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Characterization of Emu Family Members

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Kidney development has often served as a model for epithelial–mesenchymal cell interaction where the branching epithelium of the ureteric bud induces the metanephrogenic mesenchyme to form epithelial nephrons. In a screen for genes differentially expressed during kidney development, we have identified a novel gene that is dynamically expressed in the branching ureter and the developing nephrons. It was designated Emu1 since it shares an N-terminal cysteine-rich domain with Emilin1/2 and Multimerin. This highly conserved EMI domain is also found in another novel protein (Emu2) of similar protein structure: an N-terminal signal peptide followed by the EMI domain, an interrupted collagen stretch, and a conserved C-terminal domain of unknown function. We identified two further secreted EMI domain proteins, prompting us to compare their gene and protein structures, the EMI domain phylogeny, as well as the embryonic expression pattern of known (Emilin1/2, Multimerin) and novel (Emu1/2, Emilin3, Multimerin2) Emu gene family members. Emu1 and Emu2 not only show a similar structural organization, but furthermore a striking complementary expression in organs developing through epithelial–mesenchymal interactions. In these tissues, Emu1 is restricted to epithelial and Emu2 to mesenchymal cells. Preliminary biochemical analysis of Emu1/2 confirmed that they are secreted glycoproteins which are attached to the extracellular matrix and capable of forming homo- and heteromers via disulfide bonding. The widespread, but individually distinct expression patterns of all Emu gene family members suggest multiple functions during mouse embryogenesis. Their multidomain protein structure may indicate that Emu proteins interact with several different extracellular matrix components and serve to connect and integrate the function of multiple partner molecules.

Key Words: **Emilin; Multimerin; kidney; Emu1; Emu2; EMI domain; EndoGlyx-1; epithelial–mesenchymal interactions; extracellular matrix; glycosylation; disulfide bonds; cysteine-rich; collagen.**

INTRODUCTION

Epithelial–mesenchymal interactions are involved in the development of many different tissues, from structures like hair and teeth to complex organs like salivary gland and kidney. The mesenchyme directs budding and invagination or sprouting and branching of the adjacent epithelial sheets or tubuli. On the other hand, signals from the epithelium may induce mesenchymal cells to transform into an epithelial phenotype. Kidney development has often served as a model for these processes: the metanephrogenic mesenchyme stimulates the branching of the ureteric bud that differentiates into the collecting ducts. The ureteric bud in turn induces the mesenchyme to convert into epithelial nephrons (Davies and Bard, 1998). These inductive interactions and differentiation processes are guided by a variety of factors, including signaling molecules and their receptors, various extracellular matrix (ECM) glycoproteins, ECM receptors (integrins, dystroglycan), and cell adhesion molecules (CAMs). Protein modules often found in these different proteins include oligomerization domains like collagen repeats or coiled-coil regions as well as elements mediating protein interaction like immunoglobulin-like or cysteine-rich domains. Oligomerization assembles multiple protein binding motifs and thereby leads to multivalency and high binding strength (Engel and Kammerer, 2000).

ECM glycoproteins exhibit specific spatiotemporal expression patterns during organogenesis, and it is thought that not only signaling between cells but also the interaction of cells with ECM components play a crucial role in tissue morphogenesis. During metanephric development,

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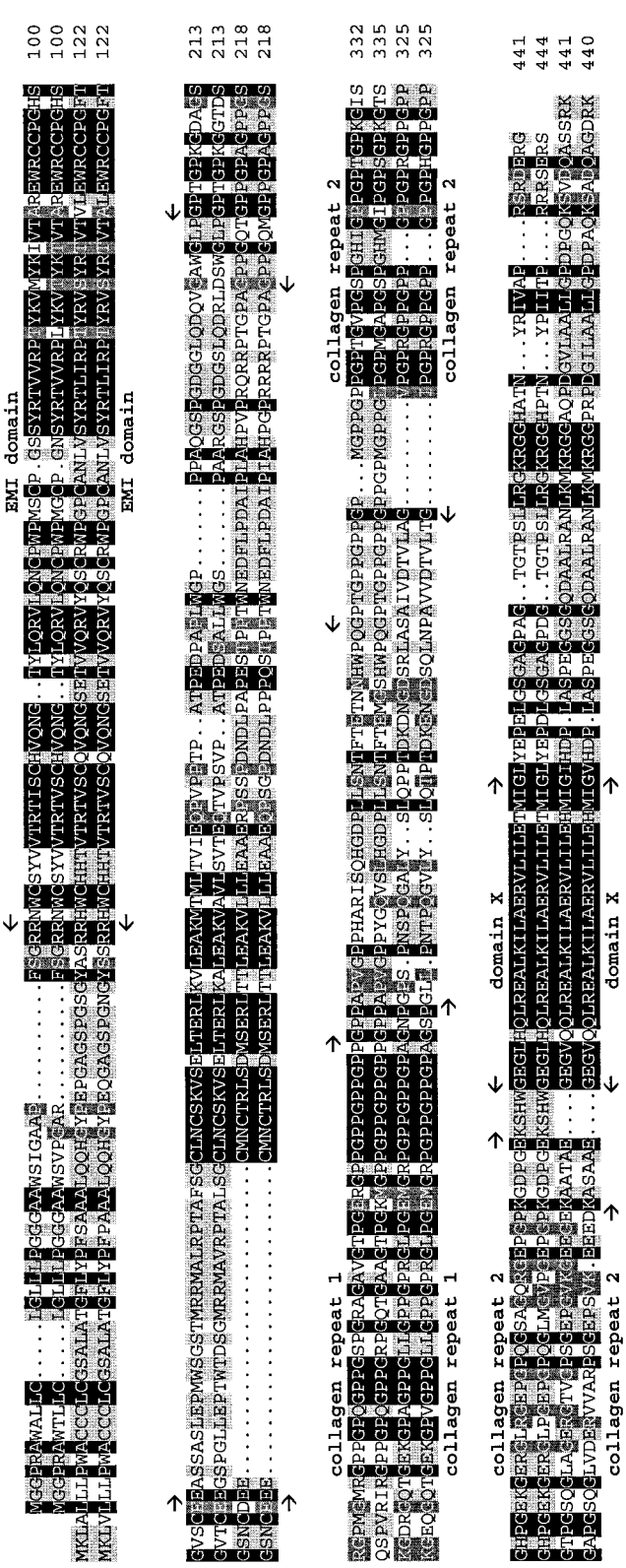


tenascin, fibronectin, and nidogen (entactin) are restricted to the mesenchyme, whereas the basement membrane components collagen IV and laminin α1 are specific for the epithelium (Wallner *et al.,* 1998). Organ culture experiments and mouse knockout studies demonstrated that the ECM is required for branching morphogenesis in the kidney (Bullock *et al.,* 1998; Davies *et al.,* 1995; Ekblom, 1981; Kreidberg *et al.,* 1996; Roskelley *et al.,* 1995). The ECM not only serves as substrate for cell migration, epithelial cell attachment, and polarization, but also contributes to the patterns of tissue growth and branching in this system. Epithelial Madin Darby canine kidney cells, for example, can only be induced to form tubules by treatment with HGF when they are embedded in collagen. When cultured on top of the matrix, they migrate away from each other (Montesano *et al.,* 1991). Likewise, branching of the salivary gland bud requires the deposition of collagen into the forming clefts (Nakanishi *et al.,* 1988). Without collagen, no clefts are formed, while inhibition of the endogenous collagenase leads to supernumerous clefts. In a similar fashion, proteoglycans not only provide a scaffold for cell movement or tissue remodeling, but are involved in signaling processes. Ligands like TGF, BMP, FGF, or Wnt are associated with the ECM and have been shown to modulate the activity of proteoglycans or vice versa (Flechtenmacher *et al.,* 1996; Kovacs *et al.,* 1994; Liu *et al.,* 1996; Ruoslahti and Yamaguchi, 1991). Heparan sulfate proteoglycans (HSPGs), for example, act as low-affinity coreceptors for several signaling molecules and thereby facilitate their dimerization, the interaction with their receptors, or alter their effective concentration (Schlessinger *et al.,* 1995). Upregulation of HSPG expression has been shown to activate Wnt activity without increasing Wnt expression levels (Perreault *et al.,* 2001). On the other hand, interference with proteoglycan synthesis leads to loss of Wnt expression (Kispert *et al.,* 1996). Integrins—large heterodimeric transmembrane receptors—have a more direct role in signaling between mesenchyme and epithelium. Different sets of integrin receptors are expressed on mesenchymal or epithelial cells; they receive signals from neighboring cells through interaction with ligands like vitronectin, tenascin, osteopontin, or nephronectin at the mesenchymal–epithelial interface and transduce this information into the cell. Thus, cell–cell and cell–ECM adhesion as well as signaling are interlinked, and it is difficult to clearly distinguish between scaffold-providing and signaling activities. Moreover, there is a steadily growing number of novel genes and gene families involved in these interactions.

