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Endogenous Meltrin α Is Ubiquitously Expressed and Associated with the Plasma Membrane but Exogenous Meltrin α Is Retained in the Endoplasmic Reticulum[1]

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Meltrin α (ADAM12) is a member of the ADAM (MDC) protein family characterized by the presence of metalloprotease and disintegrin domains. ADAM proteins contain single transmembrane domains, and the processed mature proteins are postulated to span the plasma membrane. It has been reported that transfection of a truncated meltrin α cDNA lacking the prodomain and metalloprotease domain promotes skeletal muscle cell fusion. We show here that meltrin α was constitutively expressed in both undifferentiated and differentiated C2 skeletal muscle cells and also in fibroblasts. Both its precursor and processed mature forms were present in these cells. Thus, meltrin α may play general roles in addition to its roles in myogenesis. Since endogenous meltrin α cannot be detected by immunofluorescence microscopy, we examined the location of the exogenously expressed protein by transfection. Unexpectedly, the exogenously expressed meltrin α was located to a network structure of the endoplasmic reticulum (ER) but not to the plasma membrane. Cell fractionation revealed that the intrinsic mature protein was associated with the plasma membrane. However, the exogenously expressed protein remained unprocessed. These results seem to imply that the exogenously expressed meltrin α is not translocated from the ER to the *trans*-Golgi network, where a processing enzyme resides, and that it is consequently not converted to the mature form. Thus, the transfected meltrin α is unlikely to exert its physiological functions. Conversely, the ER may serve as a reservoir of the latent form of intrinsic meltrin α.

Key words: ADAM (MDC) family, endoplasmic reticulum, meltrin α (ADAM12), plasma membrane, proteolytic processing.

To date, more than 20 members of the ADAM (MDC) protein family have been identified in vertebrates, insects, and nematodes. These proteins contain characteristic domains: a prodomain preceded by an N-terminal signal peptide, metalloprotease, disintegrin, cysteine-rich, EGF repeat, transmembrane, and cytoplasmic domains (*1–5*). The physiological roles of the metalloprotease activity of several ADAM proteins as proprotein convertases have recently been documented. ADAM17 is a tumor necrosis factor-α (TNF-α)-converting enzyme (TACE) and proteolytically converts the membrane-bound precursor of TNF-α into the soluble mature TNF-α (*6, 7*). Kuzbanian (Kuz, ADAM10) has been reported to be required for proteolytic processing of Notch, and mediates lateral inhibition during neurogenesis in *Drosophila* and vertebrates (*8*). A more recent study has revealed that Kuz is involved in cleavage of transmembrane protein Delta rather than Notch to produce a soluble form of Delta as a ligand for Notch (*9*). MDC9 (meltrin γ, ADAM9) in combination with protein kinase Cδ (PKCδ) participates in shedding of the ectodomain of heparin-binding EGF-like growth factor (HB-EGF) to generate soluble HB-EGF (*10*).

Fertilin α and β are the first characterized ADAM proteins existing on the sperm head cell surface, and are considered to mediate sperm–egg attachment and fusion during fertilization (*11*). Since sperm bind to integrin α6β1 present on the egg plasma membrane and fertilin β disintegrin peptide analogs prevent this binding, fertilin β is postulated to play essential roles through its disintegrin domain in the attachment of sperms to eggs (*12*). This is corroborated by the finding that fertilin β-deficient sperm is unable to bind to the egg surface (*13*). The recombinant disintegrin domain peptide of MDC15 (metargidin, ADAM15) can interact specifically with integrin αvβ3 (*14*). This suggests that MDC15 is involved in cell–cell interaction through binding to integrin αvβ3.

Fertilin α contains in its cysteine-rich domain a sequence similar to a potential fusion peptide of E2 glycoprotein of rubella virus (*11*). As fusion peptides of viral fusion proteins are presumed to mediate virus–cell membrane interactions that lead to fusion, the fusion peptide-like sequence of fertilin α is assumed to be involved in sperm–egg

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Abbreviations: ER, endoplasmic reticulum; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; mAb, monoclonal antibody; MelCyt, meltrin α cytoplasmic domain; MHC, myosin heavy chain; pAb, polyclonal antibody; PKC, protein kinase C; RT-PCR, reverse transcription–PCR; 10T1/2, C3H/10T1/2.

membrane fusion. Indeed, synthetic putative fusion peptides of fertilin α cause mixing of lipid vesicles *in vitro* (*15*, *16*). Meltrin α has been cloned from a myogenic cell line and also harbors a putative fusion peptide similar to the fusion peptide of Sendai virus (*17*). In addition, Yagami-Hiromasa *et al.* (*17*) have shown that transfection of a truncated meltrin α cDNA lacking the prodomain and metalloprotease domain to C2 skeletal muscle cells induces disorganized cell aggregation and facilitates cell fusion. Accordingly, they have postulated that meltrin α is involved in cell–cell or cell–matrix interactions during skeletal muscle differentiation. Despite this fascinating assumption and suggestive evidence, however, there has been no direct evidence that fertilin α and meltrin α function as fusion proteins (*2*, *3*). Thus, it is necessary to determine the mechanisms for cell fusion by these proteins and the physiological functions of these proteins other than cell fusion, if any.

