine oxidase activity) and bovine plasma monoamine oxidase (for monoamine oxidase activity) were used as positive controls (18, 21) against putrescine and benzylamine, respectively, and the activities of these controls were abolished by heating. To test whether mVAP-1 activity against benzylamine could be inhibited by semicarbazide or hydroxylamine, two known inhibitors of copper-containing amine oxidases (16), these inhibitors were incubated separately with the mVAP-1-transfected CHO cell lysates before addition of the substrate. In the presence of 0.1 mM semicarbazide or 5 μM hydroxylamine, mVAP-1 showed no activity against benzylamine (Table II). In conclusion, mVAP-1 has a clear activity against benzylamine but not against putrescine (a diamine), and the activity is inhibitable with the known copper-containing amine oxidase inhibitors.

Production of an anti-mVAP-1 mAb

To study the expression of mVAP-1 and the molecular size of mVAP-1, an anti-mVAP-1 mAb was produced. First, a C-terminal 117-bp fragment of the mVAP-1 cDNA (coding for amino acids 726–765 in the mVAP-1 protein sequence) was amplified with PCR and cloned into the pGEX3X vector to produce a GST-mVAP-1 fusion protein. After isopropylthiogalactoside induction and purification of the resulting recombinant protein from E. coli, part of the sample was run on an SDS–PAGE gel to test its purity and solubility. Because the resulting recombinant protein was soluble and of the expected size, the rest of this material was used to immunize rats from which polyclonal lymph node lymphocytes were collected after 3 wk and fused with Sp2/0 mouse myeloma cells. The resulting hybridoma supernatants were screened by immunohistologic staining of frozen tissue sections. Based on the staining
FIGURE 1. The complete nucleotide sequence of the mVAP-1 cDNA and the predicted amino acid sequence. Potential N-glycosylation sites (asparagines) are circled, and potential O-glycosylation sites are boxed. The putative transmembrane part of mVAP-1 in residues 6 to 26 is boxed and shaded. The important tyrosine for the enzyme function in residue 471 (see Discussion) is in bold and underlined together with the consensus sequence NYD in residues 470 to 472. The three conserved histidines that act as a ligand for copper are also shown in italics and underlined in residues 520, 522, and 684. The 3' polyadenylation signal is in bold and underlined. The sequence data have been submitted to the EMBL/GenBank databases under accession number AF054831.
reactivity of all anti-hVAP-1 mAbs with smooth muscle in addition to endothelial cells, we decided to test the hybridomas on mouse gut sections. One of the hybridoma supernatants positively stained these gut sections, while the negative controls showed no reactivity, and the staining pattern resembled that of anti-hVAP-1 mAbs on human gut sections.

To test the specificity of the new mAb, designated TK10-79, lysates from CHO mVAP-1 transfectants were tested in immunoblotting. Staining of the filter with mAb TK10-79 revealed specific bands with apparent molecular masses of 110 and 220 kDa from CHO mVAP-1 transfectants under nonreducing conditions (Fig. 4, lane 1), while the mock transfectants (Fig. 4, lanes 2 and 4) and control Ab stainings were negative. These results confirmed that mAb TK10-79 is specific for mVAP-1.

Expression of mVAP-1 in gut and PLN
The resulting new anti-mVAP-1 mAb TK10-79 was used in immunoperoxidase stainings to characterize the expression of mVAP-1 in gut and PLN.

FIGURE 2. A multiple alignment of the predicted mVAP-1 protein sequence with hVAP-1 and other cloned members of the copper-containing amine oxidase family. The numbers on the left of each row correspond to the first amino acid of the lane. The putative transmembrane part of mVAP-1 (residues 6–26) is 100% identical with the transmembrane residues of the rat membrane-bound amine oxidase (residues 6–26). PDAO1, human placental diamine oxidase 1 (35); PDAO2, human placental diamine oxidase 2 (35); BSAO, human placental diamine oxidase (36); RatDAO, rat diamine oxidase (37); RatAO, rat membrane amine oxidase (partial sequence) (20). The sequence alignments were performed with GCG Pileup, and residues with identity to mouse VAP-1 were identified using GCG Boxshade.

FIGURE 3. Northern blot analysis of mVAP-1 mRNA. Poly(A)^+ RNA isolated from the indicated tissues (a mouse multiple tissue Northern blot from Clontech) was probed with an 800-bp mVAP-1 fragment. No other mRNA species than the 4.4-kb form were detected even after prolonged exposure. Each lane contains approximately 2 μg of poly(A)^+ RNA.
mVAP-1 in mouse PLN and gut. In mouse gut sections, mAb TK10-79 stained smooth muscle cells (Fig. 5A) and faintly stained vessels of the lamina propria (Fig. 5B). The staining pattern of mVAP-1 in noninflamed gut resembles the staining pattern of hVAP-1 in human gut, with hardly any staining of mucosal HEV in Peyer’s patches. In mouse PLN, anti-mVAP-1 mAb reactivity was mainly seen on HEV (Fig. 5D) where the initial lymphocyte binding to endothelium and the transmigration take place. mAb TK10-79 did not stain any lymphocytes present in PLN. Thus, mVAP-1, like hVAP-1 (8), is present in both endothelial cells and smooth muscle cells.

