Identification and Characterization of AMACO, a New Member of the von Willebrand Factor A-like Domain Protein Superfamily with a Regulated Expression in the Kidney*

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The genes coding for human and mouse AMACO, an extracellular matrix protein containing VWA-like domains related to those in Matrilins and Collagens, were detected in databases, the cDNAs were cloned, and the primary structures were deduced from the nucleotide sequences. The genes consist of 14 exons and have a similar exon/intron organization. The protein consists of a signal peptide sequence, an N-terminal VWA domain connected to two additional, tandem VWA domains by a cysteine-rich sequence and an epidermal growth factor (EGF)-like domain. The C terminus is made up of another EGF-like domain followed by a unique sequence present in mouse, but absent in human. The predicted molecular weight of the proteins is 79,485 in human and 83,024 in mouse. Full-length AMACO was expressed in 293-EBNA cells, purified by use of an affinity tag and subjected to biochemical characterization. Both monomers and aggregates of AMACO were recovered, as shown by electron microscopy and SDS-PAGE. AMACO was found in the media of a variety of established cell lines of both fibroblast and epithelial origin. In the matrix formed by 293-EBNA cells overexpressing the protein, AMACO was deposited in patchy structures that were often cell-associated. Affinity-purified antibodies detect expression in cartilage and expression associated with certain basement membranes. In the kidney of adult mice, a second promoter located in intron 4 is active. If the resulting transcript is translated it could not yield a secreted protein because of the lack of a signal peptide sequence. The developmental switch from an AMACO mRNA, expressed by the newborn kidney, to the truncated transcript found in the adult kidney indicates an unusual regulation of AMACO expression.

The von Willebrand Factor A-like domain (VWA or vWFA) is a well studied protein module present in a great variety of extracellular proteins, e.g. the prototype von Willebrand factor, the complement factors B and C2, trypsin inhibitors, collagens, matrilins, and integrins. Recently even intracellular members of the protein superfamily have been identified (for review see Ref. 1). It is generally assumed that VWA domains mediate protein-protein interactions and extracellular VWA-domain-containing proteins are involved in cell-cell, cell-matrix, and matrix-matrix interactions and thereby play roles in important physiological processes such as immunity, hemostasis, and wound healing (2). X-ray crystallography revealed a structure made up from alternating α helices and a central β sheet also known as the Rossmann fold. Some VWA domains contain a conserved MIDAS (metal ion-dependent adhesion site) sequence motif, which can be involved in ligand binding. The structures of the A1 domain (3) and the A3 domain (4) of the von Willebrand factor itself, and the VWA domains (I domains) of the integrin α subunits α5 (5), α6 (6), αγ (7), and α8 (8), as well as that of the integrin β3 subunit (8) have been resolved. The binding sites of the vWFA1 domain for GpIb (9) and those of integrin α5 (10) and vWFA3 domain (11) for collagen have been determined and show differences in location and structure. Indeed, the collagen-binding site of the vWFA3-domain, located on the rather hydrophobic front face of the A3-domain, is clearly distinct from that of the homologous integrin α5 I-domain, where the MIDAS motif on top of the domain is involved in binding.

The matrilins constitute a recently discovered family of extracellular matrix proteins (for review see Ref. 12). Matrilin-1 and -3 are expressed almost exclusively in skeletal tissues, whereas matrilin-2 and -4 have a much broader distribution. In the extracellular matrix of cultured cells matrilins are deposited in fibrillar networks. All matrilins have a similar modular composition with VWA-like and epidermal-growth factor (EGF)-like domains followed by a C-terminal coiled-coil domain. It is thought that matrilins play a role in mediating interactions between major components of the extracellular matrix, such as collagens and proteoglycans (13), and indeed matrilin-1, -3, and -4 are associated with native collagen type VI microfibrils extracted from rat chondrosarcoma tissue (14). The matrilins are bound to the small leucine-rich repeat proteins biglycan and decorin, which in turn interact with the N-terminal globular domains of the collagen VI molecules. At the periphery of the microfibrillar complex matrilins were found to mediate interactions with aggrecan or collagen II.

A subgroup within the large family of collagens contains VWA domains in addition to triple helical structures. Among growth factors; ESI Q-TOF, electrospray ionization quadrupole/time-of-flight; MIDAS, metal ion-dependent adhesion site; ncRNA, non-coding RNA; RT, reverse transcription; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag; UTR, untranslated region; nt, nucleotide(s); p.c., post coitus.