To gain further insight into the cellular functions of meltrin α, we examined its expression and localization in both skeletal myocytes and nonmyogenic cells. Meltrin α was constitutively expressed during myogenic differentiation. In addition, the processed mature protein was detected in fibroblasts as well as in myogenic cells. These results suggest universal roles of meltrin α in addition to its roles in myogenesis. The endogenous mature meltrin α was associated with the plasma membrane. In contrast, exogenously expressed meltrin α was not converted to the mature form and was exclusively located to the ER but not to the plasma membrane. Thus, the ectopic meltrin α is unlikely to be physiologically active. Conversely, the endogenous unprocessed meltrin α may reside in the ER.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse C2 skeletal muscle cells (*18*) were cultured as described (*19*). Proliferating myoblasts were maintained at 37°C in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal bovine serum (growth medium). To induce terminal differentiation, ~2 × 10^5 cells were plated in the growth medium on a 100-mm dish (~20% confluency) and maintained for 16–24 h, and then the medium was replaced with DME medium supplemented with 5% horse serum (differentiation medium). Myotubes developed extensively by 96 h after the shift to the differentiation medium. Mouse C3H/10T1/2 (10T1/2) fibroblasts (*20*) were cultured in the growth medium.

Quantitative Reverse Transcription (RT)-PCR—Cytoplasmic RNA was prepared from C2 and 10T1/2 cells as described previously (*21*), and poly(A)$^+$ RNA was isolated by using Oligotex-dT30 Super (Roche Diagnostics). The first strand cDNA was synthesized with SuperScript II RNase H(–) reverse transcriptase (Gibco BRL) to 2 μg of the template poly(A)$^+$ RNA primed with an oligo(dT) primer. One twentieth of each cDNA was used as a template for PCR amplification. The amplification reaction was carried out on Zymoreactor II (Atto) with Taq DNA polymerase (Qiagen) according to a step program (94°C for 40 s, 60°C for 30 s, and 72°C for 60 s). The sequences of the primers used were:

meltrin α 5′ primer, 5′-GGAGAAGAGTGTGACTGCGGA-3′;
meltrin α 3′ primer, 5′-CCTTCTGCACACTTTGTTCC-3′;
myogenin 5′ primer, 5′-GAGCTGTATGAGACATCCCC-3′;
myogenin 3′ primer, 5′-GTAAGGGAGTGCAGATTGTG-3′;
GAPDH 5′ primer, 5′-GTGAAGGTCGGTGTGAACGG-3′;
GAPDH 3′ primer, 5′-GATGCAGGGATGATGTTCTG-3′.

After 25 cycles of amplification, these products were in the linear range. The PCR products were analyzed by agarose gel electrophoresis.

cDNA Cloning—To clone a 1,979-bp cDNA fragment of meltrin α corresponding to nucleotides 962–2940 of DDBJ/EMBL/GenBank accession number D50411, RT-PCR was carried out to C2 myotube poly(A)$^+$ RNA. The C2 myotube cDNA library constructed in λZAPII (*22*) was screened with this cDNA fragment labeled with [α-^{32}P]dCTP (> 111 TBq/mmol; ICN Biomedicals) by using a BcaBEST labeling kit (Takara Shuzo). A 2,922-bp cDNA including the entire coding region of meltrin α (nucleotides 209–3130) was cloned. Cloned cDNAs in the pBluescript SK(–) phagemid were obtained by *in vivo* excision. The nucleotide sequences of the cDNAs were determined with LI-COR 4000 automated DNA sequencing system by use of SequiTherm Long-Read Cycle Sequencing Kit-LC (Epicentre Technologies).

Epitope-Tagging and Transfection—A 2,612-bp fragment of the meltrin α cDNA (nucleotides 209–2820) was subcloned into the *Nhe*I site of the pCMVMyc vector (*22*) (pCMVMelαMyc). This construct coded for meltrin α fused with the Myc-tag at its C-terminus. The cDNA containing the entire coding region of meltrin α was subcloned into the pBK-CMV vector (Stratagene) (pCMVMelα). pCMVMelαΔPM was constructed by deleting the *Bam*HI–*Nhe*I fragment (nucleotides 382–1422) corresponding to the prodomain and metalloprotease domain from the cDNA in pCMVMelα. 10T1/2 cells cultured on glass coverslips in the growth medium were transiently transfected with these plasmids by the calcium phosphate-mediated method as described (*23*). Lipofectamine (Gibco BRL) was applied for the transfection to C2 cells because the transfection efficiency as to C2 cells with these plasmids was extremely low with the calcium phosphate-mediated method.

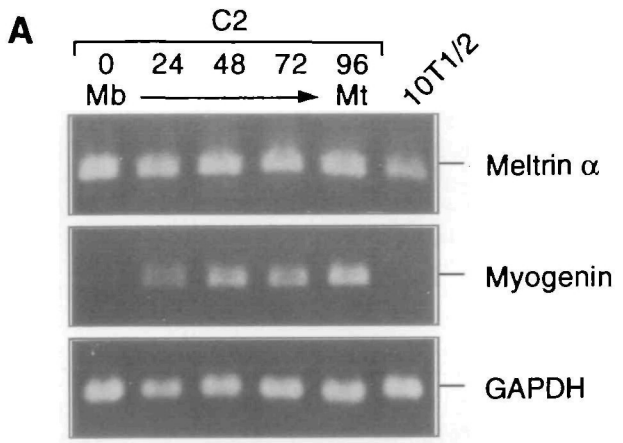
Production of Polyclonal and Monoclonal Antibodies—A *Sma*I fragment of meltrin α cDNA (nucleotides 2525–2940) encoding most of the cytoplasmic domain (amino acids 769–903) was fused in frame to a glutathione *S*-transferase (GST)-tag in pGEX-2T (Amersham Pharmacia Biotech). The recombinant protein of the GST-tagged meltrin α cytoplasmic domain (MelCyt) was expressed in *E. coli* strain XL1-Blue and affinity-purified on glutathione-Sepharose 4B (Amersham Pharmacia Biotech). The GST moiety was removed from the tagged protein by digestion with 10 U/ml bovine thrombin (Sigma). The cytoplasmic domain was separated by SDS-PAGE and recovered from the gel. The protein emulsified with Freund's complete adjuvant (Difco Laboratories) was subcutaneously injected into a New Zealand white rabbit. The protein emulsified with Freund's incomplete adjuvant was used for the second and third injections. After the third injection, whole blood was collected and the IgG fraction was isolated. The polyclonal antibody (pAb) was affinity-purified on HiTrap NHS-activated (Amersham Pharmacia Biotech) coupled with the recombinant MelCyt.

