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Novel Metalloprotease–Disintegrin, Meltrin ϵ (ADAM35), Expressed in Epithelial Tissues During Chick Embryogenesis

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Members of the ADAM (a disintegrin and metalloprotease) family are involved in fertilization, morphogenesis, and pathogenesis. Their metalloprotease domains mediate limited proteolysis, including ectodomain shedding of membrane-anchored growth factors and intercellular-signaling proteins, and their disintegrin domains play regulatory roles in cell adhesion and migration. In screening for cDNAs encoding chicken ADAM proteins expressed during muscle development, we identified Meltrin ϵ as a novel member of this family. To elucidate its functions, we investigated its expression during development by using antibodies raised against its protease domain. In the somites, Meltrin ϵ protein was specifically expressed in the myotomal cells, which delaminate from the dermomyotome to form epithelial sheets. It was also found in the surface ectoderm, lens placodes, otic vesicles, and the gut epithelia. Basolateral localization of Meltrin ϵ in these epithelial cells suggests its unique roles in the organization of the epithelial tissues and development of the sensory organs and the gut. *Developmental Dynamics 230:557–568, 2004.* © 2004 Wiley-Liss, Inc.

Key words: ADAM; metalloprotease; disintegrin; epithelium; lens; myogenesis; morphogenesis

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INTRODUCTION

Skeletal muscle cells are derived from mesodermal cells in the somites during embryogenesis. These somitic cells differentiate to form the dermomyotome, a dorsal epithelial sheet formed from the somites. The paraxial dermomyotomal cells, in turn, delaminate from its dorsal lip to generate the myotome, an internal epithelial layer that contains myoblasts. Another group of muscle precursor cells that delaminates from the ventral lip of the dermomyotome migrates into the limbs to generate muscles. Thus, skeletal myogenesis requires programmed cell fate determination, proliferation, migration, and myotube formation. Although various myogenic transcription factors and adhesion and signaling molecules are known to play roles in these processes to some extent, the molecular mechanisms underlying these dynamic cellular processes are still elusive.

In this context, ADAM (a disintegrin and metalloprotease) is an attractive family of multidomain proteins that can modulate intercellular signaling and adhesion (Wolfsberg and White, 1996; Seals and Courtneidge, 2003). Some of the ADAM proteins play regulatory roles in cell–cell interactions through proteolytic processing of the extracellular domains

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of membrane-anchored proteins such as growth factors, intercellular signaling molecules, and adhesion molecules (Black and White, 1998; Werb and Yan, 1998; Primakoff and Myles, 2000). Kuzbanian (ADAM10), for example, is involved in neurogenesis and in other morphogenetic processes through the proteolytic regulation of notch signaling (Fambrough et al., 1996; Rooke et al., 1996; Pan and Rubin, 1997; Qi et al., 1999; Lieber et al., 2002). A tumor necrosis factor (TNF) -α–converting enzyme (TACE/ADAM17; Black et al., 1997; Moss et al., 1997; Peschon et al., 1998) converts various membrane proteins such as TNF-α and transforming growth factor (TGF) -α from their membrane-bound to their soluble forms. On the other hand, disintegrins are polypeptides originally isolated from various viper venoms. The viper disintegrins work as inhibitors of platelet aggregation. Their actions are mediated by inhibition of the interaction between integrin αIIb β3 on platelets and fibrinogen in plasma (Niewiarowski et al., 1994; McLane et al., 1998). The disintegrin domains of ADAM proteins likely regulate cell–cell interactions and cell migration (Cho et al., 1998; Smith et al., 2002; Huang et al., 2003).

Previously, we identified Meltrin α (also known as ADAM12), which plays a role in myogenesis in vitro (Yagami-Hiromasa et al., 1995). Although the involvement of Meltrin α in myogenesis in vivo was confirmed by generating mice lacking (Kurisaki et al., 2003) and overexpressing (Kronqvist et al., 2002) Meltrin α, the *meltrin α* gene is dispensable for myogenesis; although muscles in the neck and shoulders are thinner in some *meltrin α* gene knockout mice, muscles in other regions such as the legs were apparently not affected in these mice. We found that C2C12 cells, an established line of muscle satellite cells, express at least three ADAMs (Yagami-Hiromasa et al., 1995); Meltrin α, Meltrin β (ADAM19), and Meltrin γ (ADAM9). Evidence suggests that these ADAM proteins are active proteases that are involved in the ectodomain shedding of ErbB ligands (Izumi et al., 1998; Shirakabe et al., 2001; Asakura et al., 2002). However, the roles of these ADAM proteins in myogenesis and other morphogenetic events remain ambiguous.

We decided to identify ADAM proteins during avian myogenesis because embryonic chicken has many advantages in investigating morphogenesis and cell–cell interactions. Especially, recent advances in technology for introducing exogenous gene structures, such as the dominant negative or the antisense constructs, into embryos by electroporation or viral infection, have enabled us to analyze gene functions in ovo.

In this study, we report the identification of Meltrin ϵ as a novel ADAM protein expressed in the myotome. In addition, several specific epithelial-derived tissues also express Meltrin ϵ. It is prominently expressed in the surface ectoderm and the epithelial tissues derived from it, such as the lens placodes, and in pharyngeal and intestinal epithelial cells. Based on its expression pattern and preferential basolateral localization during invagination of these epithelia, plausible roles of Meltrin ϵ in development are discussed.

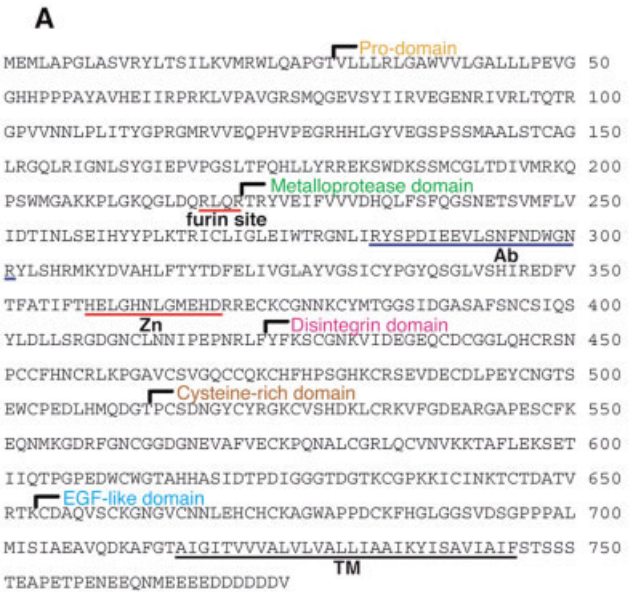
RESULTS

Cloning a Chicken cDNA Encoding a Novel ADAM, Meltrin ϵ

To identify complementary DNAs (cDNAs) encoding ADAM proteins, cDNAs prepared from the myotome of chick embryos at embryonic day (E) 6 were amplified with primers for amino acid sequences that are shared by mouse Meltrin α, β, and γ. In addition to chicken Meltrin α and γ, a novel cDNA was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) when two of the primer sets (9 and 10 and also 1+2 and 10) were used in the reaction. A cDNA clone encoding an open reading frame was subsequently surveyed by 5′ and 3′ rapid amplification of cDNA ends (RACE), followed by RT-PCR to identify a full-length cDNA that covers the entire open reading frame (775 amino acids (a.a.)) as described in the Experimental Procedures section. The nucleotide sequence and the deduced a.a. sequence are available under GenBank accession no. AB088841. Determination of the cDNA sequence revealed an open reading frame for a type-I membrane protein that shares these domain structures with other ADAM proteins: pro-do-