Initiated by a differential display screen for genes regu-

FIG. 1. Alignment of the deduced amino acid sequences of human and mouse Emu1 and Emu2. The EMI domain, collagen repeat 1, collagen repeat 2, and the novel domain X are indicated. Domain boundaries are marked with arrows. Conserved residues are highlighted by shaded boxes.

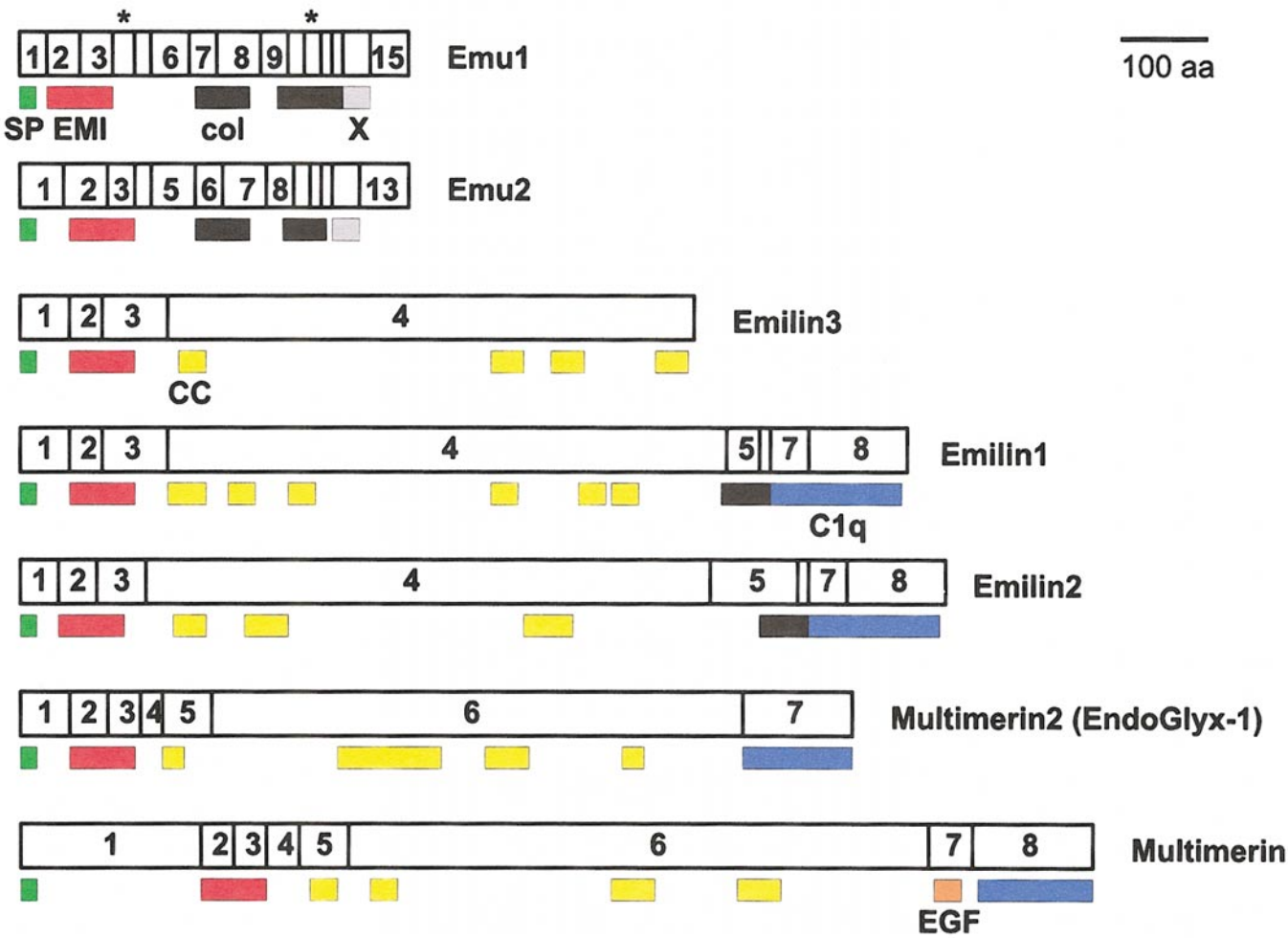


FIG. 2. Exon–intron and protein domain organization of human Emu family members. White boxes with numbers depict the numbers and relative sizes of exons. Asterisks above Emu1 exons 4 and 10 indicate that corresponding exons are missing in Emu2. Colored boxes below exons highlight protein domains: signal peptide (SP) in green, EMI domain in red, collagen repeats in black (col), coiled-coil regions (CC) in yellow, domain X in gray, Clq domain in blue, and EGF repeat in orange. Scale bar, 100 amino acids (aa).

lated during early kidney development, we have isolated two novel genes named Emu1 and Emu2. Their proteins share a cysteine-rich domain (EMI domain) with the ECM glycoproteins Emilin and Multimerin. Comparative expression analysis has been performed for the entire EMI domain family of genes. Further biochemical studies showed that Emu1 and Emu2 encode glycosylated proteins that are secreted into the extracellular space, where they form homo- or heteromers to exert their function.

MATERIALS AND METHODS

Assembly of Emu Gene Sequences

Emu gene and amino acid sequence analyses were performed by using the following software packages and Web-accessible programs: NCBI Blast, NIX, GCG, ClustalX, Treeview, and SMART. DNA sequence assemblies were confirmed by sequencing the respective I.M.A.G.E. cDNA clones or subcloned PCR fragments, which were amplified with primers flanking questionable sequences. Human and mouse Emu1 and Emu2 sequences were submitted to the EMBL database (Accession Nos. AJ416090–AJ416093).

Cloning of Emu Sequences for Expression Studies

Forward and reverse oligonucleotides coding for Myc- or HA-epitopes bearing *Xho*I adapter sites were annealed and cloned into the *Xho*I site of the vector pCS2+ (http://sitemaker.med.umich.edu/dlturner.vectors) to get the tagging vectors pCSmyc and pCSHA. The open reading frames of Emu1 and Emu2 as well as the first 202 amino acids of Emu1 containing the signal peptide and the EMI domain were amplified from cDNA of stage E12.5 mouse embryos by using the Pfx polymer-

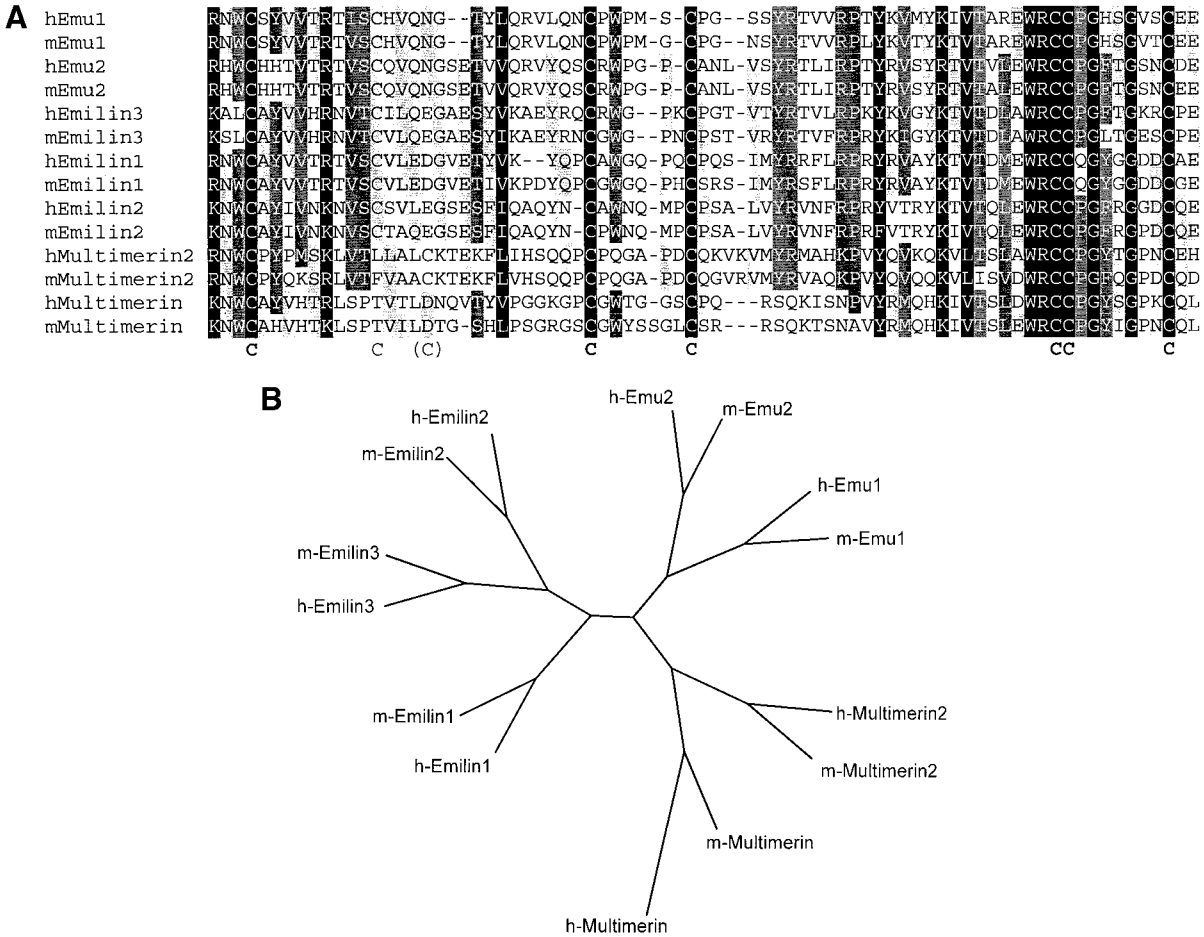


FIG. 3. (A) Amino acid sequence alignment of human and mouse EMI domains. Conserved cysteine residues are indicated below. Except for Multimerin, all Emu family members contain seven cysteine residues in the EMI domain. The third cysteine is shifted in Multimerin2. (B) Phylogenetic analysis of Emu proteins. The unrooted tree is based on the EMI domain alignment, and was generated by the Clustal X neighbor joining algorithm. The Treeview program (http://taxonomy.zoology.gla.ac.uk/rod/rod.html) was used to draw the tree.