An *Nco*I fragment of meltrin α cDNA encoding its cytoplasmic domain (amino acids 744–903) was fused in frame

to His-tag in pQE-31 (Qiagen). The recombinant protein of His-tagged MelCyt was expressed in XL1-Blue and affinity-purified on Talon metal affinity resin (Clontech Laboratories). The recombinant protein was injected into a BALB/c mouse intraperitoneally for immunization. After the third injection, splenocytes from the mouse were fused with mouse myeloma X63Ag8.653 cells with polyethylene glycol 1500 (Roche). Hybridomas were cloned by limiting dilution and screened by enzyme-linked immunosorbent assay (ELISA) and immunoblotting. Monoclonal antibodies (mAbs) were recovered in the cell culture medium.

Immunofluorescence Microscopy—Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and then processed for immunofluorescence microscopy as described (*24*). They were incubated with anti-myogenin mAb F5D (*25*) (Developmental Studies Hybridoma Bank), anti-sarcomeric myosin heavy chain (MHC) mAb MF20 (*26*), anti-human c-Myc mAb Myc1-9E10 (*27*) (American Type Culture Collection), anti-Grp78 (BiP) KDEL sequence mAb 10C3 (StressGen Biotechnologies), or anti-MelCyt mAbs or the pAb. They were then incubated with fluorescein isothiocyanate- or rhodamine-conjugated goat anti-mouse or anti-rabbit IgG (affinity-purified; Cappel Research Reagents). Specimens were observed under a Zeiss Axioskop microscope equipped with phase-contrast and epi-fluorescence optics.

Immunoprecipitation and Immunoblotting—Cultured cells or recombinant proteins were treated with the SDS sample buffer and then subjected to SDS-PAGE. For immunoprecipitation, cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% Na deoxycholate, and 0.1% SDS), and the lysates were incubated with anti-MelCyt mAb MC11. The binding proteins were recovered with Protein G–Sepharose 4FF (Amersham Pharmacia Biotech), treated with the SDS sample buffer, and then subjected to SDS-PAGE. The resolved proteins



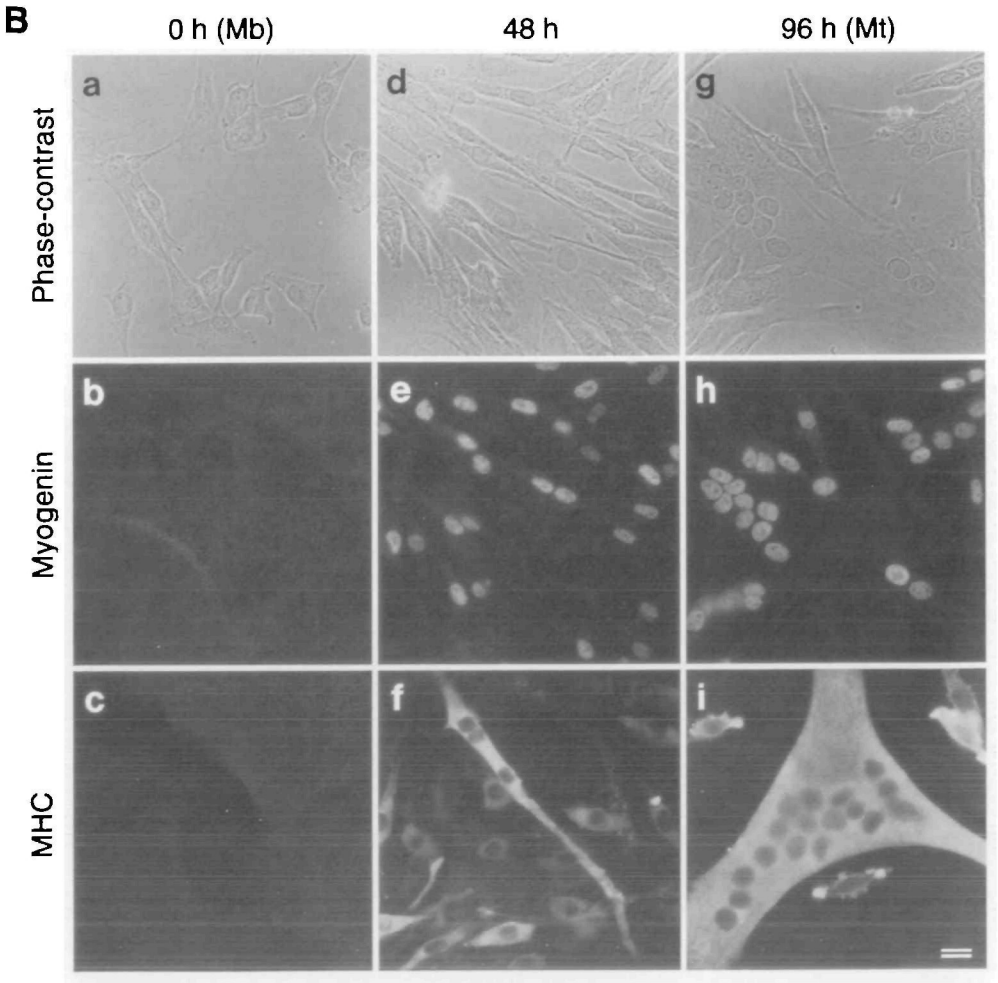


Fig. 1. **Constitutive expression of meltrin α in C2 cells.** (A) Determination of relative mRNA levels by quantitative RT-PCR. Poly(A)$^+$ RNA prepared from C2 cells cultured in the differentiation medium for 0 h (proliferating myoblasts in the growth medium), 24, 48, 72, and 96 h (fully differentiated myotubes), and from 10T1/2 fibroblasts was used for quantitative RT-PCR. The mRNA levels of meltrin α, myogenin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined. (B) Induction of muscle differentiation marker proteins during the differentiation of C2 cells detected by immunofluorescent staining. C2 myoblasts (a–c), and the cells cultured in the differentiation medium for 48 h (d–f) and 96 h (g–i) were processed for immunofluorescence microscopy. (a, d, g) Phase-contrast micrographs. (b, e, h) Immunofluorescent staining with anti-myogenin mAb F5D. (c, f, i) Immunofluorescent staining with anti-sarcomeric MHC mAb MF-20. Bar, 20 μm.