Fig. 1. Predicted amino-acid sequence of Meltrin ϵ and its domain structures. **A:** Labels above the sequence indicate the start of each structural domain. Furin site: furin protease recognition site; Ab: the region of peptides used for Meltrin ϵ antibody production; Zn: a potential zinc-chelating site in the metalloprotease domain; TM: putative transmembrane region. This sequence has been submitted to GenBank under the accession no. AB088841. **B:** Schematic diagrams of chick (C) Meltrin ϵ (775 amino acids (a.a.)), human (H) Meltrin γ (819 a.a.), and H ADAM21 (722 a.a.). The numbers are the percentages of amino acid sequence identity between C Meltrin ϵ and H Meltrin γ or H ADAM21 in the indicated domains. SP, signal peptide; PRO, pro-domain; MP, metalloprotease domain; D, disintegrin domain; C, cysteine-rich domain; E, epidermal growth factor-like domain; TM, transmembrane region; CP, cytoplasmic region.

Fig. 3. Characterization of the Meltrin ϵ antibody. **A:** The specificity of the polyclonal antibody raised against the metalloprotease region of Meltrin ϵ (Melϵ) was tested with COS1 cells mock-transfected or transfected with full-length meltrin ϵ cDNA by Western blot analysis. **B:** Immunofluorescence analysis of DLD1 human colon cancer cells that express chicken Meltrin ϵ. DLD1 cells were transfected with pCAGGS–LacZ (a–c) as a negative control or pCAGGS-meltrin ϵ (d–f). Ba,d: Both cells were cotransfected with green fluorescent protein (GFP) reporter to monitor the efficiency. Bb,e: These cells were labeled with the anti–Meltrin ϵ antibody and detected with the Cy3-conjugated secondary antibody. Fluorescence is detected only in the cells transfected with meltrin ϵ. Bc,f: Merged images of a and b, and d and e, respectively. **C:** Transverse cryosections of Hamburger–Hamilton stage 15 chick embryo were labeled with control IgG (a) or anti–Meltrin ϵ antibody (b), and Cy3-conjugated secondary antibody. The section passes through the otic vesicles (ot), metencephalon (mc), pharynx (ph), and notochord (nc). Scale bars = 20 μm in B, 80 μm in C.



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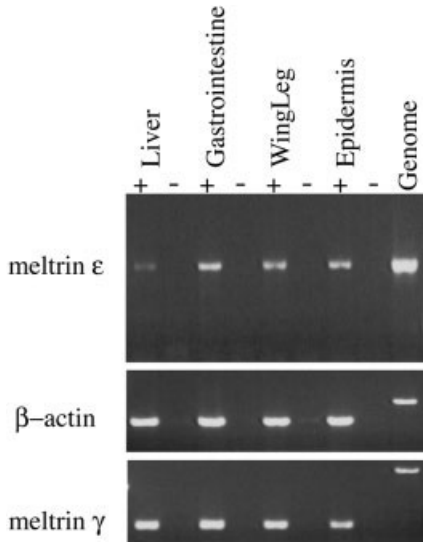

Fig. 2. Expression of Meltrin ε mRNA of chick tissues. RNA was isolated from the indicated tissues of embryonic day 7 chick embryos. Chick genomic DNA isolated from the same stage embryos was also used as a template. Primers specific for meltrin ε (see Experimental Procedures section) were used in the reverse transcriptase-polymerase chain reaction analysis to produce a 665-bp product. Plus (+) and minus (−) indicate presence and absence of reverse transcriptase in the reaction mixture, respectively. Chick β-actin and meltrin γ primers were used as controls. The size of the bands for β-actin and meltrin γ amplified with genomic DNA as the templates was different from those for which cDNA template was used, because of the presence of intronic sequences.

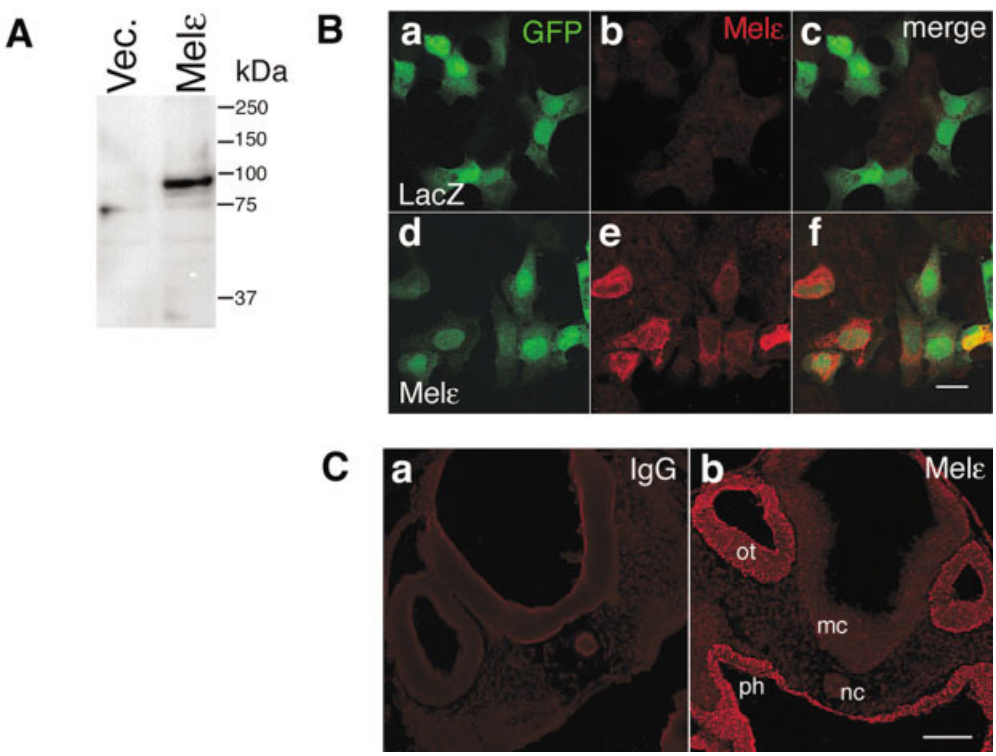

Fig. 3.

main, metalloprotease, disintegrin, a cysteine-rich domain, an epidermal growth factor-like domain, a transmembrane region, and a cytoplasmic region (Fig. 1A). Its metalloprotease domain contained an active zinc-chelating site whose sequence is conserved among various metalloproteases (Wolfsberg et al., 1995). A recognition sequence for furin protease occurs between the pro-domain and the metalloprotease domain. Five N-glycosylation sites are found in the extracellular domains. Orthologs in other species have not been found, so we designated this novel gene product Meltrin ϵ (ϵ for epithelia). Meltrin ϵ is most similar to Meltrin γ (ADAM9; Yagami-Hiromasa et al., 1995; Weskamp et al., 1996), ADAM21, and ADAM20 (Hooft van Huijsduijnen, 1998; Poindexter et al., 1999; Fig. 1B). Their similarity, however, is much less than the similarity between mouse and chick Kuzbanian (88% identity; Hall and Erickson, 2003). Thus, sequence analysis indicates that Meltrin ϵ is a novel ADAM family protein.