ase (Invitrogen) and primers with *Bgl*II or *Eco*RI adapter sequences. PCR products were cloned in frame into pCSmyc or pCSHA to yield pCSEmu1myc, pCSEmu2HA, and pCSEMI1myc. All inserts were sequenced to exclude PCR mutations. Oligonucleotides used for cloning are as follows: Myc epitope: myc-for-Xho, TCGAGGAACAGAAGCTGATTAGCGAAGAAGATCTGC; myc-rev-Xho, TCGAGCAGATCTTCTTCGCTAATCAGCTTCTGTTCC. HA epitope: HA-for-Xho, TCGAGTACCCATACGACGTGCCAGACTACGCTC; HA-rev-Xho, TCGAGAGCGTAGTCTGGCACGTCGTATGGGTAC. Emu1: m-cgene-Bgl-ATG, GCGAGATCTGCGGTGGGGACGACAGCAT; cfullAP2-Eco, CGCGAATTCCGTCTCCTGGGGGTGATGAT. Emu2: Emu2Bg1ATG, GCGAGATCTGGAGAGCGGTGCAAAATG; Emu2Eco, CGCGAATTCCTGTCGCCAGCCTGGTCT. EMI domain of Emu1: m-cgene-Bgl-ATG (see above); cemil-Sal, CGCGTCGACTAAGGAAGCCCCCAAGAATCAA.

Cell Culture, Transfection, and Western Blot Analysis

HEK293T cells were cultivated in DMEM medium supplemented with 10% fetal calf serum, 2 mM GlutamaxI, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated on 100-mm plates and transfected with the expression constructs by using the calcium phosphate method. After transfection, cells were cultivated in serum-free medium (SFM II, Invitrogen) for 36–48 h. The proteins from supernatants were precipitated with chloroform/methanol. Glycosylation was analyzed by EndoHf digestion of proteins from transfected cells or supernatants according to the manufacturer's protocol (NEB). For oligomerization studies, cell lysates were prepared in protein sample buffer (50 mM Tris–HCl, pH 6.8, 10% glycerin, 2% SDS, 1.5 mM bromphenolblue) with or without 100 mM DTT (reducing/nonreducing). Protein samples

were separated by SDS–PAGE in 6 or 10% gels and transferred to nitrocellulose membranes (Schleicher & Schuell). Monoclonal antibodies against c-Myc (9E10) or HA (12CA5) (Sigma) were used as primary antibodies, and goat anti-mouse antibody conjugated with horseradish peroxidase was used as secondary antibody. Blots were developed by using enhanced chemiluminescence (ECL; Amersham Pharmacia).

For the production of stably transfected cell lines, HEK293 and 3T3 cells were cotransfected with PJ6Ωpuro (Morgenstern and Land, 1990) and pCSEmu1myc or pCSEmu2HA in a 1:5 ratio. After puromycine selection, single colonies were picked and expanded.

In vitro transcription and translation of pCSEmu1myc (full-length Emu1) and pCSEMI1myc (EMI domain of Emu1) were performed by using a coupled rabbit reticulocyte lysate system (Promega). Products were visualized by Western blot analysis with antibodies against the c-Myc epitope.

Indirect Immunofluorescence

For immunohistochemical studies, stably transfected cells were grown on cover slips and fixed in 4% paraformaldehyde/PBS. To stain for intracellular localization of Emu1/2 proteins, cells were permeabilized with 0.2% Triton X-100/PBS for 10 min. Emu1myc fusion protein expression was detected with 9E10 as primary and Cy2-conjugated goat anti-mouse (Dianova) secondary antibody. Emu2HA was stained with HA-probe (Y-11 rabbit sc-805; Santa Cruz Biotechnology) as primary and Cy2-conjugated goat anti-rabbit (Dianova) as secondary antibodies.

In Situ Hybridization

Mouse embryos of different stages were derived from timed matings of CD1 outbred mice (Charles River Laboratories). *In situ* hybridization of whole-mount embryos and of tissue sections using digoxigenin-labeled antisense riboprobes was performed as described (Leimeister *et al.*, 1998). After color development, some tissue sections were counterstained with nuclear fast red (Sigma). Riboprobes were generated from the following constructs: Emu1, coding sequence amplified from cDNA of stage E12.5 mouse embryos cloned into pBluescript KS (primer sequences: m-cgene-Bgl-ATG, GCGAGATCTGCGGTGGGGACGACAGCAT; m-cgene-Stop-EcoRI, CGCGAATTCGCCTGCAGCCTCTCAGCTCCTC); Emu2, IMAGE clone (GenBank Accession No. AA028535); Emilin3, IMAGE clone (GenBank Accession No. AW701511); Multimerin2, cDNA insert of IMAGE clone (GenBank Accession No. AI006581) subcloned into pBluescript KS; Emilin1, IMAGE clone (GenBank Accession No. W59438); Emilin2, partial cDNA amplified from cDNA of stage E12.5 mouse embryos cloned into pCS2 (primer sequences: Emil4-5′Bam, GCGGGATCCGGAATGTGCCACGCGATCT; Emil4-3′Eco, GCGGAATTCGGATGGACAGGGCATCTGGT); Multimerin, partial cDNA amplified from cDNA of stage E12.5 mouse embryos cloned into pDK101 (primer sequences: m-Multim2-5′Bam, GCGGGATCCGTCTCTCGATT CCCCATGAA; m-Multim2-3′code, CGGAGTAGCCGGGGCARCANCKCCA). Primers for cDNA amplification of Emu1 coding sequence, Emilin2, and Multimerin EMI domains were designed according to mouse IMAGE clone or HTG sequences.

RESULTS

Identification of a Family of Secreted Proteins Sharing an N-Terminal Cysteine-Rich Domain

In a screen for genes regulated during metanephrogenic development, we have previously identified a genetag (C0–5, GenBank EST W80149), which is dynamically expressed in the branching ureter and the developing nephrons (Leimeister *et al.*, 1999). Based on predictions from human and mouse genomic and EST sequences (e.g., AL031186, Y07848, AC005528), we designed flanking primers to amplify the entire coding region of this gene. Sequence analysis of the cloned RT-PCR product from mouse E12.5 RNA confirmed that it corresponds to the genetag from the ddPCR screen. The presence of an upstream CpG island extending into exon 1 and the good agreement of the predicted transcript size with estimates from Northern blots suggest that we have identified the complete exon–intron structure. There is a high similarity (82% of nucleotides) between human and mouse exon sequences. The deduced protein sequence contains an N-terminal signal peptide followed by a cysteine-rich domain, an interrupted collagen stretch, and a C-terminal domain without any homology to known protein sequences (Fig. 1). Since a similar, characteristic cysteine-rich domain, the EMI domain (Doliana *et al.*, 2000), was also found in Emilin1, Emilin2, and Multimerin, this novel protein was designated Emu1. Further database searches yielded three additional novel Emilin/Multimerin-related genes present in human and mouse, designated Emu2, Emilin3, and Multimerin2. Emu2 has an exon–intron and protein domain organization very similar to Emu1 (Figs. 1 and 2). Moreover, phylogenetic analysis of their EMI domains suggests that Emu1 and Emu2 genes arose from a single ancestral gene through a recent duplication event (Fig. 3B). The other two novel proteins Emilin3 and Multimerin2 were likewise named according to their grouping by protein domain structure, exon–intron organization, and phylogeny (see below). Multimerin2 was independently identified in a pursuit to find new antigenic markers of human vascular endothelium and designated there as EndoGlyx-1 (Christian *et al.*, 2001). For all human genes, the entire coding region was either available or could be reconstructed from genomic and EST sequences (Fig. 2, Table 1). All exon–exon junctions were verified by correctly spliced ESTs or sequencing of RT-PCR products. For each of the corresponding mouse sequences, the EMI domain could be also identified, but there are only genomic shotgun sequences and no proven contiguous cDNA sequence information available for the carboxy-terminal regions of Emilin2, Multimerin, and Multimerin2 (EndoGlyx-1).