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¶ The abbreviations used are: VWA, von Willebrand factor A; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; EGF, epidermal growth factor; ESI Q-TOF, electrospray ionization quadrupole/time-of-flight; MIDAS, metal ion-dependent adhesion site; ncRNA, non-coding RNA; RT, reverse transcription; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag; UTR, untranslated region; nt, nucleotide(s); p.c., post coitus.

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these, collagens XII, XIV, XX, and XXI are fibril-associated collagens with an interrupted triple helix and are thought to bind to other extracellular matrix components by their N-terminal, VWA-containing segments (1). The homotrimeric collagen VII forms a cross-like structure where the three globular N-terminal domains, consisting of VWA- and FN3 domains, form the short arms. By these domains a variety of ligands, e.g. collagens I and IV and laminins 5 and 6, are bound (1). The microfibril-forming, heterotrimeric collagen VI also contains VWA domains in each subunit, and these have been shown to interact with fibrillar collagen I (15).

In the present study we have characterized a new extracellular member of the VWA domain family, AMACO. The AMACO VWA domains share homology with those found in matrilins and collagens.

**EXPERIMENTAL PROCEDURES**

RT-PCR and 5’ RACE—RT-PCR was used to clone the mouse and human AMACO cDNAs. Primers were designed according to EST and genomic sequences (Table I). To prevent mutations in the RT-PCR we used the Expand High Fidelity PCR system (Roche Applied Science). RT-PCR was used to clone the mouse and human AMACO cDNAs. Primers were designed according to EST and genomic sequences (Table I). To prevent mutations in the RT-PCR we used the Expand High Fidelity PCR system (Roche Applied Science). RT-PCR was used to clone the mouse and human AMACO cDNAs. Primers were designed according to EST and genomic sequences (Table I). To prevent mutations in the RT-PCR we used the Expand High Fidelity PCR system (Roche Applied Science).

| Name         | Sequence 5’–3’                                                                 | Position |
|--------------|-------------------------------------------------------------------------------|----------|
| Mouse primers|                                                                                   |          |
| AMm1         | TTGGACAGTGAGCCGAGCGAG                                                         | 1–19 (f) |
| AMm2         | GCGAGATCGCTGTCCCAAGATCACC                                                     | 2653–2662 (r) |
| AMm6         | TGAGGACAGTGAGCGAG                                                            | 2902–2904 (f) |
| AMm15p       | CAAGGAGCAAGCTGAGGAG                                                          | 935–954 (f) |
| RACE3        | TGGATGAGCGAGCCGAG                                                             | 889–842 (r) |
| AMm13        | GAGGAGGAGACCAGGGAAG                                                         | 951–969 (f) |
| Human primers|                                                                                   |          |
| Amh2         | AGGCGCTGCTGTTTCTCTG                                                          | 274–293 (f) |
| Amh5         | TGGTGAGACAGAGATCCTGAG                                                        | 1776–1775 (r) |
| Amh3         | AGATCCAGGCCAGCCAGCCAG                                                        | 1736–1735 (f) |
| Amh4         | GAGAGGAGACAGACTCTGAG                                                         | 2647–2637 (r) |
| Amh10        | TCCAGCAGGGGCCATTGCGAGCAAG                                                   | 1031–1006 (r) |
| Amh9         | AGCTCCCTTTCCCCAGGCGTGGTAGACC                                                  | 449–422 (r) |

* f, forward; r, reverse.

1 Primer AM15p was 3’-phosphorylated.

For the determination of the AMACO N terminus, a cut-out gel band was reduced with 20 mM dithiothreitol for 15 min at 37°C and alkylated with 50 mM iodoacetamide for 15 min at 37°C in 50 mM ammonium bicarbonate, pH 8.0. The in-gel digestion with trypsin was carried out overnight at an enzyme concentration of 12.5 ng/μl in 50 mM ammonium bicarbonate, pH 8.0, at 37°C. Subsequent elution was performed in two steps with 0.1% trifluoroacetic acid/acetone (2:3) for a total of 30 h. The supernatants were pooled and dried in a vacuum centrifuge, and the peptides were dissolved in 5% formic acid. ESI Q-TOF mass spectrometry was used to sequence a peptide containing the N-terminal 13 amino acid residues of mature AMACO.