Meltrin ϵ mRNA Expression

We examined the tissue distribution of Meltrin ϵ mRNA by RT-PCR analysis. Total RNA and control genomic DNA were isolated from E7 chick embryos. A set of specific primers for meltrin ϵ cDNA was used, a PCR product of the expected size, 665 bp, was found in liver, gastrointestine, limb, epidermis, brain, lens, and myotome (Fig. 2 and data not shown). The transcript of the *meltrin ϵ* gene was clearly detectable but at different levels in contrast to *meltrin γ* and *β-actin*.

Generation of an Antibody Against the Metalloprotease Domain of Meltrin ϵ

To further examine expression of Meltrin ϵ in development, chick embryos were immunostained with an antibody (ME26-1) raised against an oligopeptide within its metalloprotease domain. The amino acid sequence of the oligopeptide is depicted in Figure 1A. Because some ADAM proteins undergo limited proteolysis to generate metalloprotease-lacking forms during development (Blobel et al., 1990; Yagami-Hiromasa et al., 1995; Schlomann et al., 2002), we needed to investigate the expression of the metalloprotease domain to delineate its roles. The antiserum ME26-1 recognized a 95-kDa protein in the Western blot analysis using with the lysate of COS1 cells in which Meltrin ϵ was transiently expressed (Fig. 3A). When COS1 cells were transfected with pCAGGS–meltrin ϵ–HA (see Experimental Procedures section), an expression plasmid for Meltrin ϵ with an HA-tag at the carboxyl-terminus, both the anti–Meltrin ϵ and anti-HA antibodies recognized bands of the same mobility in the Western blot (data not shown), indicating that the antiserum recognizes Meltrin ϵ. The apparent molecular weight of the 95-kDa protein is considerably larger than that predicted from the deduced sequence (85 kDa), probably because of glycosylation.

When the pCAGGS–meltrin ϵ was cotransfected with the green fluorescent protein (GFP) reporter plasmid into human colon carcinoma DLD1 cells, the anti–Meltrin ϵ antiserum ME26-1 specifically recognized the transfected cells (Fig. 3B). The antiserum did not recognize transfectants of pCAGGS–LacZ control vector or pCAGGS–meltrin ϵ ΔMP (see Experimental Procedures section), a derivative of Meltrin ϵ lacking its metalloprotease domain (Fig. 3B and data not shown). Thus, the antiserum specifically recognizes the metalloprotease domain of Meltrin ϵ, as expected.

Figure 3C shows transverse cryosections of a Hamburger–Hamilton stage 15 (Hamburger and Hamilton, 1951; E2.5) chick embryo that pass through the otic vesicles (ot), metencephalon (mc), pharynx (ph), and notochord (nc). The otic vesicles and pharyngeal and surface ectoderm were stained in the section labeled with anti–Meltrin ϵ antiserum but not with the control IgG.

Expression Patterns of Meltrin ϵ in the Chick Embryo

The immunostaining analyses were performed on chick embryos from E2 to E6, which are critical stages for muscle development and organogenesis. During somitogenesis, expression of Meltrin ϵ protein is activated during myotome formation. In contrast to its prominent expression in the myotome, it is undetectable in undifferentiated epithelial somites, the dermomyotome, sclerotome, and dermatome (Fig. 4a–c). It is also expressed in embryonic limb muscles (Fig. 4e), indicating that the expression of Meltrin ϵ is activated in the myoblast and continues during skeletal myogenesis. In addition to its expression in the skeletal muscle lineage, Meltrin ϵ is expressed strongly in some other epithelial tissues, including those of ectoderm and endoderm origin. The surface ectoderm and the otic vesicles, which derive from it, express Meltrin ϵ, but it is scarcely detectable in neural ectoderm or notochord (Figs. 3C, 4). The gastrointestinal (Figs. 3C, 4f,h–k) and respiratory (Fig. 4f,g) epithelia also express Meltrin ϵ during development.

Expression Analysis of Meltrin ϵ in Detail

In the myotome, Meltrin ϵ-positive cells also express desmin, a marker for the muscle cell lineage, including myoblasts and differentiated muscle cells (Fig. 5a–d). In contrast, the expression of myogenin, a myogenic transcription factor essential for terminal differentiation and a marker for the muscle cells at the onset of differentiation, was observed in part of these Meltrin ϵ-positive cells in the myotome (Fig. 5e–h). These results suggest that the activation of the *meltrin ϵ* gene precedes the muscle cell differentiation.

Meltrin ϵ is also seen in endodermal pharyngeal epithelium. Strong expression of Meltrin ϵ was detected at the basal edges and lateral membranes of the epithelial cells, compared with α-catenin proteins accumulated at the apical edges of cell–cell boundaries (Fig. 6).

Figure 7 shows the expression of Meltrin ϵ during early stages of lens development. The cranial surface ectoderm expresses a low level of Meltrin ϵ. After the optic vesicles extend toward the surface ectoderm from the head ectoderm, the sur-