were tansferred to an Immobilon PVDF transfer membrane (Millipore), incubated with the pAb or MC11, and then with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse IgG (Bio-Rad). The reacted protein bands were detected by means of either chromogenic reaction with diaminobenzidine or chemiluminescent reaction with Renaissance Western blot chemiluminescence reagent (NEN Life Science Products).

Cell Surface Biotinylation and Cell Fractionation—The cell surface of attached C2 myoblasts was biotinylated with nonpermeable NHS-LC-biotin (Pierce) according to the manufacturer's instruction manual. Plasma membranes and microsomes were prepared from these cells according to Bilan *et al.* (*28*). Briefly, cells were homogenized with a Dounce homogenizer in buffer I (250 mM sucrose, 20 mM HEPES-NaOH, pH 7.4, 2 mM EGTA, 3 mM NaN_3, and protease inhibitors). The homogenate was centrifuged at 700 $\times g$ for 5 min and the supernatant was centrifuged at 31,000 $\times g$ for 60 min. The supernatant was centrifuged at 177,000 $\times g$ for 60 min, and the pellet was resuspended in buffer I and used as microsomes. The 31,000 $\times g$ pellet was resuspended in buffer I and subjected to ultracentrifugation on a discontinuous sucrose density gradient (32, 40, and 50% w/w, in 20 mM HEPES–NaOH, pH 7.4). The 32% layer, and 32/40% and 40/50% interface fractions were collected. These fractions were dialyzed against 20 mM HEPES–NaOH, pH 7.4, and 2 mM EGTA, and centrifuged at 23,000 $\times g$ for 60 min. The pellet was lysed with RIPA buffer and immunoprecipitated with MC11. Proteins resolved by SDS-PAGE were transferred to Immobilon and immunoblotted with the pAb. Biotinylated proteins on Immobilon were detected with HRP-conjugated Z-avidin (Zymed Laboratories). Because the cell surface biotinylation was retained mostly at the 32/40% interface, this was regarded as the plasma membrane fraction.

RESULTS

Constitutive Expression of Meltrin α during C2 Cell Differentiation and in Fibroblasts—Meltrin α was originally cloned from skeletal myocytes and has been postulated to be involved in skeletal muscle cell fusion. The previous study showed that the amount of meltrin α mRNA was very low in proliferating C2 skeletal muscle myoblasts and remarkably increased in response to the induction of differentiation in a manner similar to in the case of myogenin mRNA (*17*). However, its mRNA and protein have been detected in several human tissues and tumor cell lines (*29*, *30*), and mouse tissues during development (*31*, *32*). We reexamined the amount of meltrin α mRNA during the differentiation of C2 cells and in 10T1/2 fibroblasts by quantitative RT-PCR. The meltrin α transcript was easily detected in proliferating C2 myoblasts, in which myogenin mRNA was hardly detected (Fig. 1A). The amount of meltrin α mRNA remained constant throughout differentiation at least for 96 h after the shift to the differentiation medium (Fig. 1A). By this time, myotubes were fully developed (Fig. 1B). On the other hand, myogenin mRNA was induced by 24 h, reached the maximal level by 48 h, and persisted after that (Fig. 1A). Although myogenin expression was absent in 10T1/2 cells, meltrin α mRNA was present in 10T1/2 cells as well, and the amount was almost equivalent to that in C2 cells (Fig. 1A). Both the myogenin and sarcomeric MHC proteins were induced during the differentiation of C2 cells and accumulated abundantly in myotubes, as detected by immunofluorescence microscopy (Fig. 1B).

To determine the amount and localization of meltrin α protein, we prepared rabbit pAb to MelCyt. We also produced hybridomas secreting mAbs to MelCyt. The affinity-purified pAb and mAbs, MC9, MC11, and MC21, reacted specifically to the recombinant protein on immunoblotting (Fig. 2). These antibodies were used to determine the amount of endogenous meltrin α. Lysates of C2 myoblasts and myotubes and 10T1/2 cells were immunoprecipitated with MC11, and then the immunoprecipitated proteins were used for immunoblotting with the pAb. Protein bands corresponding to 115 kDa and 86 kDa were detected for all these three types of cells in equivalent amounts (Fig. 3). The 115 kDa protein was more abundant than the 86 kDa protein in these cells. These two bands likely represent glycosylated unprocessed meltrin α (pro-meltrin α) and the proteolytically processed mature form, respectively (*17*). These results indicate that meltrin α is constitutively expressed during the differentiation of C2 cells and even in fibroblasts. It is not coordinately induced with myogenin or certain muscle-specific genes including MHC.

Localization of Exogenously Expressed Meltrin α to the ER—Most, if not all, ADAM family proteins have been considered to span the plasma membrane, probably because the first characterized ADAM proteins, fertilin α and β, have been shown to be present on the cell surface (*33*), and because some of them are thought to be required for cell attachment or fusion. However, immunostaining data have shown that certain ADAM proteins including exogenously expressed ADAM13 (*34*) and MDC15 (*35*) are distributed in the cytoplasm rather than on the cell surface. Furthermore, endogenous ADAM17 is predominantly localized to an intracellular perinuclear compartment (*36*). Thus, to deter-

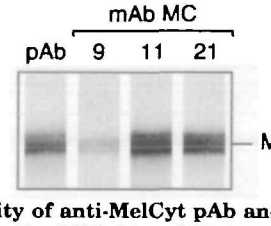


Fig. 2. **Reactivity of anti-MelCyt pAb and mAbs.** The reactivity of the pAb and mAbs (MC9, 11, and 21) to recombinant MelCyt in *E. coli* lysates was analyzed by immunoblotting.