An alignment of all EMI domains from human and mouse is shown in Fig. 3A. Each EMI domain, except in Multimerin, contains seven conserved cysteine residues, opening the possibility for dimerization. Phylogenetic analysis of these sequences confirmed the grouping of human and

TABLE 1
Sequence Characteristics of Emu Genes and Proteins

	Human chromosome	Exons	Human protein (aa)	Mouse protein (aa)	Human gene size (kb)	Human cDNA	Human genomic	Mouse cDNA	Mouse genomic
Emu1	22q21.1	15	441/439	444/442	54	AJ416090	AL031186/ Y07848	AJ416093	AC005528*
Emu2	7q22.1	13	441/439	440/438	196	AJ416091	AC004953/ AC006329/ AC004965	AJ416092	AC083889*/ AC083857*
Emilin1	2p23.2	8	1016	1017	8	NM_007046	AC013403	BC005481	shotgun trace sequences
Emilin2	18p11.32	8	1053	incompl.	67	NM_032048	AC015958*	AA145049#	shotgun trace sequences
Emilin3	20q12	4	713/766	711/758	7	M802331#	AL031667	BC002161#	AL590389/ AL590430
Multimerin	4q22.1	8	1229	incompl.	60	NM_007351	AC093759*	AK014984#/ codehop-PCR	shotgun trace sequences
Multimerin2	10q23.2	7	949	incompl.	22	NM_024756	AC025268*	BB618429 (5′)/ AA624706 (3′)	shotgun trace sequences

*, draft sequences.
#, partial sequence.

mouse sequences as evident by pairwise clustering (Fig. 3B). For each human/mouse protein pair, the amino acid similarity was in the 80–90% range. The excellent spatial conservation of cysteines and additional highly conserved amino acid residues together with an overall similarity of 50–70% between all members suggest that EMI domains assume quite similar three-dimensional structures in these proteins.

The Emu Gene Family Comprises Structurally Different Members of Genes and Proteins

Emu domain proteins are expected to be secreted since they start with a signal peptide, which is followed by the adjacent EMI domain. Aside from these common features, the proteins are divergent and can be subdivided into 2 classes (Fig. 2). Emu1 and Emu2 proceed with two collagen stretches and a novel C-terminal domain. Interestingly, the latter is highly conserved between the two genes and between human and mouse, including a stretch of 17 amino acids identical in all 4 proteins. Alternative splicing at the 3′ splice site of intron 2 can lead to loss of 6 nucleotides (amino acids SY) in human and mouse Emu1/2. Two presumably orthologous genes with the same protein domain organization can even be found in Fugu rubripes genome sequences and in other vertebrate genomes (data not shown).

All other EMI domain proteins are significantly larger than Emu1/2 and contain coiled-coil domains. The collagen triple helix and the α-helical coiled-coil represent the two basic supercoiled multistranded protein motifs capable of forming rod-like structures that may separate the presumed globular domains of the N and C termini of all EMI-domain proteins. With the exception of Emilin3, proteins of the second class have a C1q domain at the C terminus that is also implicated in protein oligomerization but may form bouquet-like arrangements of trimers or higher oligomers (Brodsky and Shah, 1995; Reid, 1989).

A similar subclassification of Emu proteins is also evident from the genomic organization of the respective genes: Emu1 and Emu2 genes are composed of 15 and 13 small exons spread over a large distance (Table 1, Fig. 2). All other genes have a central or terminal very large exon encoding the coiled-coil regions that span 600 or more amino acids. In Emilin1, Emilin2, and Emilin3, the EMI domain is encoded in exon 2 and only the first half of exon 3. While Emilin1 and Emilin2 possess a collagen repeat followed by a C1q domain in exons 5–8, this feature is absent from Emilin3. In Emilin3, the first coiled-coil region may further be shortened through the use of an alternative splice site in exon 4 (Table 1).

The more distant members Multimerin and Multimerin2 again share the feature of an EMI domain spread over two complete exons, but they contain the C1q domain in a single exon. These data suggest that the pairs Emu1/2, Emilin1/Emilin2, and Multimerin/Multimerin2 are likely derived from a common ancestor each, with Emilin3 exhibiting strongest similarity to the Emilin1/Emilin2 pair.

Expression of the Novel and Known Emu Genes during Mouse Embryogenesis

To elucidate a potential role of Emu proteins during embryogenesis, we analyzed the expression of the murine genes in E9.5–E17.5 embryos. Early stages of expression were explored by whole-mount *in situ* hybridization, but E14.5 and subsequent stages were analyzed on tissue sections.

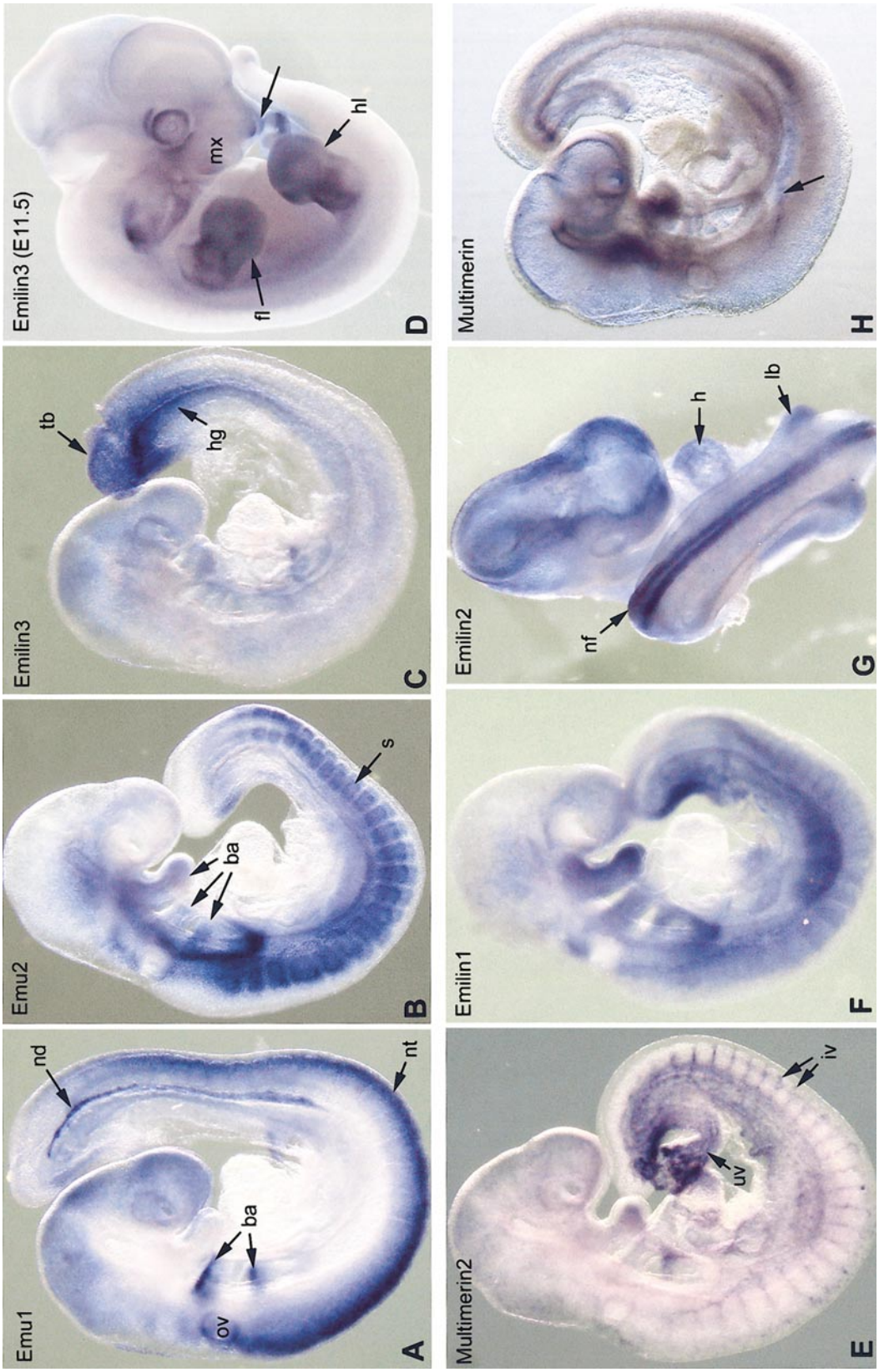
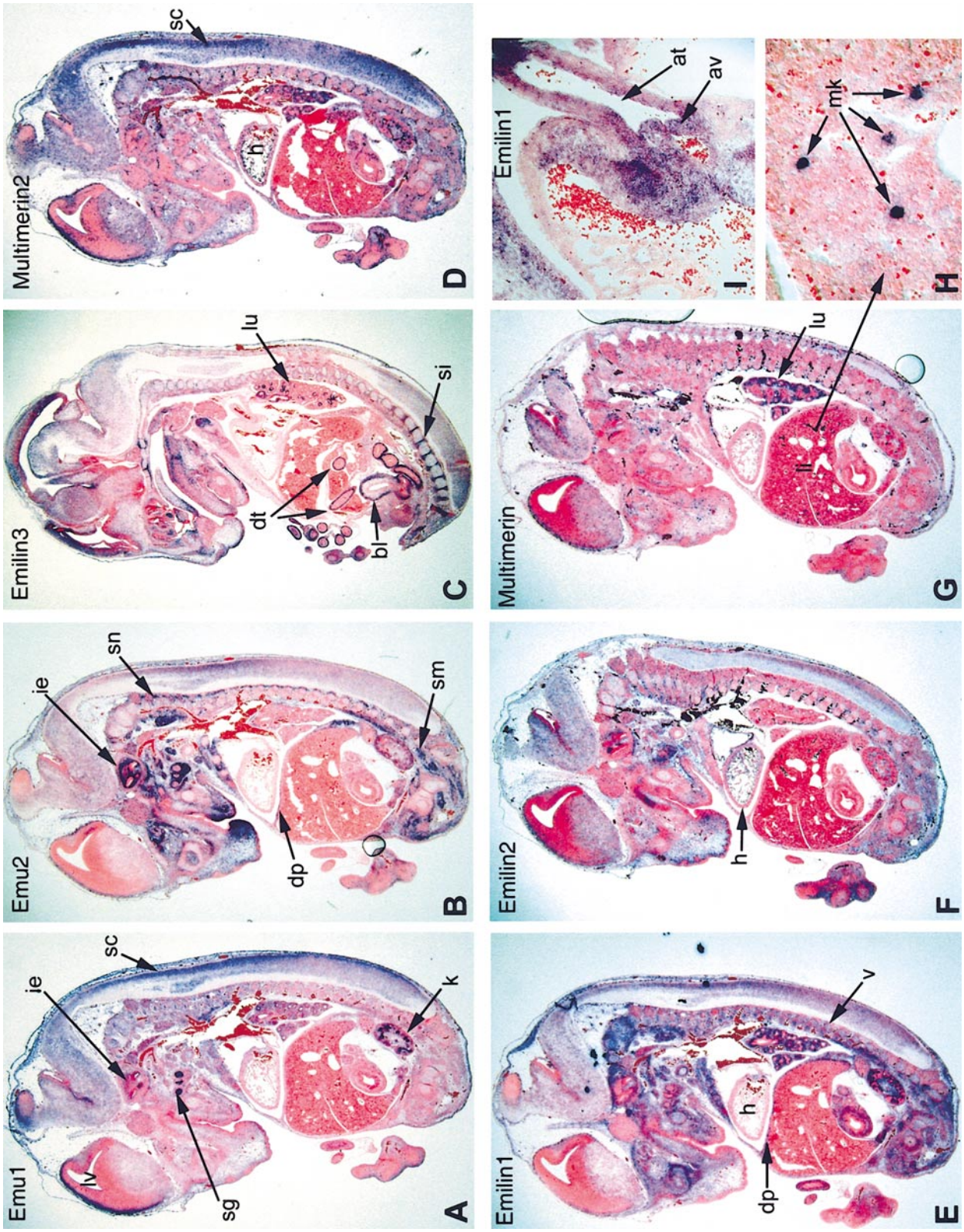