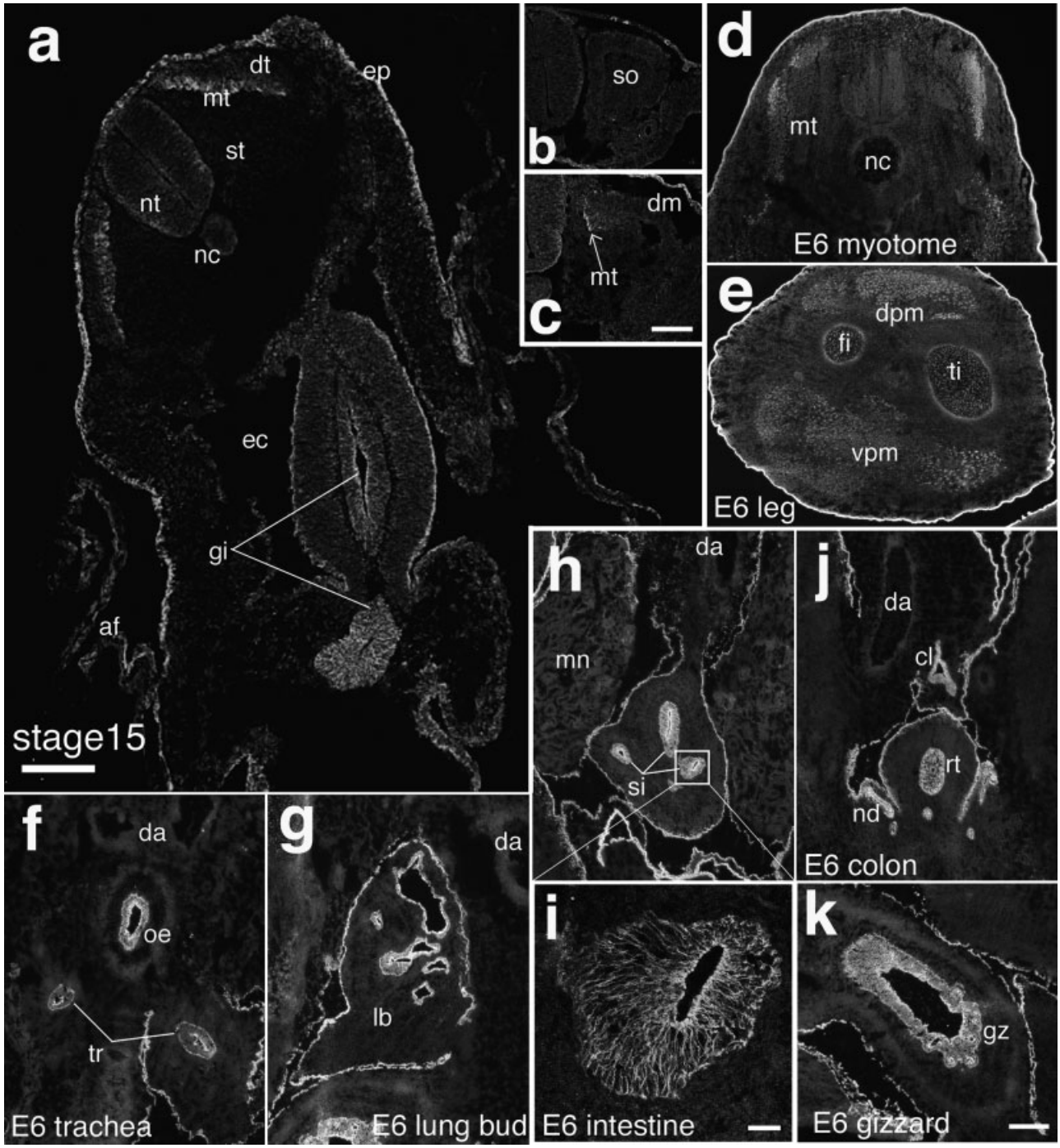

Fig. 4. Meltrin ϵ is expressed in the myotome and some of other epithelial tissues of the developing chick. Immunohistochemistry of cryosections at stage 15 (a–c) and embryonic day (E) 6 embryos (d–k). **a:** At stage 15, prominent expression was seen in the myotome, epidermis, amnionic fold, and the gastrointestinal epithelia. **b,c:** During somitogenesis, Meltrin ϵ was detected in the myotome as soon as it was formed but not in the somites and dermomyotome. **d,e:** Its expression continued through E6 myotome and premuscles. **f–k:** Meltrin ϵ was also expressed in various epithelial tissues. i: A magnified view of h. ep, epidermis; dt, dermatome; mt, myotome; st, sclerotome; nt, neural tube; nc, notochord; ec, embryonic coelom; gi, gastrointestine; af, amnionic fold; so, somite; dm, dermomyotome; dpm, dorsal premuscle mass; vpm, ventral premuscle mass; fi, fibula; ti, tibia; da, dorsal aorta; oe, esophagus; tr, trachea; lb, lung bud; mn, mesonephros; si, small intestine; cl, colon; rt, rectum; nd, nephric duct; gz, gizzard. Scale bars = 100 μm in a, 80 μm in c (applies to b,c), 200 μm in k (applies to d–h,j,k), 20 μm in i.

face ectoderm over the optic vesicles starts thickening to form lens placodes, in which the expression of Meltrin ϵ is enhanced in the basal region of the epithelial cells at HH stage 13 (E2; Fig. 7a,b; note that the expression of Meltrin ϵ is modest in the surface ectoderm, on both sides of the lens placode). Then the lens

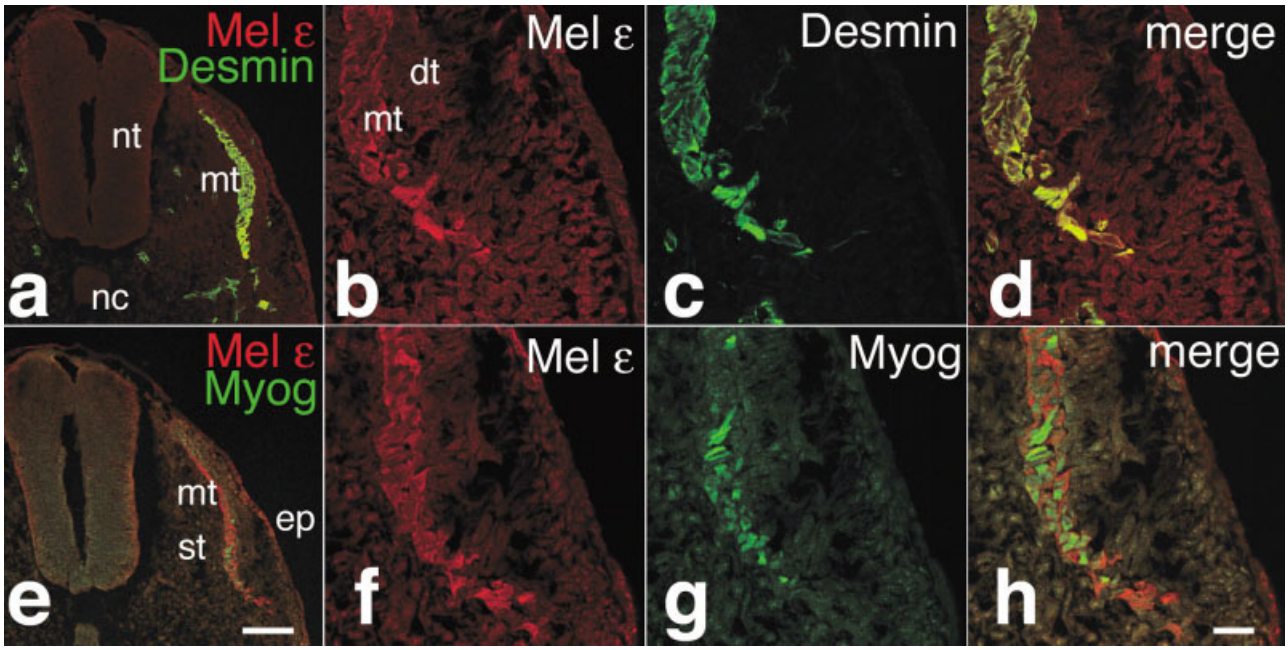

Fig. 5.

placodes invaginate to form lens vesicles, which continue to show polarized localization of Meltrin ϵ in the basal side of the epithelial layer. Figure 7c shows a section that was immunostained with anti–Meltrin ϵ antiserum and an antibody against N-cadherin. In contrast to N-cadherin that is strongly expressed in the optic cup but is faint in the lens vesicle at this stage, Meltrin ϵ is markedly expressed in the basal region of the lens vesicle. This basal localization of Meltrin ϵ is transient, because Meltrin ϵ is also found in the apical side of

Fig. 5. The expression of Meltrin ϵ and muscle markers at stage 18 myotome. Double immunostaining for Meltrin ϵ and muscle markers performed with serial transverse sections. **a–d:** Meltrin ϵ (Mel ϵ, red) is expressed in desmin-positive myoblasts (green). **e–h:** Myogenin (Myog, green), a terminal differentiation marker of myotome, is not expressed in all of the Meltrin ϵ-positive cells (red). nt, neural tube; nc, notochord; mt, myotome; dt, dermatome; st, sclerotome; ep, epidermis. Scale bars = 80 μm in e (applies to a,e), 20 μm in h (applies to b–d,f–h).