In Situ Hybridization—Tissues from newborn mice were fixed overnight with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at 4°C, washed overnight with PBS at 4°C, dehydrated, and embedded in paraffin. Sections of 7 μm were cut, mounted on SuperFrostPlus glass slides, dewaxed in xylene, and rehydrated. After washing in PBS these were digested with 200 milliunits/ml proteinase K postfixed, and acetylated with 0.25% acetic anhydride. The sections were hybridized overnight at 55°C with digoxigenin-labeled riboprobes covering the nucleotides 322–1158 of the AMACO cDNA. After hybridization, the sections were washed in 50% formamide, 2× SSC (0.3 M NaCl containing 0.03 mM sodium citrate) for 30 min at 55°C, digested with RNase A, washed once with 2× SSC, and twice with 0.2× SSC for 20 min at 55°C. The immunological development of the digoxigenin-labeled sections was carried out according to the instructions of the manufacturer (Roche Applied Science) with additional use of polyvinyl alcohol in the detection solution (19).

Immunohistochemistry—Immunohistochemistry was performed on frozen and paraffin-embedded sections of fetal, newborn, 3-week-old, and adult mice. The frozen sections were prefixed with 0.5% paraformaldehyde in PBS for 10 min. Deparaffinization ensued through incubation for 30 min in Rotihistol (Carl Roth GmbH). After rehydration, the sections were digested with hyaluronidase (Sigma, paraaffin sections 500 units/ml, cryostat section 50 units/ml in 0.1 M NaH2PO4, 0.1 M sodium acetate, pH 5.0) at 37°C for 30 min. The sections were blocked...
**FIG. 1.** Nucleotide and deduced amino acid sequences of mouse and human AMACO and domain structure of mouse AMACO (next page). The upper lines represent the mouse sequences. Except for part of the 5'-UTR, the differences in the human sequences are indicated below. Arrows mark the signal peptide cleavage sites. For explanation of symbols in the domain structure, see key below figure.
Analysis of the AMACO VWA Domain Sequences—The MIDAS (metal ion-dependent adhesion site) motif is fully conserved only in the A1 domain (Fig. 2). Nevertheless, a search of the module databases SMART (smart.embl-heidelberg.de) and PFAM (www.sanger.ac.uk/software/pfam/) returned scores for a VWA domain of 6.90e-35 and 5.70e-37 for the A1 domain, 1.26e-28 and 8.20e-21 for the A2 domain, and 1.55e-37 and 1.40e-31 for the A3 domain. Sequence alignment of the mouse AMACO VWA domains with their counterparts in matrilins and selected collagens shows the homology (Fig. 2). The sequence identity between the mouse AMACO VWA domains ranges between 21.6% for A2- and A3 domains and 26.5% for the A1- and A2 domains. Higher identity values were obtained for the A2 domain of mouse matrilin-4 and the mouse AMACO A1 domain (35.8%) and the A4 domain of chicken collagen XII α1 and the mouse AMACO A3 domain (31.4%). However, it was not possible to determine the relationship between the AMACO VWA domains and those of matrilins and collagens with statistical significance by phylogenetic analyses (not shown), which is consistent with an early divergence.

Recombinant Expression of Mouse AMACO—cDNA encoding the sequence of mouse AMACO was cloned into the pCEP-Pu vector utilizing the intrinsic AMACO secretion signal sequence and a C-terminal strepII tag (17). The recombinant plasmid was introduced into human embryonic kidney 293-EBNA cells and maintained in an episomal form. The recombinant AMACO protein was secreted into the cell culture medium and was subsequently purified by affinity chromatography on a Strep-tactin column (Fig. 3A). The purified protein appeared in non-reducing SDS-PAGE as a broad band with an apparent molecular mass in the range expected for monomeric AMACO. Bands corresponding to defined higher oligomeric forms of AMACO were not detected, but additional protein was present as very large protein aggregates that hardly entered the gel. Such aggregates were also seen in the original cell culture supernatant (Fig. 3A), showing that they are not formed during the purification. The correct usage of the predicted signal peptide cleavage site was confirmed by ESI Q-TOF mass spectrometry sequencing after tryptic digestion of the purified recombinant strepII-tagged AMACO.