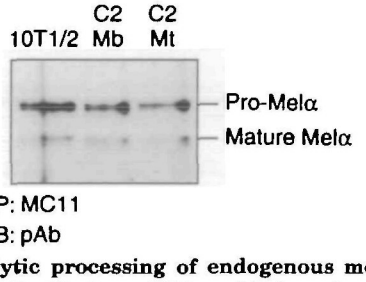


Fig. 3. **Proteolytic processing of endogenous meltrin α.** Lysates of 10T1/2 fibroblasts, and C2 myoblasts and myotubes were immunoprecipitated with MC11 and anti-MHC mAb MF20 (negative control), and then immunoblotted with the anti-MelCyt pAb. Protein bands of unprocessed pro-meltrin α (115 kDa) and processed mature meltrin α (86 kDa) were detected.

mine the subcellular localization of meltrin α, we used the anti-MelCyt pAb and mAbs for immunofluorescent staining of C2 myoblasts and myotubes and 10T1/2 cells. However, none of these antibodies detected the endogenous protein, possibly due to the low level of expression.

Accordingly, we next exploited the epitope-tagging method to determine the localization. The recombinant plasmid, pCMVMelαMyc (Fig. 4), encoding meltrin α fused with a Myc-tag at its C-terminus was transfected to C2 myoblasts and 10T1/2 fibroblasts. Staining of the cells with the mAb, Myc1-9E10, recognizing the Myc-tag located the exogenously expressed meltrin α to a cytoplasmic network structure in both types of cells (Fig. 5, a and d). Remarkably, the staining was absent on the cell surface. To identify the network structure, the transfected cells were doubly stained with the anti-MelCyt pAb and anti-KDEL mAb 10C3, which recognizes several ER resident proteins containing the ER-targeting KDEL motif (*37*). The localization of the exogenous meltrin α coincided with the anti-KDEL staining (Fig. 5, b and c; e and f). The transfected cells were also doubly stained with the anti-Myc mAb and tetramethylrhodamine-conjugated wheat germ agglutinin (WGA), which recognizes the distal face of the Golgi apparatus (*38*). The WGA staining did not coexist with the anti-Myc stain-

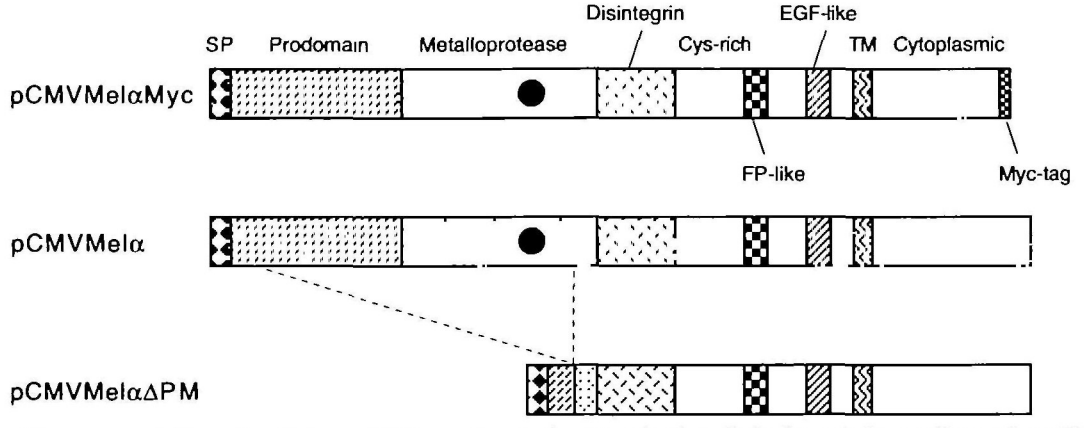


Fig. 4. **Schematic representation of meltrin α cDNA constructs for transfection.** Only the meltrin α-coding region of the cDNA and Myc-tag are shown, and the untranslated regions or the plasmids are omitted. SP, signal peptide; FP-like, fusion peptide-like sequence; TM, transmembrane domain. The solid circle in the metalloprotease domain represents the catalytic site.