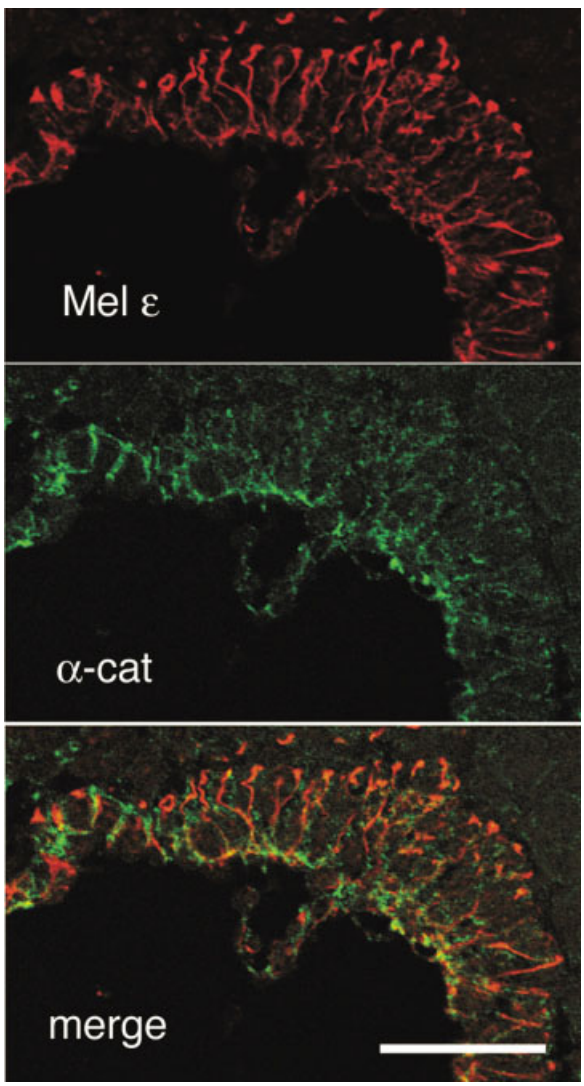

Fig. 6.

Fig. 6. Expression of Meltrin ϵ (Mel ϵ) in pharyngeal epithelia. Pharyngeal cross-sections of stage 14 chick embryos were stained with anti–Meltrin ϵ antibody (red) and anti–α-catenin antibody (α-cat, green). Both proteins were expressed on lateral membranes in pharyngeal epithelia, separately. Scale bar = 20 μm.

the lens vesicle after they take on a ball-like structure (see Fig. 8).

Overexpression of Full-Length and Protease-Deleted Meltrin ε in the Lens

Because expression of Meltrin ε is prominent in the lens, we examined the effects of overexpression of full-length or protease-deleted Meltrin ε (ΔMP) in the lens placodes (Fig. 8). When full-length Meltrin ε with an HA tag (pCAGGS–meltrin ε –HA) was expressed in the lens placodes, anti-HA and anti–Meltrin ε antibodies show similar intracellular distribution, indicating that the tagged Meltrin ε can be localized in the basolateral side (Fig. 8e,f). In the embryo injected with pCAGGS ΔMP–HA (see Experimental Procedures section), HA expression was not concentrated at the basal side in contrast to endogenous Meltrin ε, and the basal localization of endogenous Meltrin ε was not disturbed (Fig. 8h,i; note: the anti–Meltrin ε antiserum does not recognize Meltrin ε ΔMP proteins). It appears that the expression of full-length and protease-deleted Meltrin ε constructs did not affect lens formation grossly, when they were assessed morphologically or immunohistochemically, for the expression of δ-crystallin (Fig. 8j–l), N-cadherin (Fig. 8m–o), E-cadherin, or L-Maf, a lens-specific transcription factor (Ogino and Yasuda, 1998; data not shown).

DISCUSSION

In this study, we described the isolation from the chick embryo myotome of a cDNA encoding a novel member of the ADAM family of proteins. We found that Meltrin ε is most similar to Meltrin γ, ADAM21, and ADAM20, except in their cytoplasmic regions. In contrast to Meltrin γ that has sequence motifs that interacts with SH-3–containing molecules (Weskamp et al., 1996; Howard et al., 1999), the cytoplasmic regions of Meltrin ε, ADAM20, and ADAM21 are extremely short and are devoid of SH-3 binding sites (Hooft van Huijsduijnen, 1998). Poindexter et al. previously described phylogenic clusters that are related to each other; one of them includes Meltrin γ, ADAM20, and ADAM21, and another includes Meltrin α and Meltrin β (Poindexter et al., 1999). Our analysis shows that Meltrin ε belongs to the former cluster (data not shown). The sequence similarity between Meltrin ε with mouse Meltrin γ and human ADAM21 are 41% and 39% identities, respectively, which are much lower than 88% between the chicken and mouse kuzbanian proteins (Hall and Erickson, 2003), indicating that Meltrin ε is a novel ADAM protein distinct from Meltrin γ. We could not find any orthologs in other organisms, but we will continue the survey of genomes of other species such as reptiles.

In this study, we succeeded in raising an antibody against the protease domain of Meltrin ε to detect the protein immunohistochemically. Because ADAM proteins sometimes undergo limited proteolysis to generate their protease-lacking forms, we tried to determine when and where potentially active forms of Meltrin, containing the protease domain, were expressed by using the antibody. Staining of E2 through E6 embryos revealed several features of the expression patterns of the *meltrin* ε gene. First, Meltrin ε shows epithelia-specific expression during development. Although both Meltrin ε and Meltrin α are expressed in overlapping patterns in the myotome, only Meltrin ε is expressed prominently in epithelial tissues. Meltrin α, in contrast, is expressed in the condensed mesenchyme giving rise to skeletal muscle, visceral organs, and bone (Kurisaki et al., 1998, 2003). Among the Meltrins that we originally identified as metalloprotease–disintegrin proteins in skeletal muscle lineages (Yagami-Hiromasa et al., 1995), Meltrin ε is named based on its marked expression in such epithelial tissues.

In the somite, Meltrin ε is activated only in the myotome, although several lineages of epithelial layers are generated from undifferentiated epithelial somites. Meltrin ε is expressed soon after the muscle precursor cells in the dermomyotome, which delaminate from their dorsal lip to generate the myotome (Fig. 4c). Coexpression of Meltrin ε in desmin-positive cells indicates that Meltrin ε was activated when the cells were committed to skeletal muscle lineage. In contrast, only part of the Meltrin ε-positive cells coexpressed myogenin, a myogenic transcription factor necessary for the differentiation of myoblasts to myotubes, which shows that expression of Meltrin ε precedes terminal differentiation of skeletal muscle cells. Expression of Meltrin ε is also prominent in the developing gut, lens, and otic epithelial cells. During lens development, Meltrin ε is activated remarkably in lens placodes, specified regions of the surface ectoderm. However, its expression is modest in the prospective epidermis, an adjacent region to these placodes, and is very low in the neuroectoderm, both of which are derived from a common ectoderm layer. Thus, Meltrin ε is expressed in some of lineage-specified, undifferentiated epithelial tissues. These expression patterns are contrasting with kuzbanian (ADAM10), which is expressed in various epithelial tissues, including the neuroectoderm and the dermomyotome (Hall and Erickson, 2003).

Another feature of the expression of Meltrin ε is its subcellular location during the development of epithelial cells. The head ectoderm expresses low levels of Meltrin ε and does not show specific subcellular localization. When the head ectoderm makes contact with optic vesicles that invaginate from the head ectoderm itself, the contacts induce local thickening of epithelial cells in the head ectoderm to give rise to lens placodes. These lens placodes start to express Meltrin ε strongly, and it is preferentially localized on the basal side of the epithelial cells (Fig. 7). Invagination of these epithelial cells accompanies spreading localization of Meltrin ε to lateral as well as basal regions of the cells (Fig. 8). After lens vesicle formation, the epithelia lose polarized subcellular localization of Meltrin ε. The basal localization of Meltrin ε was also found in the initial stage of myotome formation (Fig. 4c).