The mVAP-1 is a 110/220-kDa molecule

To determine the molecular size of the Ag that the mAb TK10-79 recognizes in tissue sections and to compare the size of this Ag to that of mVAP-1 CHO transfectants, lysates from mouse gut smooth muscle and from CHO mVAP-1 transfectants were run in parallel on SDS-PAGE and used for immunoblotting with the mAb TK10-79. From mouse gut smooth muscle cell lysate, mAb TK10-79 revealed, under nonreducing conditions, a band of approximately 110 kDa and a smeared band of around 220 kDa (Fig. 5, lanes 2 and 3), no specific bands were seen. Molecular mass standards are indicated on the left in kilodaltons.

Discussion

In this work we have cloned and characterized a previously unknown mouse molecule and used the cloned cDNA to produce a TABLE I. mVAP-1 is a monoamine oxidase

<table>
<thead>
<tr>
<th>Sample</th>
<th>Benzyamine</th>
<th>Putrescine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-mVAP</td>
<td>1449 ± 73.1</td>
<td>2.28 ± 0.1</td>
</tr>
<tr>
<td>CHO-mock</td>
<td>−0.80 ± 3.75</td>
<td>−0.03 ± 0.04</td>
</tr>
<tr>
<td>DAO</td>
<td></td>
<td>18.3 ± 1.2</td>
</tr>
<tr>
<td>MAO</td>
<td>528 ± 22.3</td>
<td></td>
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* CHO-mVAP = CHO cells transfected with pcDNA3 containing mVAP-1 cDNA; CHO-mock = CHO cells transfected with pcDNA3; DAO = porcine kidney diamine oxidase, a positive control for diamine oxidase activity; MAO = bovine plasma monoamine oxidase, a positive control for monoamine oxidase activity.

specific mAb against this novel protein. The protein is named mVAP-1 because it represents the mouse homologue of hVAP-1 based on the following criteria. 1) The predicted protein sequence from the isolated cDNA has significant identity (83%) to hVAP-1 protein. 2) The anti-mVAP-1 mAb TK10-79 that was raised against the C-terminal end of the molecule detects in immunoblotting 110- and 220-kDa proteins, indicating that mVAP-1 is probably, like human VAP-1, a dimer and composed of two identical subunits. 3) In immunoperoxidase stainings, the anti-mVAP-1 mAb stained cells in mouse gut smooth muscle and mouse PLN tissue sections in a manner similar to anti-hVAP-1 mAb staining on corresponding human tissues. 4) In Northern blot experiments, the tissue distributions of mVAP-1 and hVAP-1 mRNA are very similar.

The hVAP-1 has been shown to be a transmembrane molecule with a short four-amino acid intracellular part and a long extracellular domain (see Footnote 5). Very recently, a new rat transmembrane protein belonging to the same copper-containing amine oxidase family as hVAP-1 has been partly cloned, and the predicted protein sequence of this cloned fragment of the molecule has 95% identity to mVAP-1 and 83% identity to hVAP-1, indicating that the published partial cDNA is probably encoding the rat homologue of mouse and human VAP-1 (20). Because the transmembrane-spanning amino acids among these three molecules (Fig. 2, residues 5–26 in hVAP-1 and 6–26 in rat AO) are highly conserved, mVAP-1 is probably also a type II transmembrane protein with the N-terminus of the molecule located intracellularly and the C-terminus located extracellularly (23). The predicted mVAP-1 amino acid sequence revealed that mVAP-1 has six potential N-glycosylation and six putative O-glycosylation sites, and
all the potential glycosylation sites are located in the putative extracellular part of the molecule. Thus, different oligosaccharides may also play a role in the function of mVAP-1 as an adhesion molecule, as has been shown for hVAP-1 (7). The functional importance of these possible oligosaccharide modifications of mVAP-1 and the adhesion properties of CHO-mVAP-1 transfectants remain to be determined, since the anti-mVAP-1 mAb TK10-79 is apparently not a function-blocking mAb in adhesion assays. This is not surprising, since the mAb was raised against a short peptide sequence of mVAP-1 that was expressed in a bacterial host.

In immunoblotting experiments the anti-mVAP-1 mAb detected 110- and 220-kDa Ag from mouse gut smooth muscle lysate and from CHO mVAP-1 transfectants. Both 110- and 220-kDa forms of the detected molecule are larger than the corresponding hVAP-1 bands (~90/170 kDa) detected by immunoblotting (7). This may be due to the species-specific glycosylation differences, since the core proteins of the molecules are almost equal in size, whereas the number of potential glycosylation sites is even higher in mVAP-1 than in the hVAP-1 protein core.