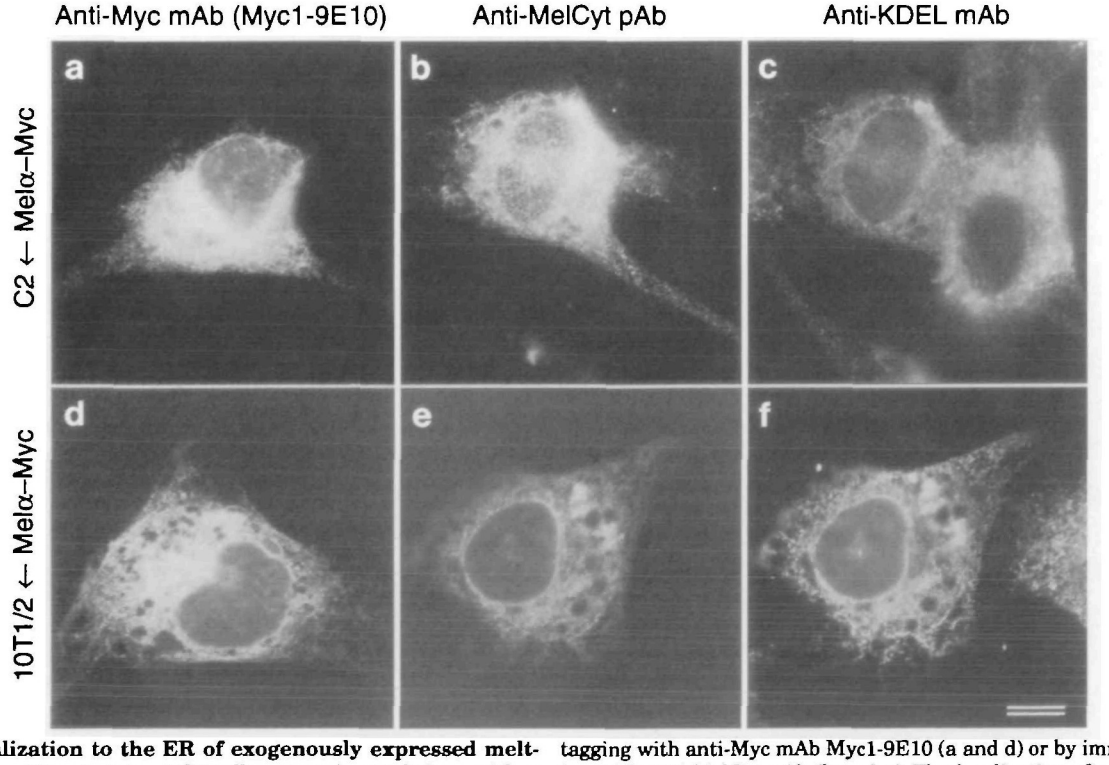


Fig. 5. **Localization to the ER of exogenously expressed meltrin α with a Myc-tag.** (a–c) C2 cells expressing meltrin α with a Myc-tag at its C-terminus (meltrin α–Myc). (d–f) 10T1/2 cells expressing meltrin α–Myc. The exogenous protein was detected by epitope-tagging with anti-Myc mAb Myc1-9E10 (a and d) or by immunostaining with anti-MelCyt pAb (b and e). The localization of meltrin α (b and e) was compared with that of the ER detected by the staining with anti-KDEL mAb 10C3 (c and f). Bar, 10 μm.

ing (data not shown). Consequently, the network structure in which Myc-tagged meltrin α was located represents the ER but not the Golgi apparatus.

To determine whether or not the Myc-tag disturbed the inherent localization of meltrin α, pCMVMelα expressing meltrin α without any tag was transfected to C2 and 10T1/2 cells, and then the cells were doubly stained with the anti-MelCyt pAb and anti-KDEL. The exogenous meltrin α was also located to the ER in both C2 and 10T1/2 cells maintained in the growth medium (Fig. 6, a and b; e and f). Thus, the C-terminal Myc-tag did not affect the localization of exogenously expressed meltrin α, but rather the exogenous meltrin α was associated with the ER regardless of the presence of the C-terminal tag. If meltrin α is involved in skeletal muscle cell fusion or any other functions in myogenic differentiation, the protein might be translocated from the ER to the plasma membrane under differentiation conditions. To assess this possibility, transfected C2 cells were shifted to the differentiation medium and maintained for 48 h. However, the exogenously expressed meltrin α remained associated with the ER (Fig. 6, c and d). This was also the case for 10T1/2 cells (data not shown). Accordingly, the localization to the ER of the exogenous meltrin α is not affected by the differentiation conditions.

The previous study showed that transfection of a truncated meltrin α cDNA lacking the prodomain and metalloprotease domain to C2 cells induced disorganized cell aggregation and facilitated cell fusion (*17*). On the other hand, transfection of the full-length meltrin α cDNA suppressed cell fusion (*17*). One possible explanation for this is that the truncated form but not the full-length protein or its physiologically processed form is translocated to where it acts and becomes functional. According to this postulation, we transfected C2 and 10T1/2 cells with pCMVMelαΔPM containing the truncated meltrin α cDNA lacking the prodomain and metalloprotease domain (Fig. 4). Immunostaining with the antibodies also located the truncated protein to the ER (Fig. 6, g and h). This finding does

