Intracellular Processing of Metalloprotease Disintegrin ADAM12*

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ADAM12 has been implicated in cell-cell interactions in myogenesis and cancer, but the structure of the mature form of ADAM12 is not known, and its localization on the cell surface has been questioned. In this report, we show that full-length ADAM12 is N-glycosylated in the endoplasmic reticulum (ER) and proteolytically processed in the trans-Golgi network to an ~90-kDa form. The ~90-kDa form, which lacks the prodomain, was the predominant form present at the cell surface. Replacement of Leu83 in the putative α-helical region in the prodomain with proline resulted in retention of ADAM12 in the ER and a complete lack of its processing. However, deletion of the entire pro- and metalloprotease domains did not affect the processing and trafficking of ADAM12. In contrast, replacement of the cytoplasmic domain of ADAM12 with that of ADAM9 or adding a c-Myc tag at the C terminus led to a significant increase in transport of the protein to the cell surface. These results suggest that the cytoplasmic domain of ADAM12 plays an important role in regulating ADAM12 exit from the ER. We conclude that properly folded mouse ADAM12, after passing a rate-limiting step of exit from the ER, is processed in the secretory pathway and reaches the cell surface, where it can mediate adhesion-mediated signaling.

ADAMs, a family of proteins containing a disintegrin and metalloprotease domain, play important roles in many biological processes involving cell-surface proteolysis and cell-cell or cell-matrix interactions (1–3). ADAMs have been implicated in many vital functions during development (4–7) and in the pathogenesis of cancer (8–10), rheumatoid arthritis (11), Alzheimer’s disease (12), and inflammatory responses (9). An exception of the ADAMTS subfamily (ADAMs with thrombospondin motifs) and alternative splice variants of several family members, ADAMs are type I transmembrane proteins expressed in all animal organisms from worm to human. A typical ADAM protein contains an N-terminal secretion signal and pro-, metalloprotease, disintegrin-like, cysteine-rich, epidermal growth factor-like, transmembrane, and cytoplasmic domains.

The metalloprotease domains of many, but not all, ADAMs contain a consensus sequence (HEXXHXXGXXH) for the active site of zinc-dependent metalloproteases (13). These ADAMs are predicted to be active proteases involved in shedding of the ectodomains of membrane proteins, which is critical for cell-surface remodeling, regulating growth factor availability, and modulating the capacity of cells to respond to extracellular stimuli (14, 15). At least six members of the ADAM family have been demonstrated to have proteolytic activity. ADAM17 (tumor necrosis factor-α-converting enzyme) releases soluble tumor necrosis factor-α from its membrane precursor (16, 17). It also cleaves the tumor necrosis factor-α receptor, transforming growth factor-α, and L-selectin (6); interleukin-1 receptor type II (18); amyloid precursor protein (12); ErbB4/HER4, a member of the epidermal growth factor receptor family (19); and Notch (20). ADAM10/Drosophila Kuzbanian is responsible for proteolytic cleavage and activation of Delta, a ligand of the Notch receptor (7); ephrin A2 (21); and amyloid precursor protein (22). ADAM9 has been implicated in shedding of the membrane-anchored heparin-binding epidermal growth factor-like growth factor (23). Members of the ADAMTS subfamily of ADAM proteins degrade the extracellular matrix proteins aggrecan (ADAMTS-4 and ADAMTS-5) and procollagen (ADAMTS-2) (11, 24). ADAM19 participates in the cleavage of membrane-anchored neuregulin (25). Finally, ADAM12 cleaves insulin-like growth factor-binding protein-3 (26) and -5 (27) and the heparin-binding epidermal growth factor-like growth factor (28).

ADAM12 has been previously implicated in differentiation of mesenchymal cells such as skeletal myoblasts (29–32) and osteoblasts (33). In mouse embryo, ADAM12 expression is temporally and spatially restricted, and it is the most prominent in the regions of skeletal muscle and bone formation (30). In adult skeletal muscle, the expression level of ADAM12 is very low in both differentiated muscle fibers and quiescent satellite cells (29, 31, 32). ADAM12 expression is, however, dramatically up-regulated in regenerating muscle, where ADAM12 is readily detected in activated satellite cells undergoing differentiation and fusion (32) and in newly formed muscle fibers (34). In addition, a strong up-regulation of ADAM12 expression has been observed in human carcinomas, raising a possibility that ADAM12 may play a role in cell-cell and/or cell-matrix interactions in cancer (35).