Appearance of AMACO in Electron Microscopy—The purified strepII-tagged full-length AMACO was submitted to electron microscopy after negative staining with uranyl formate (Fig. 4). The protein particles were heterogeneous in size, and a closer examination revealed that both monomers and higher aggregates were present in the sample, in agreement with the results from SDS-PAGE analysis. Most of the monomers form compact folded structures, but extended monomeric molecules could occasionally be seen (Fig. 4). In both forms, globular domains were resolved, arranged in a tandem array. Each globule had a diameter of 3–4 nm, which is consistent with that expected for a single VWA domain.

Structure of the AMACO Genes—The human and mouse AMACO genes map to syntenic regions on chromosomes 10q26.11 and 19 (19D2), respectively, and, in both cases, lie between the genes coding for the tudor domain containing protein 1 and a hypothetical protein containing a pleckstrin homology domain. Both the AMACO genomic sequences (human: AC005383, NT_030059; mouse: AC125150, NT_039692) are contained in the public databases. We identified exons by flanking consensus splice signals and by comparison with the respective cDNAs. The exon/intron organization of both genes is very similar (Table II). The human gene is 51 kb, the mouse gene 37 kb long, and both AMACO genes consist of 13 exons that code for the translated part of the mRNA. In addition, in
mouse and human an exon 0 exists that codes for the 5′-untranslated region. Exon 1 codes for the major part of the signal peptide, whereas the signal peptide cleavage site is encoded on exon 2. The first VWA domain is encoded by exons 3–6, the cysteine-rich domain by exons 7 and 8, and the EGF1, VWA2, VWA3, and EGF2 domains by the single exons 9, 10, 11, and 12, respectively. In mouse, exon 13 codes for the unique C-terminal domain and the 3′-UTR. In contrast, in the human gene a 5-bp deletion of GAGAG, at the beginning of exon 13, leads to a frameshift followed by a stop codon. This leads to the lack of the unique C-terminal sequence in humans, and the last exon of the human gene encodes mainly the 3′-UTR.

AMACO Gene Expression—We studied the expression of the AMACO gene in different mouse tissues by Northern hybridization with total RNA. Positive signals could be detected in uterus (4.2 kb), kidney (3.7 kb), and skin (4.2 kb) of adult mice, but not in other tissues tested (Fig. 5A). The length of the cDNA cloned by us is markedly shorter than the mRNAs detected on Northern blot and contains no polyadenylation signal. A search in the databases detected a variety of 3′ AMACO EST clones that, when compared with the genomic sequence, contain a polyadenylation signal 1317 bp after the stop codon and end 12–14 bp after the polyadenylation signal and also a 5′ EST clone (BY734959) that extends 78 bp upstream of the sequence presented in Fig. 1A. On the basis of this information a total mRNA length of 3959 nt could be calculated, in good agreement with that detected by Northern blot.

To determine which part of the mRNA is lacking in the shorter transcript present in the kidney, we performed Northern blots on RNA prepared from both newborn and adult mouse kidney using either probes hybridizing to the 5′ or the 3′ end (Fig. 5B). The shorter 3.7-kb message was present only in the adult, as the 5′ probe only detected the 4.2-kb band in RNA from newborn kidney, whereas the 3′ probe detected the 3.7-kb band in adult kidney as well as the 4.2-kb band in newborn kidney. We then performed 5′ RACE to determine the end of the AMACO mRNA present in mature kidney (Fig. 5C). An mRNA was cloned that lacked exons 0–4 and started within

Fig. 2. Amino acid alignments of the VWA domains of AMACO, matrilins, and selected collagens. The sequences were aligned by the PILEUP program of the GCG package, using the default parameters. The conserved metal ion-dependent adhesion site (Lee et al. (2)) and the conserved hydrophobic moieties (Perkins et al. (33)) are denoted with (□) and (○), respectively.
intron 4, 32 bp upstream of the splice acceptor site. In the 32 bp there is no start codon, and the next in-frame ATG conserved between human and mouse is located toward the end of the sequence coding for the second VWA domain. If this ATG was used, the N terminus of the resulting protein would lack the sequence coding for the second VWA domain. If this ATG was chosen, it would therefore be highly unlikely to reach the endoplasmic reticulum.

By a more sensitive RT-PCR procedure, AMACO mRNA could be detected also in intestine and lung of adult mice, in calvaria, femur, brain, heart, intestine, skeletal muscle, and lung of newborn mice (Fig. 5D) and in human placenta and 293-EBNA cells (not shown).