The ligands of both hVAP-1 and mVAP-1 are unknown. The predicted protein sequence of hVAP-1 (see Footnote 5) revealed at residues 726 to 728 an RGD motif that is an important integrin binding site (24) present in a variety of integrin ligands. At present, the significance of this RGD motif for the function of hVAP-1 is unknown. Although this motif is not completely conserved between hVAP-1 and mVAP-1, residues 726 to 728 in mVAP-1 (REG) may also represent a possible integrin binding site, since a
cyclic peptide containing the sequence RE has been shown to be able to interact with the same or an overlapping binding site in the \(\alpha_\beta\) integrin as RGD (25). In principle, in RE, the basic group of arginine is one amino acid closer to the acidic group of the aspartate than in RGD, making the sequences REG and RGD closely related in spatial terms (E. Ruoslahti, unpublished observations).

In addition to the high homology to hVAP-1, the isolated mVAP-1 cDNA showed significant identity in database comparisons to enzymes belonging to the family of copper-containing amine oxidases, but no similarity to FAD-containing amine oxidases, which include monoamine oxidases A and B. The copper-containing amine oxidases are enzymes that catalyze the oxidative deamination of various biogenic amines, and they have widely differing substrate specificities (21). They are composed of two identical subunits, each containing one copper atom and one covalently bound carbonyl-containing cofactor, the chemical identity of which is still controversial. It is not clear whether the cofactor is the same in each member of the family or whether the function of all these enzymes is actually copper dependent (26). The cofactor at the active site of BSAO has been identified as topa-quinone (6-hydroxydopa) (27). The topa-quinone is formed from a precursor tyrosine that has to be part of a consensus sequence Asn-Tyr-Asp/Glu to become post-translationally modified to topa-quinone (28). This active site consensus sequence is also found in the mVAP-1 sequence in residues 470 to 472. The crystal structure of one copper-containing amine oxidase (E. coli copper amine oxidase) has been published (29), and it confirmed the importance of the modified tyrosine residue in the active site and the existence of three histidines that also have to be conserved for enzyme activity (27, 30). These three histidines that coordinate copper binding in the protein core can also be found in mVAP-1, and they are located in positions 520, 522, and 684 in mVAP-1 cDNA sequence (Fig. 1).

In addition to identification of the structural requirements for the enzyme activity in mVAP-1, we have directly shown that mVAP-1 is enzymatically active. The mVAP-1 cDNA-transfected CHO cell lysates had activity against benzylamine, but not against putrescine, indicating that mVAP-1 has activity at least against monoamines but probably not against diamines. Our results differ from previous reports in which tissue-bound amine oxidase from mouse white adipose tissue homogenate has been shown to possess activity not only against benzylamine but also against acetylputrescine (31). Tissue-specific substrate differences may be one explanation for this detected difference, whereas the most likely explanation is that crude homogenate of the white adipose tissue contains in addition to their structural identity. This finding is of great importance, as much species-dependent variation in substrate specificity and enzyme kinetics of these enzymes is available for BSAO and rat SSAO. We have cloned a novel mouse molecule with activity against monoamines that is inhibitable by carbonyl reagents such as semicarbazide and hydroxylamine. Thus, the cloned mouse molecule most likely belongs to the same SSAO subgroup of amine oxidases as hVAP-1 and represents the first cloned mouse SSAO.

Relatively little is known about the biologic role of SSAO, especially in higher eukaryotes. One problem has been the different substrate specificities, which can limit the value of studies with different laboratory animals in attempts to define the physiologic role of primary amine oxidases in man. The identification and cloning of a mouse molecule with similar enzyme activity to the human homologue will be a valuable tool in future studies on the biologic role of these enzymes. Now it will be possible to investigate in a mouse model whether enzyme activity and lymphocyte adhesion are linked or independent functions.

Although the significance of hVAP-1 as a functionally important endothelial cell adhesion molecule that can mediate early interactions between endothelial cells and lymphocytes in the normal recirculation of lymphocytes as well as in the extravasation of lymphocytes into sites of inflammation has been well established in vitro (6-9, 11), only limited information on the functional significance of this endothelial cell adhesion molecule in vivo is available. With intravital microscopy, VAP-1 has been shown to be involved in the initial interaction of lymphocytes and endothelial cells in inflamed rabbit mesenterial venules (11). However, a mouse model would be extremely useful for further analyses of the site of VAP-1 in the multistep adhesion cascade and of the significance of VAP-1 in vivo. In this work we have isolated a cDNA encoding mVAP-1, produced an Ab against it, and characterized with this mAb the mouse homologue of hVAP-1. The cloning of the mVAP-1 gene is now in progress, and the targeted disruption of the gene will give valuable information about the role of this adhesion molecule in vivo.

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References