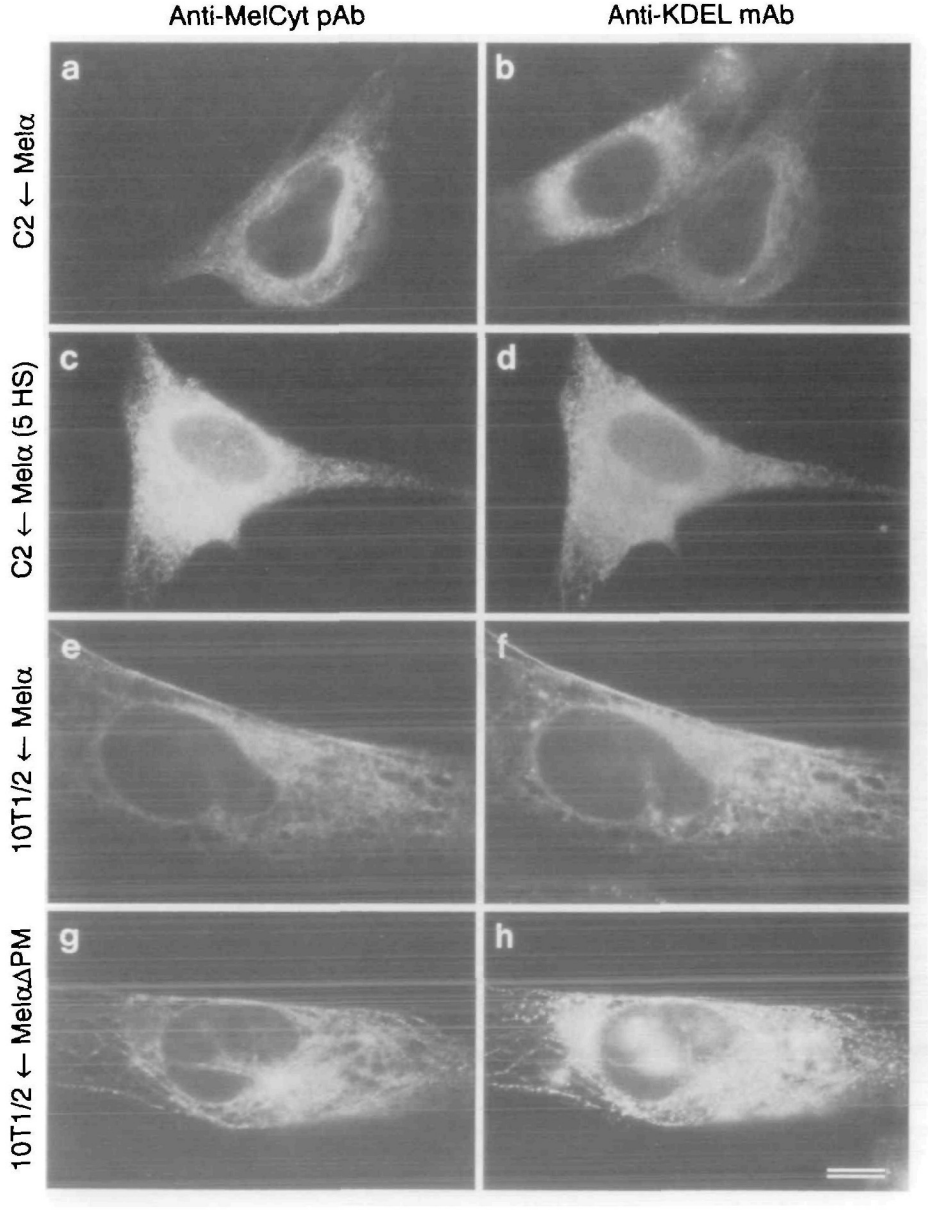


Fig. 6. **Localization to the ER of exogenously expressed full-length and truncated meltrin α without an epitope tag.** (a and b) C2 cells expressing full-length meltrin α cultured in the growth medium. (c and d) C2 cells expressing full-length meltrin α cultured for 48 h in the differentiation medium. (e and f) 10T1/2 cells expressing full-length meltrin α. (g and h) 10T1/2 cells expressing meltrin α lacking the prodomain and metalloprotease domain (MelαΔPM). The localization of meltrin α detected with the anti-MelCyt pAb (a, c, e, and g) was compared with that of the ER detected with the anti-KDEL mAb (b, d, f, and h). Bar, 10 μm.

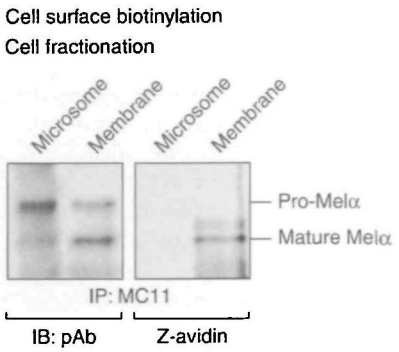


Fig. 7. **Association of endogenous processed meltrin α with the plasma membrane.** Cell surface biotinylated C2 myoblasts were fractionated into microsomes and plasma membrane (32/40% interface of the sucrose density gradient). Each fraction was immunoprecipitated with MC11 and then immunoblotted with the anti-MelCyt pAb (left panel). Biotinylated cell surface proteins were detected with HRP-conjugated Z-avidin (right panel). Only mature meltrin α in the plasma membrane fraction was labeled by cell surface biotinylation.

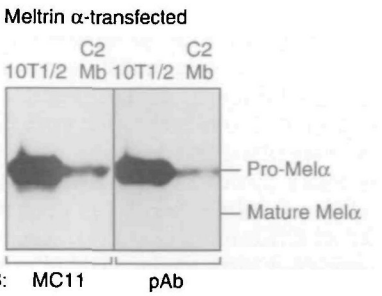


Fig. 8. **Lack of processing in exogenously expressed meltrin α.** Lysates of meltrin α-transfected 10T1/2 and C2 cells were subjected to immunoblotting with MC11 and with the anti-MelCyt pAb. Only the unprocessed pro-meltrin α (115 kDa) band was detected, and the mature meltrin α (86 kDa) band was undetectable. The difference in transfection efficiency between 10T1/2 and C2 cells was reflected on the difference in the strength of the bands between these cells.

not argue for the above postulation. Thus, it remains to be determined how the truncated form but not the full-length protein induces myogenic cell aggregation and fusion.

Lack of Processing of Exogenously Expressed Meltrin α— Because immunofluorescent staining was not suitable for determining the subcellular localization of endogenous meltin α, we applied cell surface biotinylation and cell fractionation for this purpose. Microsome and plasma membrane fractions of C2 myoblasts were subjected to immunoprecipitation and immunoblotting with the antibodies to MelCyt. The microsome fraction contained both pro-meltrin α and its mature form, and the former was more abundant than the latter (Fig. 7, left), as in whole cell lysates (see Fig. 3). In contrast, the mature protein was predominant in the plasma membrane fraction (Fig. 7, left). The mature form but not the unprocessed protein in the plasma membrane fraction was labeled by cell surface biotinylation (Fig. 7, right). These results imply that the processing occurs before the protein reaches the plasma membrane and that only the processed form is associated with the plasma membrane.

We next examined whether exogenously expressed meltrin α, which was exclusively present in the ER, was processed. C2 myoblasts and 10T1/2 cells transfected with pCMVMelα were analyzed by immunoblotting with MC11 and the pAb. Only unprocessed meltrin α was detected, *i.e.* the processed protein was undetectable in both cell lysates (Fig. 8). Together, these results indicate that endogenous meltrin α is proteolytically processed into the mature active form, whereas the exogenously expressed protein is retained in the ER and remains in an unprocessed inactive form.