Detection of AMACO in Cell Culture Supernatants from Established Cell Lines—The native purified protein was used to immunize a rabbit and, after affinity purification on the anti-strepII-coupled to Sepharose, the antibodies reacted specifically with AMACO in immunoblots (Fig. 3B). Without reduction, both monomeric and aggregated material was seen, and with reduction, only monomers of apparent $M_r$ 87,000. Cell culture media from a variety of established cell lines were submitted to SDS-PAGE and immunoblotted as in B. strepII-tagged AMACO. The overview (Fig. 3B) shows aggregates as well as monomeric molecules, and B shows selected monomeric particles. Both compact and extended monomeric molecules are seen, the resolution being sufficient to allow visualization of the individual globular domains. The bar corresponds to 25 nm in A and 5 nm in B.

SDS-PAGE (A) and immunoblot analysis of recombinant AMACO (B) and endogenous AMACO in cell culture supernatants of established cell lines (C). A, cell culture medium of 293-EBNA cells transfected with C-terminally strepII-tagged AMACO (CM) and affinity-purified AMACO (AP) were submitted to SDS-PAGE without prior reduction and stained with Coomassie Brilliant Blue. B, cell culture medium of 293-EBNA cells transfected with C-terminally strepII-tagged AMACO (CM) and affinity-purified AMACO (AP) were submitted to SDS-PAGE with and without prior reduction, transferred to nitrocellulose, and developed with the affinity-purified antibody against AMACO. C, media from a variety of established cell lines were submitted to SDS-PAGE and immunoblotted as in B. EBNA, embryonic kidney fibroblast; SCC25, oral squamous carcinoma; L132, fetal lung fibroblast; A431, vulvar squamous carcinoma; CaCo2, colon adenocarcinoma; Pan212, mouse keratinocytes; ATY1D, breast cancer; OVCAR, ovarian carcinoma; Pys2, mouse endoderm-like embryonic cells; and A375, melanoma. Recombinant AMACO was used as a control. In all cases separation was carried out using 8% polyacrylamide gels.

AMACO is a New Member of the VWA Domain Protein Superfamily

AMACO Is Present in Both Loose and Dense Connective Tissue—The tissue distribution of AMACO was studied by immunohistochemistry using affinity-purified antibodies on paraffin and cryostat sections of embryonic and newborn mice. AMACO was first detected at days 7.5–8 p.c., when it is weakly expressed around the developing mesodermal cells (not shown). At day 10.5 p.c. AMACO was detected in the heart and the condensing somites (not shown), and at day 14.5 p.c. it is present in the choroid plexus (Fig. 6A), the cochlea (Fig. 6B), the terminal bronchi of the lung (Fig. 6C), the heart (Fig. 6C), the skin, and in the cartilage primordium of the developing skeleton as well as in the interdigital spaces (Fig. 6D). Strong staining was seen in the condensed mesenchyme forming the edge of the developing teeth budding into the branchial arch and coinciding with the basement membrane that underlies the stratified squamous epithelia in the oral cavity (Fig. 6E).

In newborn mice, the protein is found in the cartilage primordium of the developing bones (Fig. 7B). It is present in the resting, proliferating, and hypertrophic zone, whereas it is absent from the perichondrium. In lung, AMACO can be detected in the epithelial layer of the alveoli and in the interalveolar walls (Fig. 7D). In dermis, AMACO (Fig. 7F) is strongly expressed in association with the basement membrane and around hair follicles. In uterus, AMACO is found underneath epithelial cells, associated with the basement membrane (Fig. 6F). Co-staining with a nidogen-1-specific antibody shows that not only the basement membrane is stained by the AMACO antibody but also the stroma of the epithelial cells. Indeed, even in the basement membrane zone the staining for nidogen-1 and for AMACO only partially coincides. In contrast to the result for nidogen-1, the smooth muscle cells do not stain for AMACO. In kidney, AMACO is mainly present in the cortex, associated with basement membrane structures (Fig. 6G). Co-staining with nidogen-1 detects AMACO associated with basement membranes of a subgroup of tubules, however, it is
AMACO, a New Member of the VWA Domain Protein Superfamily

We report on the initial characterization of human and mouse AMACO, new members of the VWA domain protein superfamily. The two genes are clearly orthologous, as their exon-intron structures. Cloning of the cDNA by RT-PCR confirmed the expression of the AMACO gene in human and mouse. These genes have previously only been incompletely reported on the basis of genome analysis (1).