Basolateral localization of Meltrin ε protease in the epithelial layers may also be relevant to its functions. The lens, otic placodes, and the gut invaginate or enlarge toward the direction of their basement without destroying their epithelial structures. The myotomal cells invaginating be-

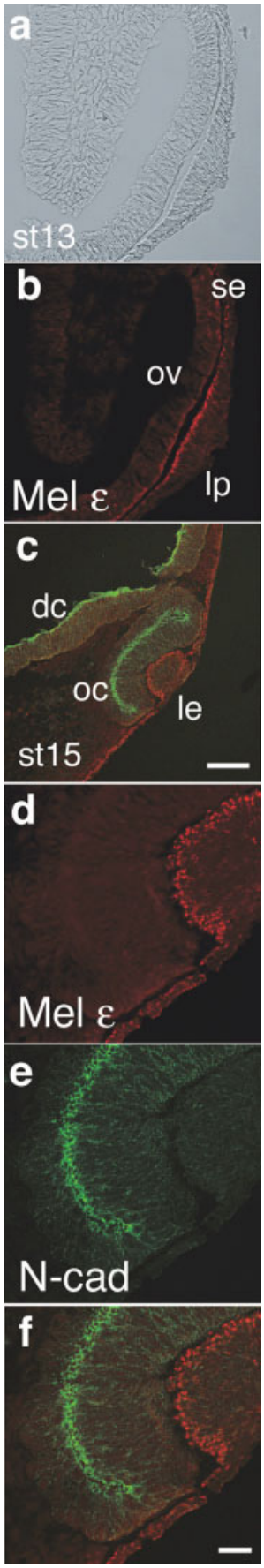

Fig. 7.

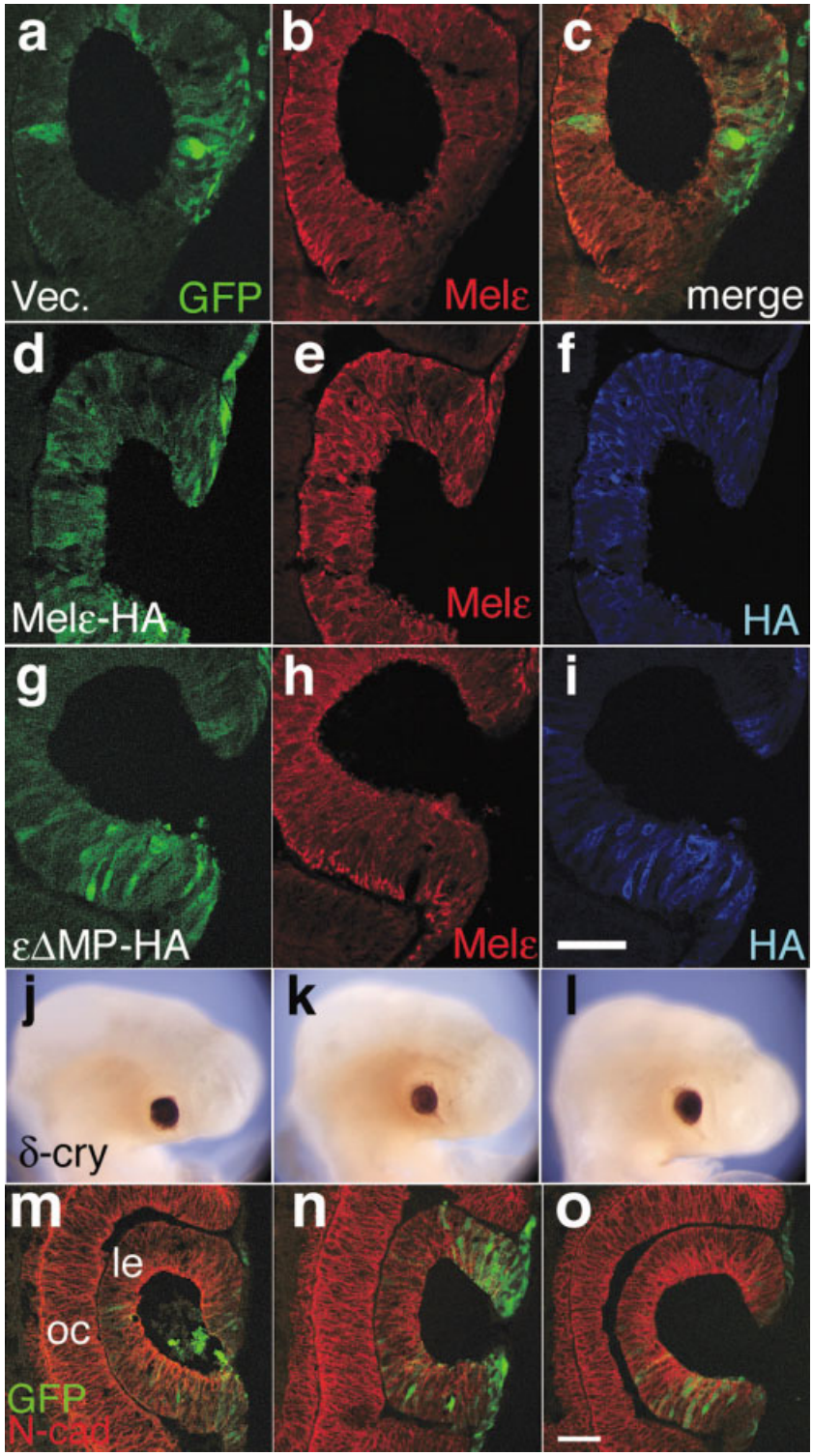

Fig. 8. Electroporation of Meltrin ϵ (Mel ϵ) vectors into the lens in ovo. **a–o:** Embryos electroporated with the control vector (Vec., a–c,j,m), or meltrin ϵ–HA (d–f,k,n), or ΔMP–HA (g–i,l,o) together with green fluorescent protein (GFP; a,d,g) were observed at stage 17 (24 hr after injection). b,e,f,h,i: Cryosections were immunostained with anti–Meltrin ϵ (red; b,e,h) and anti-HA (blue; f,i). b,e,h: Anti–Meltrin ϵ detects endogenous Meltrin ϵ (b,e,h) and exogenous Meltrin ϵ–HA (e), but it cannot recognize ΔMP–HA (h). f,i: The anti-HA antibody detects exogenous Meltrin ϵ –HA (f) and ΔMP–HA (i). c: Merged a and b. h,i: In the lens expressing ΔMP–HA, expression of the endogenous Meltrin ϵ was not disturbed (h), although ΔMP–HA was not concentrated at the basal side (i). j–l: Whole-mount immunostaining for δ-crystallin (δ-cry) in these embryos after the electroporation. m–o: Immunostaining with anti–N-cadherin (N-cad; red) and coinjected GFP (green). N-cadherin expression looks normal in these embryos. le, lens vesicle; oc, optic cup. Scale bar = 40 μm in a–i; 40 μm in m–o.