DISCUSSION

As meltrin α was originally cloned from skeletal myocytes and has been postulated to be involved in skeletal muscle cell aggregation or fusion, its inducible expression during myogenesis was suggested (*17*). But we showed here that meltrin α was constitutively expressed in C2 cells. The mRNA and protein were already present in proliferating myoblasts and persisted throughout differentiation, and thus its expression was not coordinated with that of myogenin or certain muscle-specific genes. Considering the reports that meltrin α is expressed in a variety of cells and tissues (*29–32*), and our finding that 10T1/2 fibroblasts also contain meltrin α mRNA and protein, the constitutive expression of meltrin α in C2 cells seems to be plausible. Thus, meltrin α is likely to play general roles in these cells and tissues in addition to its specific roles in myogenesis. Expression of meltrin α in myoblasts does not necessarily argue against the possibility that meltrin α participates in myogenesis, because the cellular functions of meltrin α could be regulated through posttranslational modifications such as phosphorylation or by translocation. The reports that the MDC9 cytoplasmic domain binds Src and PKCδ (*10*, *39*) may suggest the involvement of phosphorylation in the regulation of some ADAM family proteins. The binding of Src is postulated to be mediated by the proline-rich Src-homology 3 (SH3)-binding motifs in MDC9 (*39*). Since the meltrin α cytoplasmic domain also contains several SH3-binding motifs and a consensus sequence for phosphorylation by PKC, its biological roles might be modulated by phosphorylation.

Although most ADAM proteins have been believed to be located on the plasma membrane, endogenous ADAM17 (*36*), and exogenously expressed ADAM13 (*34*) and MDC15 (*35*) are tentatively thought to be located to cytoplasmic perinuclear compartments. To determine the location of meltrin α, we tried to detect endogenous meltrin α by immunostaining with the anti-MelCyt pAb and mAbs. However, none of these antibodies detected the endogenous protein, possibly due to either its low level of expression or its conformation or location, to which the antibodies have no access. Thus, we determined the location of meltrin α exogenously expressed by transfection. The protein was located to the ER but not to the plasma membrane regardless of whether or not the epitope tag was added. The exogenous pro-meltrin α was not processed. On the other hand, endogenous meltrin α that had been processed into the mature form was predominantly associated with the plasma membrane and also in part with microsomes, as shown by cell surface biotinylation and cell fractionation.

Taken together, these results indicate that the endogenous meltrin α eventually becomes associated with the plasma membrane, whereas the exogenously expressed protein is neither translocated from the ER nor processed.

The ER is a quality control station preventing proteins that have not been folded correctly or have not gained their mature quaternary structure from targeting the cell surface (*40–42*). Since fertilin α and β are both present on the sperm surface as a heterodimer (*11*), endogenous meltrin α is possibly associated with another ADAM protein. The heterodimerization of this ADAM protein and meltrin α might be required for trafficking from the ER to a *bona fide* destination. If meltrin α is overexpressed by transfection, most of the protein will remain alone due to a limited number of the heterodimeric partner protein molecules. This would explain the unexpected ER residency of transfected meltrin α.

Human meltrin α has been shown to become an active metalloprotease through cleavage of its prodomain by furin or a furin-like endopeptidase (*43*), which serves as a proprotein convertase. Not only meltrin α but also other ADAM proteins, including MDC9 and MDC15, contain potential furin cleavage sites (R-X-K/R-R) between their prodomains and metalloprotease domains (*35*, *43*, *44*). Their prodomains are likely to maintain the enzymes in a latent state *via* the cysteine switch mechanism (*43–45*), in which coordination of Zn^{2+} in the active site of the metalloprotease catalytic domain by a cysteine residue in the prodomain is critical for inhibition of the protease. In this situation, the unprocessed ADAM proteins are held as inactive metalloproteases. Furin is concentrated and exerts its endopeptidase function in the *trans*-Golgi network (*46*, *47*). Thus, meltrin α retained in the ER cannot be processed by furin and consequently remain inactive. This accounts for the finding that the exogenous meltrin α is not processed. The exogenous protein will therefore be unable to exert its physiological functions. Conversely, unprocessed endogenous meltrin α, at least in part, possibly resides in the ER.

Although the low serum differentiation conditions did not induce translocation of the exogenous meltrin α, we cannot rule out the possibility that some conditions or stimulation is responsible for the translocation. According to this possibility, the ER may serve as a reservoir of the latent form of meltrin α. This may represent one of the regulatory mechanisms for its activity. We need to address the possibility that the translocation as well as the cellular activity after the processing is induced by phosphorylation by Src family protein tyrosine kinases or PKC, as discussed above. In this context, it is worth noting that certain Src family kinases and PKC isozymes are also associated with the ER (*48–50*).

The previous study showed that overexpression of full-length meltrin α suppressed cell fusion in C2 cells, whereas the truncated protein lacking the prodomain and metalloprotease domain (MelαΔPM) facilitated cell fusion (*17*). In addition, it has been suggested that a secreted form of the human meltrin α (ADAM12-S) minigene product lacking these domains is involved in myogenesis (*29*). But we showed here that overexpressed MelαΔPM as well as the full-length protein were retained in the ER. Mutations in the metalloprotease domain result in dominant negative effects on the metalloprotease activity of MDC9 (*10*). Furthermore, because a furin cleavage site is not present except for between the prodomain and metalloprotease domain, a processed form corresponding to the MelαΔPM or ADAM12-S minigene product may not be physiologically generated. Thus, the mechanism by which these truncated proteins but not the full-length protein facilitate myogenesis remains to be elucidated. Identification of proteins involved in the signal transduction pathways leading to cell–cell interaction, cell fusion, or the other functions of meltrin α may be required to answer this question.

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