A published mouse cDNA (AK029297) represents the AMACO cDNA, but due to a sequencing error nt 2291 and 2292 are missing, leading to a frameshift causing the second EGF-like domain and the C-terminal unique sequence to be only partially predicted (hypothetical protein 4832416E03; NP_766428.1). The human protein sequence (XP_291673.1) was predicted from NCBI contig NT_030059 by automated computational analysis using gene prediction method Genomescan (25). The predicted protein lacks the signal peptide sequence and parts of the cysteine-rich sequence and the first EGF-like domain were incorrectly assembled, because the last intron was not identified and therefore the short C-terminal stretch of amino acid residues not detected.

The bioinformatics analysis assigns AMACO to the VWA domain protein superfamily. It is the only member of a new subfamily, because no additional AMACO-like gene is contained in the completely sequenced human and mouse genomes. Therefore AMACO belongs to the minority of VWA domain members that have no paralog, together with matrilins and of matrilins, the phylogenetic analysis showed that AMACO could not be assigned to any of these two sub-branches of unconventional collagens could be misleading, because AMACO does not contain any triple helical domain. Although the VWA domains clearly have similarities to those of collagen and of matrilins, the phylogenetic analysis showed that AMACO could not be assigned to any of these two branches with statistical significance.

In contrast to the matrilins, AMACO does not contain an α-helical coiled-coil oligomerization domain and the MIDAS

† H, human; M, mouse; CR, cysteine-rich.
‡ The length of the 3′-UTR is not included.

### Table II

| Exon | Domain | Size | Splice donor | Intron | Size | Splice acceptor | Codon phase | Amino acid interrupted |
|------|--------|------|--------------|--------|------|----------------|-------------|-----------------------|
| 0H   | 5′-UTR | 240  | TTCCGttagtt  | 0      | 9,135| gtctcttagTTA   | I           | Val (18)              |
| 0M   | 5′-UTR | 163  | ACCAGttagg   | 6,547  | tctcttagTGTC | I           | Val (18)              |
| 1H   | SigPep | 62   | CAGAGttagg   | 4,885  | tctcttagTGTC | I           | Met (43)              |
| 1M   | SigPep | 62   | CAGAGttagg   | 3,836  | tctcttagTGTC | I           | Leu (43)              |
| 2H   | 75     | CAAAAGttacc | 1,189  | tctctcctagTGA | I          |                    |
| 2M   | 75     | CAAATttagtt | 1,782  | ctgctctcTagTAA | I          |                    |
| 3H   | VWA1 A | 134  | AGAGGttagg   | 5,157  | ctgctctcTagTGC | I          |                    |
| 3M   | VWA1 A | 134  | GCCAGttagg   | 3,675  | ctgctctcTagTGC | I          |                    |
| 4H   | VWA1 B | 110  | TTCCGttagtt  | 11,444 | ctgctctcTagAGG | II         | Lys (124)            |
| 4M   | VWA1 B | 110  | TTCCGttagtt  | 3,998  | ctgctctcTagAGG | II         | Lys (124)            |
| 5H   | VWA1 C | 195  | CCAGGttagg   | 4,979  | ctgctctcTagAGG | II         | Arg (189)            |
| 5M   | VWA1 C | 195  | CCAGGttagg   | 3,463  | ctgctctcTagAGG | II         | Arg (188)            |
| 6H   | VWA1 D | 134  | GCCAGGttagg  | 6,717  | ctgctctcTagAGG | I          | Arg (234)            |
| 6M   | VWA1 D | 134  | GCCAGGttagg  | 574    | tctctctcTagAGC | I          | Arg (333)            |
| 7H   | CR A   | 133  | TACAGttggttt | 7,366  | tctctctcTagCTG | II         | Ser (275)            |
| 7M   | CR A   | 133  | TACAGttggttt | 1,098  | tctctctcTagCTG | II         | Ser (277)            |
| 8H   | CR B   | 56   | GCCAGttagg   | 2,991  | tctctctcTagGCC | I          | Gly (297)            |
| 8M   | CR B   | 56   | GCCAGttagg   | 1,956  | tctctctcTagGCC | I          | Gly (296)            |
| 9H   | EGF1   | 108  | CTGTTttagtt  | 968    | atctctctcTagCC | I          | Ala (333)            |
| 9M   | EGF1   | 108  | CTGTTttagtt  | 804    | atctctctcTagCC | I          | Ala (322)            |
| 10H  | VWA2   | 573  | GCCAGttagg   | 2,426  | tctctctcTagGCT | I          | Gly (524)            |
| 10M  | VWA2   | 573  | GCCAGttagg   | 1,589  | tctctctcTagGCT | I          | Gly (525)            |
| 11H  | VWA3   | 552  | TTGGAGttagtt | 750    | tctctctcTagAG | I          | Glu (708)            |
| 11M  | VWA3   | 552  | TTGGAGttagtt | 306    | tctctctcTagAG | I          | Glu (707)            |
| 12H  | EGF2   | 126  | GAACGttggttt | 852    | tctctctcTagGAT | I          | Arg (750)            |
| 12M  | EGF2   | 126  | GAACGttggttt | 799    | tctctctcTagGCA | I          | Arg (749)            |