FIG. 4. Embryonic expression of Emu genes in early mouse embryogenesis at E9.5 (A–C, E–H) and E11.5 (D). Gene names are indicated. (A) Emu1 transcripts are detected in the nephric duct (nd), the dorsal neural tube (nt), the epithelia of the branchial arches (ba), and the otic vesicle (ov). (B) Emu2 is expressed in the somites (s) and in mesenchymal cells of the head and the branchial arches (ba). (C) At E9.5, Emilin3 expression is only observed in the tailbud (tb) and the hindgut (hg). (D) At E11.5, Emilin3 is additionally expressed at sites of bone formation, i.e., surrounding mesenchymal condensates in the fore- and hindlimbs (fl, hl), the nose (arrow), the maxilla (mx), and the other parts of the jaw. (E) At E9.5, as during all stages of development, Multimerin2 shows an endothelial-specific expression, e.g., in the umbilical vessels (uv) and the intersomitic vessels (iv). (F) Emilin1 shows a widespread expression in mesenchymal cells of the head and the trunk. (G) Emilin2 is strongly expressed in the neural fold (nf), the limbbuds (lb), and the heart (h). (H) Multimerin transcripts are only observed as a faint stripe on both sides of the embryo (arrow).



At E9.5, Emu1 is expressed in the dorsal neural tube, the nephric duct, the otic vesicle, and the branchial cleft epithelium (Fig. 4A). Emu2 is expressed in the somites, the mesenchyme of the branchial arches, and the head (Fig. 4B). At this early stage, Emilin3 expression is only visible in the caudal part of the embryo, and vibratome sectioning localized the staining in the gut (Fig. 4C; and data not shown). With the onset of skeletal formation, Emilin3 transcripts are additionally found at sites of cartilage and bone formation in the trunk and skull, i.e., surrounding mesenchymal condensations in fore- and hindlimbs as well as the jaw (E11.5, Fig. 4D). Multimerin2 is specifically expressed in small and large blood vessels during embryogenesis (Fig. 4E). Emilin1 is expressed in mesenchymal cells throughout the body, but especially in the trunk and the branchial arches (Fig. 4F). Emilin2 expression is more restricted with the main expression sites at the neural folds, the limb buds, and the heart (Fig. 4G). Even after prolonged staining, Multimerin transcripts are only observed in cells of unknown origin forming two lines on both sides of the embryo (Fig. 4H).

In E14.5 embryos, like in earlier ones, Emu1 is expressed in the dorsal spinal cord and the brain, where it is restricted to the proliferating ependymal and cortical cell layers (Fig. 5A). Additionally, Emu1 expression is observed in smooth muscles of the digestive tract (Fig. 6G) as well as in the epithelia of the salivary gland, the inner ear, and the developing nephrons of kidney. In these latter organs, Emu2 is expressed in the adjacent mesenchyme — thus showing a complementary expression pattern to Emu1 (Fig. 5B). Emu2 mRNA is further detected in the spinal nerves and ganglia, in the head mesenchyme, and in skeletal muscles. Emilin3 is expressed in the nervous plexus of the digestive tract and the main branches of the bronchi (Fig. 5C). Transcripts are also found in the segmental interzone and surrounding many sites of ossification, especially in the skull. As in early embryos, Multimerin2 mRNA is observed in the vascular endothelium and additionally in the spinal cord and the brain (Fig. 5D). Emilin1 transcripts are seen throughout the mesenchyme, in the diaphragm, in cartilage (Fig. 5E), and in the outflow tract of the heart (Fig. 5I). Emilin2 is expressed in interstitial cells throughout the embryo and in the trabecular layer of the embryonic heart (Fig. 5F). A speckled expression of Multimerin is observed throughout the embryo which is very prominent in the lung and often associated with blood vessels (Fig. 5G). In the embryonic liver, Multimerin transcripts are restricted to a few large cells with multilobulated nuclei, which are most likely megakaryocytes (Fig. 5H).

At E17.5, Emu genes are expressed in the same tissues as shown for E14.5 embryos (data not shown).

Comparison of Emu1 and Emu2 Gene Expression

Interestingly, Emu1 and Emu2 not only share a similar gene and protein organization but are complementary expressed in several tissues developing through epithelial–mesenchymal interactions. In early embryos, Emu1 is expressed in the epithelium of the branchial arches, whereas Emu2 is localized to the adjacent mesenchyme (Figs. 4A and 4B). At E14.5, Emu1 is restricted to the epithelium, but Emu2 to the surrounding mesenchyme in the developing kidney, the salivary gland, and the inner ear (Figs. 5A and 5B). This separation is also observed in these organs at later stages (Figs. 6A–6F). However, in the advanced developing kidney (at E15.5 and later), transcripts of both genes are complementary only at the kidney periphery with Emu1 in the epithelium of the developing nephrons and Emu2 in the surrounding mesenchyme, but their expression overlaps in the collecting duct epithelium (Figs. 6A and 6B). In some tissues with comparable epithelial–mesenchymal interactions, transcripts of both genes are absent (e.g., the lung) or only Emu2 is expressed (e.g., the tooth). Moreover, in a variety of other tissues, either Emu1 or Emu2 is expressed: Emu1 in the spinal cord and in the smooth muscle cells of the digestive tract (Fig. 6G) and Emu2 in the spinal ganglia and the skeletal musculature (Fig. 6H). Thus, Emu1 and Emu2 expression is complementary in some tissues, but overlapping or exclusive in others.