tween the dermatome and sclerotome also maintain their epithelial nature. ADAM proteases participate in the ectodomain shedding of membrane anchored growth and differentiation factors (Black and White, 1998; Qi et al., 1999; Shirakabe et al., 2001; Lieber et al., 2002; Sunnarborg et al., 2002; Chesneau et al., 2003). Assuming that Meltrin ϵ also works as a protease to regulate action of growth factors, the protease activity on the basal side of epithelial layers might contribute to delivering these factors efficiently to basolateral regions of the epithelial layers. Functional significance of other domains of Meltrin ϵ should be also taken into consideration. Fertilin β (ADAM2), for example, is one ADAM protein that encodes an inactive metalloprotease. Genetic and cell biological analyses indicate that the disintegrin domain of fertilin β is involved in fertilization (Cho et al., 1998). The *C. elegans* gene *unc-71* (*adm-1*, ADAM14), another ADAM with an inactive metalloprotease domain, participates in motor axon guidance and sex myoblast migration. The disintegrin and cysteine-rich domains of *unc-71* are required for UNC-71 function (Huang et al., 2003). Thus, disintegrin and cysteine-rich domains of ADAM proteins also modulate intercellular adhesion or adhesion to extracellular matrix proteins. Basolateral localization of Meltrin ϵ may have regulatory roles for adhesion to basal lamina of the epithelial cells or for organization of the epithelial tissues.

To determine the function of Meltrin ϵ, we overexpressed full-length or protease-deleted meltrin ϵ constructs in the lens placodes. Expression of protease-deleted derivatives of ADAM proteins causes dominant-negative effects on morphogenesis and inhibits the ectodomain shedding of membrane-anchored growth factors (Yagami-Hiromasa et al., 1995; Pan and Rubin, 1997; Shirakabe et al., 2001). If Meltrin ϵ plays a role as a protease involved in the invagination of placodes, protease-deleted meltrin ϵ may perturb lens formation. However, we could not find gross alteration in lens formation in this tissue. More prolonged disturbance of Meltrin ϵ during development than we examined in this study may be required to find the effects of these constructs. Further analyses from different aspects or with other methods will be needed to evaluate the functions of Meltrin ϵ. Alternatively some ADAM proteins and Meltrin ϵ may have redundant functions in lens formation. Meltrin γ, ADAM20, and ADAM21 that are structurally similar to Meltrin ϵ are good candidates.

In conclusion, we identified Meltrin ϵ, a novel ADAM protein. An antibody raised against its protease domain revealed that it is prominently expressed in several undifferentiated epithelia during development. Among those epithelia, we focused on the myotome and lens development and found transient but strong basal localization of Meltrin ϵ. This spatially and temporally regulated expression may reveal new insights to clarify the mechanism of organization of polarized epithelial tissues.

Fig. 7. Meltrin ϵ expression in lens vesicles. **a:** Phase-contrast photograph of a cryosection of a stage 13 chick embryo. **b:** Immunofluorescence of Meltrin ϵ (Mel ϵ) in the same section as in a. Expression was highest at the basal ends of the lens placode. **c–f:** At stage 15 embryo. **c:** A section double stained for Meltrin ϵ (red) and N-cadherin (green). **d–f:** A magnified view of c; single-color images of Meltrin ϵ (d) and N-cadherin (e), and a merged image (f). se, surface ectoderm; ov, optic vesicle; lp, lens placode; dc, diencephalon; oc, optic cup; le, lens vesicle. Scale bars = 80 μm in c (applies to a–c), 20 μm in f(applies to d–f).

EXPERIMENTAL PROCEDURES

Cloning of Chick *meltrin ϵ* cDNA

Fertilized eggs (Shiroyama Poultry Farm, Kanagawa, Japan) were incubated at 38.5°C and 100% humidity. Embryos were staged as described by Hamburger and Hamilton (1951). The chick myotome was collected from Hipeco chick embryos at E6 as follows. The dorsal region of the trunk was retained after cutting off the head, ventral internal organs, and ventral skin. The neural tube and dorsal root ganglia were discarded, and after dividing what remained into three parts along the long axis, the side parts including the dermomyotomes were collected. Total RNA was isolated with TRIzol (Gibco BRL, Rockville, MD) and used as a template for cDNA synthesis with the SuperScript Preamplification System (Gibco BRL). Three sets of primers for isolating meltrin cDNAs were designed (primer 1+2 for HELGHNF/LG, which corresponds to mouse Meltrin α a.a. residues 348–355; primer 9 for LVGVEVW, corresponding to 259–265 a.a.; and complementary primer 10 for GEECDCG, corresponding to 433–439 a.a.). PCR was performed with AmpliTaq Gold (Applied Biosystems, Foster City, CA) by using 0.2 μg of each primer and 0.5 μl of myotome cDNA in 20-μl total volume, under the following conditions: preincubation at 94°C for 9 min; 31 cycles at 94°C for 15 sec, 50°C for 30 sec, and 72°C for 1 min; and then a final incubation at 72°C for 9 min. The DNA amplified with primers 9 and 10 was reamplified with primers 1+2 and 10. The PCR products were cloned into pT7Blue Vector (Novagen, Madison, WI). The 5′ end of the cDNA encoding a novel Meltrin, designated cMeltrin ϵ, was obtained by a 5′ RACE system (Gibco BRL) using total RNA prepared from the myotome. To obtain the 3′ end of meltrin ϵ cDNA, mRNA was purified by using the Micro-FastTrack 2.0 Kit (Invitrogen Corp., Carlsbad, CA) on E6 myotome, and cDNA was synthesized with oligo dT primer. To amplify the 3′ end, internal primers for the meltrin ϵ cDNA and a primer of 17 tandem Ts were used for PCR. After subcloning the PCR products, multiple clones were sequenced. Ultimately, the entire coding sequence of meltrin ϵ was amplified with the primers S7 (TTTGAATTCTGCCACATCACAACATGGCGGCCAGGAG) and A7 (TTTGAATTCAGTTGATGAGTTTCGCAGTGCTTTCATG) and subcloned into the *Eco*RI sites of pBluescript II SK- (Stratagene, La Jolla, CA) and pCAGGS (Tokui et al., 1997) to generate pBS–cmeltrin ϵ and pCAGGS–meltrin ϵ, respectively.

The hydrophobicity of the sequence was deduced by using MacVector version 6.5 software (Oxford Molecular, presently Accelrys,

San Diego, CA); the molecular weight of the protein was predicted with GENETYX-MAC version 11.2.0 (Software Development Co., Tokyo). A BLAST search was performed to find sequences similar to chick Meltrin ϵ. Sequence similarity between Meltrin ϵ and either human Meltrin γ or ADAM21 was determined with GENETYX-MAC version 11.2.0.

RT-PCR Analysis

Total RNA was extracted from various E7 embryonic tissues with the RNAgents Total RNA Isolation System (Promega, Madison WI) and was used as templates for reverse transcription in the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). RNA samples were digested with DNase I (Sigma, St. Louis, MO) before RT-PCR. Control genomic DNA for RT-PCR was prepared from the E7 neck. PCR was carried out with AmpliTaq Gold DNA polymerase. The conditions were as follows: preincubation at 94°C for 9 min; 37 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 1 min; and finally at 72°C for 9 min. Primers for meltrin ϵ were e-1s (5′-CAAGCTGGATGGGGGCAAAAAAG-3′) and 4-a1 (5′-ACAATCTGTTGGGCTCGGGG-3′), yielding a 665-bp product. As positive controls, we used the primer sets for chick β-actin (β-s, 5′-CTGTGCCCATCTATGAAGGC-3′; β-a, 5′-CAGACAGCACTGTGTTGGCA-3′; 416 bp from cDNA or 722 bp from genomic DNA; Kost et al., 1983) and chick meltrin γ (unpublished observations; G-F2s, 5′-CGAGAGAAGAACCTTGTTTTGCG-3′; G-B1a, 5′-TGCTCCACAGTGACAGACCCTTTC-3′; 240 bp from cDNA or approximately 900 bp from genomic DNA) for RT-PCR under the latter conditions described above except that the number of cycles was 32.