* H, human; M, mouse; CR, cysteine-rich.
† The length of the 3′-UTR is not included.
motif is conserved only in the A1 domain. The presence of a MIDAS motif does not prove the presence of a binding site for divalent cations, because the crystallization of the von Willebrand factor A3 domain revealed no bound metal ion, although the motif is present (27). On the other hand, an incomplete MIDAS motif must not exclude the binding of divalent cations.

The AMACO A1 and A3 domains contain three cysteine residues, and it is generally assumed that the N- and the C-terminal cysteines in VWA domains form a disulfide bridge. Accordingly, the additional cysteine residues, located in the first α-helix, may be able to form another disulfide bond, and these unpaired cysteines may be the cause of the aggregation of AMACO, seen under non-reducing conditions. A cysteine residue in a similar position has been described in the VWA domain of complement factor B and leads to dimerization and aggregation of the recombinantly expressed complement factor B VWA domain (28). The two unpaired cysteines in AMACO may form an intramolecular disulfide bond and thereby stabilize the compact, folded structure seen for most of the monomeric AMACO molecules in electron microscopy.

On the other hand, the existence of two different conformations of monomeric AMACO is reminiscent of VWA domain containing integrins, which can assume either an extended and active, or a folded and inactive, conformation. Like integrins AMACO also contains a cysteine-rich module. In integrins this module forms a fulcrum for the rearrangement after activation (29).

In mouse kidney, we found an unusual form of developmental regulation resulting in the sequential expression of an mRNA coding for a secreted AMACO protein and a probably non-coding transcript from the same gene, leading to a functional inactivation of the gene in adult animals. Shortly after

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**Fig. 5. Analysis of AMACO mRNA species in various mouse tissues.** A, Northern hybridization of 6 µg of total RNA from different tissues of newborn and adult C57BL/6J mice. B, Northern hybridization of 6 µg of total RNA from skin, uterus, and kidney of adult and from kidney of newborn mice using a 3′ (left panel) and a 5′ (right panel) riboprobe. Blot A and the left blot in B were hybridized with a digoxigenin-labeled riboprobe (nt 1159–2308) from the 3′ end of the AMACO cDNA; the right blot in B was hybridized with a digoxigenin-labeled riboprobe (nt 1–478) from the 5′ end of the cDNA. Below the ethidium bromide-stained ribosomal 18 S and 28 S RNAs are shown. C, map of the murine AMACO gene; exons are numbered and shown as black bars. Arrowheads show the transcription starts of the longer and shorter AMACO mRNA. The positions of the riboprobes used in A and B are given below. Enlarged, the 5′ end sequence of the shorter AMACO mRNA is given in lowercase letters for the intronic sequences, in uppercase letters for the exonic sequences, and below the partial amino acid sequence of exon 5. D, RT-PCR analysis was performed using primer pair Am2 and Am6. Template RNA was isolated from newborn (upper panel) and adult mice (lower panel). As a marker a 1-kb ladder from Invitrogen was used.
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Fig. 6. Tissue distribution of AMACO. Immunohistochemistry was performed on paraffin-embedded (A–F) or frozen (F–I) tissue from a day 14.5 p.c. mouse embryo (A–I), newborn (G and I), or adult (H) mice. For immunohistochemistry, tissues were incubated with an affinity-purified antiserum against AMACO (A–I) and a rat monoclonal antibody against nidogen-1 (F and I) followed by either biotin-SP-conjugated goat anti-mouse IgG and alkaline phosphatase-conjugated streptavidin (A–E, G, and H), or by Cy7-conjugated donkey anti-rabbit IgG (F, I, and red), or an AlexaFluor 488-conjugated goat anti-rat IgG (F, I, and green), respectively. In the choroid plexus (cp, A) of a day 14.5 p.c. mouse embryo AMACO is located around capillaries and epithelial cells. In the cochlea (co, B) AMACO was strongly stained in a basement membrane structure next to the inner surface and more weakly in the surrounding tissue. In lung (lu, C) AMACO is present in epithelial cells of the developing bronchii and in the underlying basement membrane. Further signals could be detected in heart muscle (he, C). In the paw (D) AMACO is expressed in the interdigital space, in the skin, and in the cartilage primordium of the long bones. In the oral cavity (E), strong signals could be detected in the developing tooth buds and coinciding with the basement membrane that underlies the stratified squamous epithelium. ll, lower lip; mo, molar; and in, incisor. In uterus of 3-week-old mice (F) AMACO (red) is present below the epithelial cell layer (ep), where it only partially colocalizes with the basement membrane marker nidogen-1 (green). Merge is seen in yellow. bm, basement membrane; sm, smooth muscle.