FIG. 5. Embryonic expression of Emu genes in sagittal sections of E14.5-old mouse embryos. Nuclei are counterstained with nuclear fast red, and specific gene expression is colored in blue. Gene names are indicated. (A) Emu1 is expressed in the future neopallial cortex (lv, lateral ventricle), the dorsal spinal cord (sc), the epithelia of the salivary gland (sg), the kidney (k), and the inner ear (ie). (B) In these latter organs, Emu2 is observed in the surrounding mesenchyme. Emu2 transcripts are further seen in the spinal nerves (sn) and ganglia, the mesenchyme of the skull, the diaphragm (dp), and the skeletal muscles (sm). (C) Emilin3 is strongly expressed in the enteric nerves of the digestive tract (dt) and the bladder (bl). Emilin3 transcripts are also observed surrounding the main branches of the alveoli (lu, lung) and surrounding sites of bone formation in skull and trunk (si, segmental interzone). (D) Multimerin2 expression is restricted to endothelia of blood vessels, the endocardium of the heart (h), and the spinal cord. (E) Emilin1 is expressed in mesenchymal cells throughout the body, in the diaphragm (dp), in skeletal muscles, and in bones of the skull and trunk (v, vertebrae). Emilin1 is not observed in the heart ventricle but in the outflow tract (see I). (F) Emilin2 transcripts are seen in the trabecular zone of the heart ventricle and in many mesenchymal cells overlapping with Emilin1. (G) Multimerin is expressed in a speckled pattern throughout the body. Transcripts are found in endothelial cells and megakaryocytes. (H) Magnification of Multimerin expression in megakaryocytes (mk) of the liver. (I) Emilin1 expression in the outflow tract of the heart (at, aortic trunk; av, aortic valve).

Emu1 and Emu2 Are Secreted N-Glycosylated Proteins

To explore protein properties, we cloned the full-length coding region of Emu1 and the EMI domain of Emu1 with C-terminal Myc epitope-tags in eukaryotic expression vectors. The coding region of Emu2 was similarly tagged with the HA-epitope.

HEK293T cells were transiently transfected with these expression constructs. Precipitated proteins from supernatants and cell lysates were analyzed by Western blotting using anti-Myc and anti-HA antibodies. Emu1 and Emu2 proteins were detected in the cell lysates and, if at all, to a lower extent in the supernatants. This suggests that they are secreted but are attached to the extracellular matrix. Therefore, the subsequent experiments were performed with complete cell lysates. As seen in Fig. 7A, full-length Emu1 and Emu2 recombinant proteins show a strong upper band of approximately 60 kDa and a very faint lower band of around 55 kDa, with the latter corresponding to the calculated molecular weight. Since there are two predicted N-glycosylation sites (Asn-Xaa-Ser/Thr consensus) within the EMI domains of Emu1 and Emu2, the slower migrating protein products probably bear carbohydrate residues. Indeed, when proteins from the cell lysates were digested with endoglycosidase H (EndoH), which removes N-linked carbohydrates, only the lower band is observed.

Analysis of cell lysates from cells transfected with the EMI domain of Emu1 (EMI1) revealed an upper glycosylated band of 30 kDa, but several lower protein bands, consistent with more than one glycosylation site (Fig. 7B). *In vitro* transcription and translation of full-length Emu1 or the EMI1 domain expression constructs only generated protein bands corresponding in size to the nonglycosylated protein products (Fig. 7C). It remains to be seen whether there is tissue- or cell-specific variation of glycosylation *in vivo*.

Oligomerization of Emu1/2 EMI Domains

The EMI domains of Emu1 and Emu2 include seven cysteine residues, raising the possibility of dimerization or oligomerization through disulfide bonds. To test whether each of these proteins is capable of forming homomers via disulfide bonding, we separated the tagged recombinant proteins from transiently transfected HEK293T cells on polyacrylamide gels under reducing or nonreducing conditions. In addition to the monomeric 60-kDa band, slower migrating bands of around 120 kDa, 180 kDa, and larger were observed for Emu1 and Emu2 under nonreducing conditions (Fig. 8A). This indicates that both proteins exist as monomers, but they can form homodimers, -trimers, or even larger complexes. The EMI1 domain of Emu1 alone also form aggregates mainly of 90 kDa (trimer) but also of 60 kDa (dimer), 120 kDa (tetramer), 150 kDa (pentamer), or higher aggregates (Fig. 8B, lane 2). When cells were cotransfected with the full-length Emu1 and the EMI1 domain, the same aggregates were observed. The contribution of Emu1 or EMI1 to the complexes cannot be distinguished because they are both tagged with the Myc epitope and their sizes are multiples of 30 kDa (Fig.8B, lane 3). We then cotransfected full-length Emu2 and EMI1 to analyze a potential heteromerization of Emu1 and Emu2. Since Emu2 was tagged with the HA- and EMI1 with the Myc-epitope, we could distinguish between the two proteins. Using anti-HA for detection, we observed two high molecular weight bands migrating between the full-length mono-, di-, and trimeric Emu2 bands (Fig. 8C, lane5). These bands were not observed when cells were transfected with Emu2 alone (Fig. 8C, lane 4) and are thus complexes of Emu2 and the EMI1 domain. This indicates that Emu1 and Emu2 are capable of forming heteromeric complexes via their EMI domains.

Extracellular Matrix Association of Emu Proteins

When HEK293T cells were transiently transfected with Emu1/2 expression constructs, we could not detect recombinant proteins in the supernatant, suggesting that they are attached to the cells or the ECM. For a more thorough analysis of Emu1/2 protein localization and in order to exclude aberrant protein processing due to the massive overexpression in transient transfections, we established stably transfected cell lines. Cells were stained by immunofluorescence with or without permeabilization. In permeabilized HEK293 and 3T3 cells stably expressing Emu1myc or Emu2HA, proteins were localized in the ER and the Golgi apparatus as well as on the cover slip, i.e., the ECM secreted by the cells (Fig. 9). Without permeabilization, only the strong extracellular fluorescence on the cover slips was observed like a trace of the migrating cells. Thus, Emu1/2 proteins are secreted and deposited in the ECM.

DISCUSSION

With Emu1 and Emu2, we have isolated two novel genes that are specifically and often complementary expressed during murine embryogenesis. The similarity of amino acid sequences and strong parallels in gene and protein organization suggest a related function. Both genes encode secreted glycosylated proteins that are attached to the ECM and are thus likely immobilized. The common EMI domain defines a subfamily of seven genes and proteins with related structure. In addition to Emu1/2, this domain is also found at the N terminus of the secreted ECM molecules Emilin1, Emilin2, and Multimerin, as well as in two novel proteins, Emilin3 and Multimerin2. Related and evolutionary conserved sequences have been detected in several vertebrate species, including Fugu and zebrafish. The genomic organization as well as protein domain structure clearly suggest an evolutionary history of gene pairs — with the exception of the singleton Emilin3 that appears truncated at the C terminus. The overall protein structure of most Emu proteins is expected to be quite similar to the dumbbell shape proposed for Emilin1, with globular N- and C-terminal domains separated by a rod-like structure that is formed by