Antibodies

Anti–Meltrin ϵ antibody was raised in a rabbit against a keyhole limpet hemocyanin-coupled peptide (RYSPDIEEVLSNFNDWGNR) corresponding to 222–241 a.a. of Meltrin ϵ (Fujiya Bioscience Laboratory, Kanagawa, Japan). The rabbit was immunized with the conjugate four times (every 2 weeks), and whole blood was collected (ME26-1) at Mitani Sangyo, Tokyo, Japan, and Pana Farm Laboratory, Kumamoto, Japan.

Other antibodies used in this study were as follows. Purified rabbit control IgG (Sigma), mouse anti-desmin monoclonal antibody D9 (Progen, Heidelberg, Germany), mouse anti-chick myogenin monoclonal antibody 5C1 (a generous gift from Dr. Yo-ichi Nabeshima, Graduate School of medicine, Kyoto University, Kyoto, Japan), rat anti-chick N-cadherin monoclonal antibody NCD2 (a gift from Dr. Masatoshi Takeichi, RIKEN Center for Developmental Biology, Kobe, Japan; Hatta and Takeichi, 1986), rat anti–α-catenin monoclonal antibody α18 (Nagafuchi et al., 1994), mouse anti-HA.11 monoclonal antibody 16B12 (BabCO, Richmond, CA), mouse anti–δ-crystallin monoclonal antibody (Sawada et al., 1993), rabbit anti–L-Maf polyclonal antibody (Ogino and Yasuda, 1998), mouse anti–E-cadherin monoclonal antibody 36 (Transduction Laboratories presently, BD Biosciences, Franklin Lakes, NJ), Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), FluoroLink Cy5-labeled goat anti-mouse IgG (Amersham Biosciences, Piscataway, NJ), Alexa Fluor 488 goat anti-rat IgG and anti-mouse IgG (Molecular Probes, Eugene, OR), biotin-SP–conjugated mouse anti-rabbit IgG (Jackson ImmunoResearch Laboratories).

Construction of Plasmids

Meltrin ϵ expression vector, pCAGGS-meltrin ϵ, was constructed as described above. pCAGGS–meltrin ϵ –HA was generated by inserting the HA epitope into the *Dra*I site at the carboxyl terminus of meltrin ϵ. pCAGGS ΔMP, lacks almost the entire metalloprotease domain (227–404 a.a.), including the recognition site of antibody ME26-1 and the metalloprotease consensus sequence. A *Sac*I site was introduced in the deleted region by using PCR when pCAGGS ΔMP was constructed. pCAGGS ΔMP–HA, in which the MP region of meltrin ϵ is replaced by an HA tag, was constructed by inserting the HA epitope into the *Sac*I site of pCAGGS ΔMP. pCAGGS–LacZ was described previously (Aihara and Miyazaki, 1998).

Cell Culture and Transfection by Plasmids In Vitro and In Ovo

COS1 cells were cultivated in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum (FBS), and human colon carcinoma DLD1 cells were cultured in RPMI 1640 containing 10% FBS. These cells were plated on and transfected with the expression constructs (as described above) and a GFP reporter plasmid (pGreen Lantern-1/Gibco BRL), by using the LIPOFECTAMINE PLUS Reagent (Gibco BRL). Twenty-four hours after transfection, the transfectants were analyzed for the expression of Meltrin ϵ by Western blot analysis and immunohistochemistry. Microelectroporation in ovo was done according to the procedure described previously (Momose et al., 1999; Shimada et al., 2003). pCAGGS–meltrin ϵ–HA, pCAGGS ΔMP–HA, or pCAGGS control vector was transfected into lens placode, the head ectoderm of stage-9–10 chick embryos. Each expression vector was mixed with pCAGGS–GFP (Ogawa et al., 1995) at a ratio of 9:1 and expression of the HA-tagged constructs was confirmed by immunostaining with anti-HA antibody. Analyses were performed 24 hr after electroporation, at approximately HH stage 17–18.

Western Blot Analysis

COS1 cells were transfected with pCAGGS-meltrin ϵ or the control vector, and cultured for 24 hr. The transfectants were scraped from dishes with a rubber policeman and lysed in 2× sodium dodecyl sulfate (SDS) buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue, and 2% 2-mercaptoethanol) supplemented with a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). After sonication, cell lysates were cleared by centrifugation and were boiled for 2 min. Protein samples were separated by SDS-polyacrylamide gel electrophoresis in 10–20% gel and transferred to polyvinylidene difluo-

ride membranes. Total blotted protein was visualized by using AuroDye Forte (Amersham Biosciences). Blots were blocked with 5% skim milk in phosphate buffered saline (PBS) containing 0.2% Tween 20 and then incubated with the antiserum ME26-1 or other primary antibodies in this solution. The membranes were washed and incubated with biotinylated secondary antibodies. After washing in PBS containing 0.2% Tween 20, bands were probed with Streptavidin–horseradish peroxidase conjugate and were detected with an ECL plus Western Blotting Detection System and Hyperfilm MP (Amersham Biosciences).

Immunohistochemistry

For immunostaining, cultured cells were washed twice with PBS, fixed in 4% paraformaldehyde (PFA)/PBS for 20 min at 4°C, and then permeabilized with Triton X-100 (0.2% in PBS) for 5 min at 4°C. The cells were blocked with 5% skim milk and 10% FBS in PBS. The primary and secondary antibody reactions were carried out in 1% skim milk/PBS for 1 hr at room temperature, and then, the excess antibodies were rinsed.

For immunohistochemistry of cryosections, embryos were dissected in PBS and fixed in 4% PFA/PBS for 10 min. (For anti–α-catenin antibody α18, fresh-frozen sections were prepared by omitting the fixation procedure.) After washing in PBS, the embryos were equilibrated in a series of sucrose solutions (10%, 20%, and 30%) and then embedded in Tissue Tek O.C.T. Compound (Sakura Finetechnical, Tokyo, Japan). Frozen embryos were cut into 10-μm-thick sections with a cryostat (Leica, Solms, Germany). Cryosections were mounted on glass slides coated with 3-aminopropyltriethoxy silane and stored at −80°C. The sections were refixed in 4% PFA/PBS for 20 min at 4°C, then in methanol for 20 min at −30°C, and finally washed in PBS. After blocking in 5% skim milk/PBS for 30 min, sections were incubated with primary antibodies in 1% skim milk/PBS for 2 hr, washed with PBS, and then incubated with secondary antibodies for 40 min. Finally, the sections were washed with PBS again.

Whole-mount immunostaining was performed as described previously (Shimada et al., 2003). Imaging was done with an Axiovert 200 fluorescent microscope (Zeiss, Oberkochen, Germany), Leica TCS-SP confocal microscope (with DM IRBE, Leica), or MZ 12 stereoscopic microscope (Leica).

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