In kidney of newborn mouse (G) AMACO is strongly expressed in the cortex, associated with basement membranes (G, enlarged), while kidneys from adult mice (H) were negative. AMACO is expressed in certain tubules (tu) in the kidney (I) where it colocalizes (yellow) with nidogen-1 (green), but is absent from other basement membranes most strikingly those of the glomeruli (g/l). The bar is 200 \( \mu \)m in D, G, and H; 100 \( \mu \)m in B, C, and E; 60 \( \mu \)m in the inset of G; 40 \( \mu \)m in A; 55 \( \mu \)m in I; and 15 \( \mu \)m in F.

Fig. 7. Comparison of the distribution of AMACO protein and mRNA. Antisense riboprobes labeled with digoxigenin were hybridized to paraffin-embedded sections of an ilium (A), lung (C), and skin (E) of newborn mice. Parallel sections were immunolabeled with affinity-purified AMACO antibody (B, D, and F). In the developing bone (A), hybridization is strong in resting (rc), proliferating (pc), and hypertrophic (hc) cartilage and corresponds well to the immunolabeling (B). In the lung, mRNA is present in epithelial cells of the alveoli (al, C) where protein can also be detected (D). Hybridization in the skin showed high transcription levels in the keratinocytes and hair follicles (E), whereas the protein is most strongly detected in association with the basement membrane (F), ed, epidermis; de, dermis. The bar is 100 \( \mu \)m in A, B, E, and F and 50 \( \mu \)m in C and D.

Fig. 8. Immunofluorescence microscopy of AMACO transfected 293-EBNA cells. Cells were transfected with strepII-tagged AMACO cDNA and cultured in the presence of ascorbate. AMACO was detected with affinity-purified antibodies followed by a Cy5-conjugated affinity-purified donkey anti-rabbit IgG (red). A vinculin mouse monoclonal antibody was used for cellular counterstaining followed by Cy3-conjugated affinity-purified goat anti-mouse IgG (green). Merge is seen in yellow. The bar is 25 nm.

silencing of the AMACO gene involves not only switching off the first promoter, but also the activation of the second, alternative one, could indicate that the non-coding AMACO transcript has an unknown function. Non-coding RNAs (ncRNAs) are far more abundant than earlier thought and have been found to play roles in a great variety of processes, including transcriptional regulation, chromosome replication, RNA proc-
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AMACO is a new member of the VWA domain protein superfamily. It is expressed in a variety of cells, including chondrocytes, lung, and uterine epithelial cells and keratinocytes. The protein is deposited in the extracellular matrix surrounding these cells and appears to be particularly concentrated in and around the basement membranes that underlie epithelial cells. This may indicate that AMACO is a structural component of some basement membranes or functions in joining basement membranes to the underlying stroma. It could be that AMACO is a part of the anchoring structures formed on a scaffold of collagen VII or fibrillin, a search for potential interaction partners is now ongoing.

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