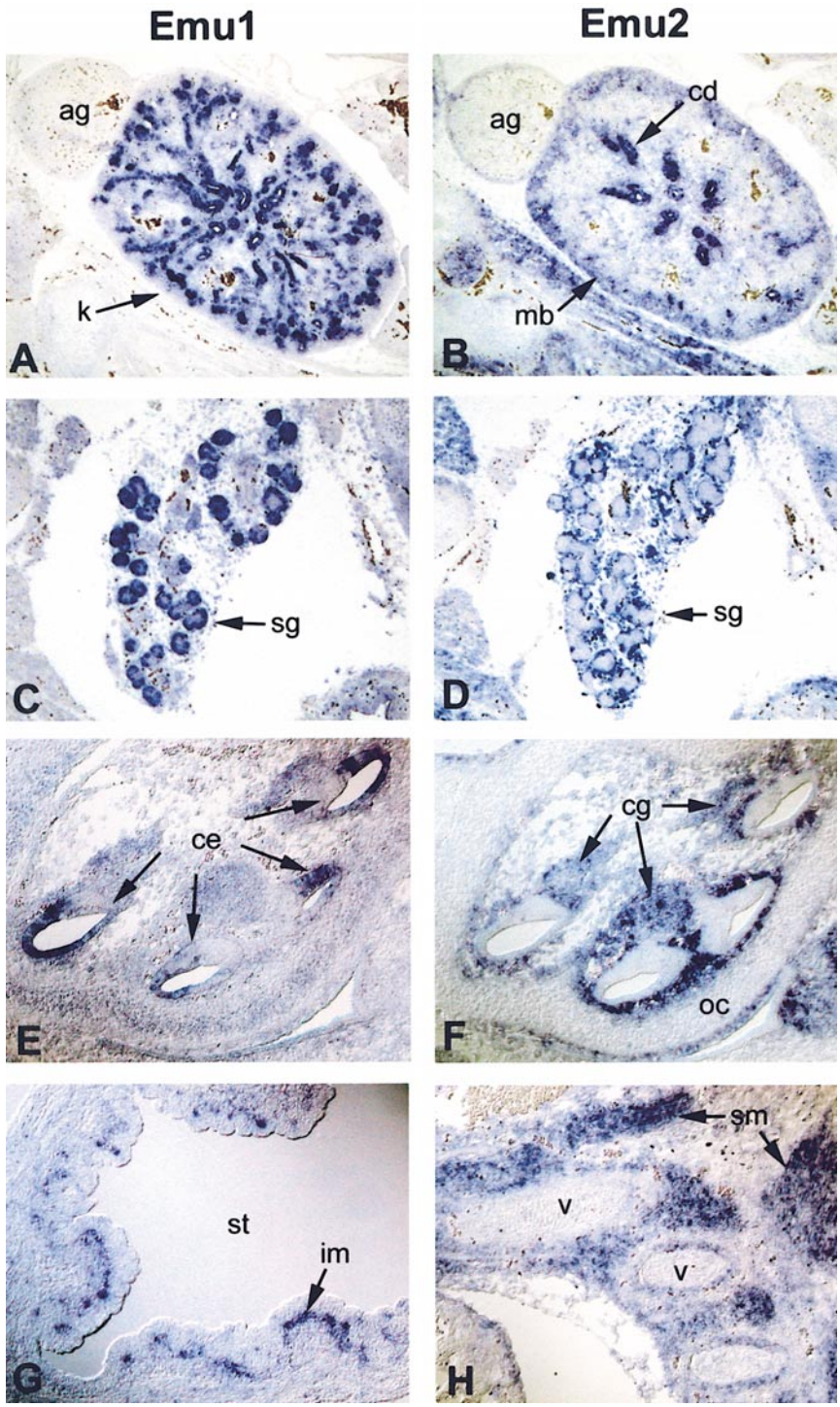


FIG. 6. Comparison of Emu1 and Emu2 gene expression in sagittal sections of E15.5 (A–G) and E14.5 (H) mouse embryos. (A, B) In the embryonic kidney at E15.5, Emu1 is expressed in the epithelia of the developing collecting duct and the nephron precursors, whereas Emu2 is expressed in the collecting ducts (cd) and the metanephrogenic blastem (mb) at the kidney periphery (ag, adrenal gland). (C, D) In the salivary gland (sg), Emu1 transcripts are seen in the epithelial branches, while Emu2 transcripts are observed in surrounding mesenchymal cells. (E) Emu1 is expressed in the cochlear epithelium (ce) of the inner ear. (F) Emu2 expression is observed in the surrounding mesenchyme as well as in the cochlear ganglia (cg). (G) Emu1 is expressed in smooth muscles of the digestive tract. Here, expression in the inner muscles (im) of the stomach (st) is shown. (H) Emu2 expression in skeletal muscles (sm) in E14.5-old embryos (v, vertebrae).

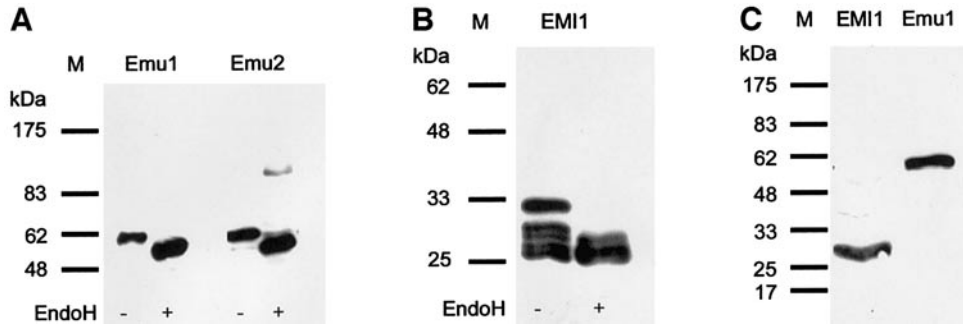


FIG. 7. N-glycosylation of Emu1 and Emu2 proteins. HEK293T cells were transiently transfected with the tagged full-length Emu1myc and Emu2HA expression constructs (A) or the EMI domain of Emu1 (EMI1myc) (B). Protein extracts were incubated with or without endoglycosidase H, separated on polyacrylamide gels under reducing conditions, and analyzed by Western blot. Molecular weight marker (M) sizes are indicated on the left. (A) Untreated Emu1 and Emu2 full-length proteins migrate around 60 kDa (lanes 1 and 3). After removal of carbohydrate residues, the sizes of Emu1 and Emu2 proteins are reduced to 55 kDa (lanes 2 and 4). (B) Without EndoH treatment, the fully processed EMI1 domain has a molecular weight of around 30 kDa, but lower migrating partially processed bands are also observed (lane 1). Digestion with EndoH removes the upper glycosylated bands (lane 2). (C) *In vitro* transcription/translation of EMI1myc (lane 1) and the full-length Emu1myc (lane 2) protein.

coiled coils or collagen repeats (Colombatti *et al.,* 2000; Mongiat *et al.,* 2000).

To provide a basis for future functional studies, we investigated the embryonic expression of Emu1/2 and compared it with the patterns of other Emu family members. In a number of tissues developing through epithelial–mesenchymal interaction, like kidney, salivary gland, inner ear, and branchial arches, expression of Emu1 and Emu2 is complementary. In the developing kidney, Emu1 is first expressed in the branching ureter and Emu2 in the surrounding mesenchyme that gets induced to form nephrons. After transition of this mesenchyme into epithelial structures, Emu1 expression is then switched on. Epithelial-specific expression of Emu1 and restriction of Emu2 transcripts to subpopulations of mesenchymal cells are also observed during the development of other organs. The complementary expression of these two structurally related proteins suggests that they may interact extracellularly at boundaries to participate in the regulation of epithelial–mesenchymal interaction and development. We detect such heteromeric complexes in transfected cells, but it remains to be seen whether Emu1 and Emu2 proteins — when made by neighboring cells types — can still interact.

There is only limited overlap of Emu1/Emu2 mRNA expression with other Emu family members. Multimerin2 transcripts are restricted to blood vessels and this endothelial specific expression was also reported for the homologous human protein EndoGlyx-1 (Christian *et al.,* 2001). Multimerin is similarly found only in endothelial cells and megakaryocytes. Emilin3 shows a very specific expression in the nervous plexus of the digestive tract and the lung as well as surrounding sites of cartilage or bone formation, where neither Emu1 nor Emu2 expression overlaps or is adjacent. Furthermore, no Emu protein aside from Emu1 is epithelium-specific. Only Emu2 and Emilin1 are coexpressed in developing skeletal muscles. Furthermore, Emu2 seems to overlap with Emilin1/2 transcripts in the mesenchymal compartment of tissues, like kidney or salivary gland. At higher resolution, however, it appears that Emilin1 is strongly and Emilin2 weakly expressed in the interstitial mesenchyme of the developing kidney, whereas Emu2 is exclusively found in the peripheral mesenchyme that transforms into nephrons and stroma. This suggests that all EMI domain proteins may provide quite unique and nonredundant functions to the respective tissue compartments.

Little is known about the function of the cysteine-rich EMI module. In yeast two-hybrid studies, it was shown that the EMI domain of Emilin1 interacts with itself and with the C1q domain of Emilin2, suggesting a role of this domain in oligomerization of Emilin1 as well as head-to-tail association with Emilin2 (Doliana *et al.,* 2000). We observed a similar homophilic interaction of the EMI domain of Emu1 in yeast two-hybrid analysis (not shown). Nevertheless, the physiological relevance of such interactions remains questionable given the fact that extracellular domains are analyzed here, which are normally characterized by glycosylation and multiple disulfide bonds. Probing such interactions in a yeast nuclear compartment may hint at some intrinsic affinity of the domains involved, but immunodetection of heteromers of tagged proteins as reported here for Emu1 and Emu2 certainly represents a much more relevant approach.

Cysteine-rich domains are frequently involved in protein–protein interactions and they are found in numerous extracellular and transmembrane proteins, from enzymes involved in blood coagulation to matrix proteins or signaling molecules like BMPs, Wnts, and their receptors or modulators. The EMI domain is characterized by the regular spacing of seven cysteines, a number found in TGF-β

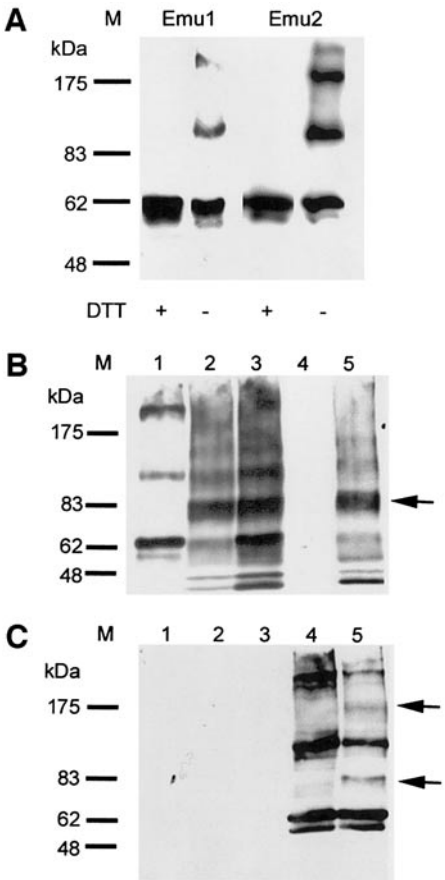


FIG. 8. Emu1 and Emu2 proteins are linked by disulfide bonds. Transiently transfected HEK293T cell lysates were denatured in reducing (+DTT) or nonreducing (−DTT) sample buffer and analyzed by Western blot. Molecular weight marker (M) sizes are indicated. (A) Homomerization was analyzed in cells transfected either with Emu1myc or Emu2HA and detected with anti-Myc and anti-HA, respectively. Under nonreducing conditions, slower migrating bands corresponding to the size of homodimers and homotrimers of Emu1 or Emu2 are seen (lanes 2 and 4, respectively). (B, C) To investigate heterodimerization of Emu1 and Emu2, cells were transfected with Emu1myc (lane1), EMI1myc (lane 2), Emu1myc plus EMI1myc (3), Emu2HA (lane 4), or Emu2HA plus EMI1myc (lane 5). (B) Staining with anti-Myc as primary antibody reveals all Myc-tagged Emu1 proteins or complexes. Lanes 1 and 2 show the monomers and oligomers of Emu1 or the EMI1 domain alone. Note that the EMI1 domain forms mainly trimers (arrow in B). (C) A parallel Western blot was analyzed with the HA antibody staining Emu2. In lysates from cells transfected with full-length Emu2 monomeric (60 kDa), dimeric (120 kDa) and trimeric (180 kDa), as well as the unprocessed monomeric bands (55 kDa) were observed (lane 4). Two additional interbands (arrows in C) appear when cells were cotransfected with full-length Emu2 and the EMI1 domain, demonstrating that heteromeric complexes between the two proteins are formed (90 and 150 kDa) (lane 5).

and several BMPs. These latter molecules form homodimers, covalently linked by one of the seven cysteines, while the other six cysteines form intramolecular disulfide-bridges (cystin-knot). It is suggestive to propose a similar mechanism of dimerization for the EMI domains that would utilize the unpaired cysteine for intermolecular bridging. Our biochemical analysis indeed shows that mammalian Emu1 and Emu2 proteins are capable of forming homo- as well as heteromers via disulfide bonds, although homomers seem to be the favored aggregation state.

An important difference to BMP dimers lies in the fact that we detect higher multimers that are held together by disulfide bridges — even with the isolated EMI domain of Emu1. Under nonreducing conditions, we found similar amounts of Emu1 and Emu2 proteins as monomers, dimers, and trimers. Since transient transfections result in a non-physiological excess of protein, it remains to be seen whether Emu1/2 proteins interact at different stoechiometry *in vivo*. On the other hand, we cannot exclude that even higher aggregates are formed that were not resolved in the protein gels used. At least the EMI domain of Emu1 is capable of forming tetra- or pentamers on its own, although trimerization appears to be preferred. This suggests that the EMI domain itself must have at least two surfaces for homomeric interactions stabilized by disulfide bridges. In this respect, it will also be interesting to analyze a potential interaction of Emu1/2 with the EMI domains of the other Emu proteins. Emilin1 and Multimerin proteins have been shown to multimerize to form very large molecular complexes of several million daltons that are highly disulfide-linked with the smallest protomer being a trimer (Hayward *et al.*, 1991; Mongiat *et al.*, 2000). Similarly, EndoGlyx-1 (Multimerin2) of HUVECs and EA.hy926 cells assembles to form a disulfide-linked complex of three subunits. Stretches of coiled-coil domains implicated in oligomerization to three-stranded or five-stranded complexes as well as triple helices of C1q that are known to further multimerize to form a bouquet-like quaternary structure may contribute to these aggregates (Brodsky and Shah, 1995; Reid, 1989). Emu1/2 instead have triple-helix-forming collagen repeats, making it likely that the central domains of Emu1 and Emu2 assemble to form trimers. As in collagens XII or XIV, there are two separate and comparatively short collagen repeats in Emu1/2. One could envisage incorporation of Emu1/2 into collagen fibers as a macromolecular interconnector, similar to FACIT collagens. In this way, both the EMI domain and the novel C-terminal domain X would be available for further interactions and network formation.

The domain X of Emu1 and Emu2, which has not been described previously, is almost identical in human and mouse and even in Fugu, tetraodon, and zebrafish sequences. This points to a conserved function that may well extend beyond that of an initiator for the assembly of the flanking collagen triple helix. We expect that *in situ* localization of Emu proteins using specific antisera, protein interaction studies, and gene knockout will provide new insight into the role of the Emu class of extracellular matrix proteins in various developmental processes.

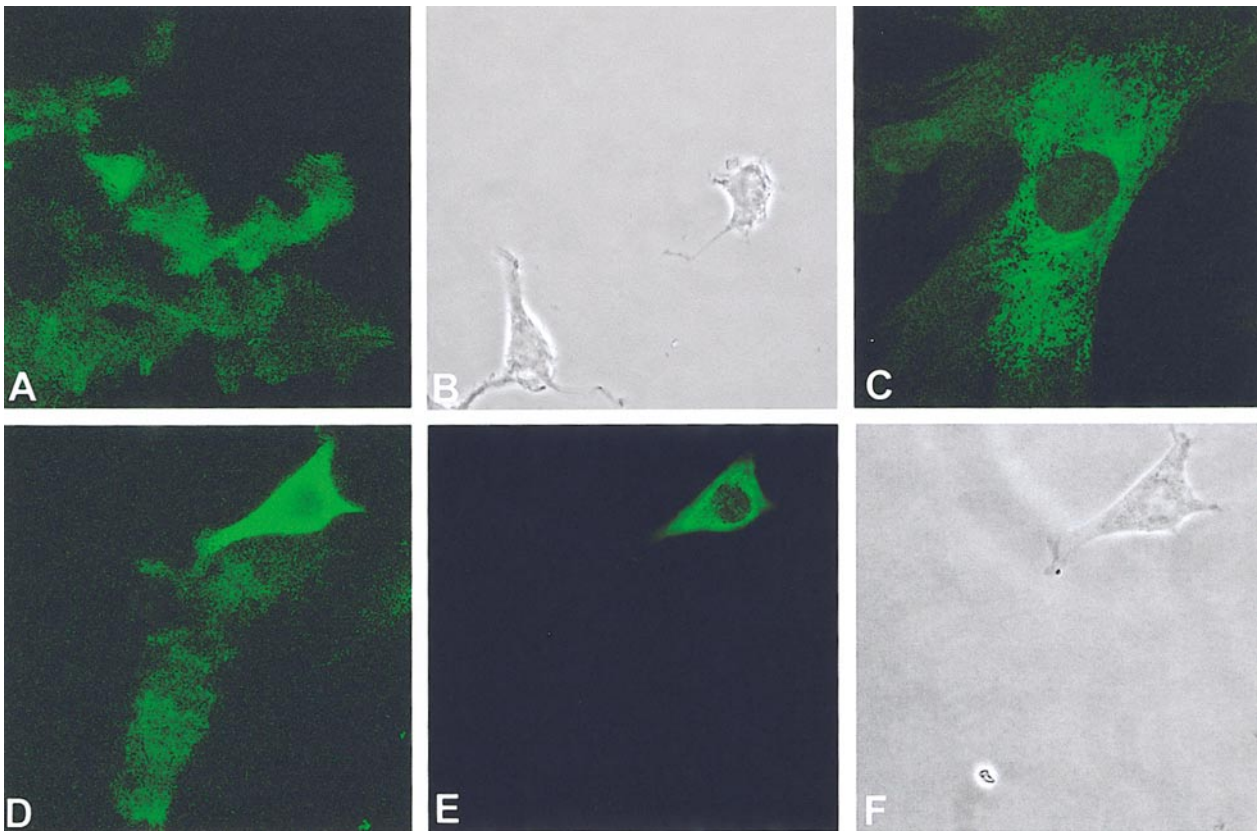


FIG. 9. Emu1 and Emu2 proteins are secreted and attached to the extracellular matrix. 3T3 cells stably expressing Emu1myc (A–C) or Emu2HA (D–F) were stained with anti-Myc or anti-HA, respectively, as primary antibodies. Photographs were taken with a Leica confocal microscope. (A) Without permeabilization, no Emu1 staining was observed within the cells but on the coverslip as a trace of the migrating cell. (B) Phase contrast photography of the same region shows that not the two cells are stained but the secreted Emu1. (C) Cells permeabilized with Triton X-100 display a strong Emu1 staining in the ER and Golgi. (D) Permeabilized 3T3 cell stably expressing Emu2: Emu2 staining was observed in the ER and Golgi within the cell and in the secreted extracellular matrix on the coverslip. (E) ER and Golgi staining is visualized better at a different confocal plane. (F) Phase contrast photography of the same region.

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