Proteolytic processing is an inherent element of maturation of ADAM proteins in mammalian cells. Most notably, many ADAMs are cleaved between the pro- and metalloprotease domains as they progress through the secretory pathway. Because the presence of the prodomain in an unprocessed ADAM protein inhibits the catalytic activity of the metalloprotease domain via a cysteine-switch mechanism (36), the cleavage of ADAMs at the boundary between the pro- and metalloprotease domains, followed by the dissociation of the free prodomain, constitutes an important step in the activation of the metalloprotease. Proteolytic removal of the prodomain has been best studied for ADAM15 (37), ADAM9 (38), ADAM17 (39), and ADAM28 (40), but it has been also observed for ADAM10 (22) and ADAM19 (25). In addition, ADAM15 (37) and ADAM2 (41) have been reported to undergo further processing, resulting in...
mature forms in which the entire metalloprotease or a large part of it was missing.

Proteolytic processing seems to play a particularly important role in the regulation of ADAM12 function. The extent of the processing and the intracellular localization of the mature form of ADAM12 are, however, unclear. According to the original report, both the pro- and metalloprotease domains of ADAM12 need to be removed in order for ADAM12 to stimulate myoblast fusion (29). Overexpression of ADAM12 lacking its pro- and metalloprotease domains in the mouse myoblastic cell line C2C12 leads to accelerated cell differentiation and fusion, whereas transfection of C2C12 cells with full-length ADAM12 results in inhibition of fusion (29). However, when expressed as a green fluorescent protein (GFP) fusion protein in COS-7 cells, full-length human ADAM12 is cleaved predominantly between the pro- and metalloprotease domains (42), suggesting that mature ADAM12 contains an intact metalloprotease domain. Surprisingly, the processed form of ADAM12-GFP is retained in the trans-Golgi network (TGN), with very little protein present at the cell surface (42). When overexpressed in C2C12 myoblasts or 10T1/2 fibroblasts, mouse ADAM12 does not undergo any proteolytic processing and localizes exclusively in the endoplasmic reticulum (ER) (43). In clear contrast, a mature form of ADAM12 is expressed at the cell surface of Chinese hamster ovary cells transfected with full-length mouse ADAM12, where it supports the interactions with integrin α9-expressing cells (44).

In this report, we have investigated the intracellular processing and localization of ADAM12 in mouse C2C12 myoblasts and COS-7 cells. We have found that full-length ADAM12 was processed in both cell lines to an ~90-kDa form. The 90-kDa form, which lacks the prodomain, was the predominant form present at the cell surface. We also found that the processing of endogenous ADAM12 in C2C12 cells was similar to the processing of overexpressed ADAM12. Immunofluorescence analysis of transfected cells using anti-ADAM12 antibody further demonstrated that ADAM12 was present both at the cell surface and in the intracellular compartments of the secretory pathway and that the cytoplasmic domain of ADAM12 was a limiting factor for the export of ADAM12 from the ER. In contrast, replacement of a leucine residue (Leu77) in the putative α-helical region in the prodomain with a helix-breaking proline resulted in retention of the ADAM12 mutant in the ER and in a complete lack of its proteolytic processing. We conclude that properly folded mouse ADAM12 is targeted to the plasma membrane, where it can mediate cell-cell adhesion or adhesion-mediated signaling.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit anti-ADAM12 and anti-ADAM9 antibodies were as described previously (45). Mouse anti-KDEL monoclonal antibody was obtained from Stresgen Biotech Corp. (Victoria, British Columbia, Canada). Mouse anti-TGN38 monoclonal antibody was from BD Transduction Laboratories (Lexington, KY). Rabbit anti-Myc tag polyclonal antibody was from Medical and Biological Laboratories (Watertown, MA). Mouse anti-penta-histidine monoclonal antibody against five consecutive histidine residues was from Qiagen Inc. (Valencia, CA).

Mammalian Expression Constructs—A schematic representation of the expression constructs used in this study is shown in Fig. 1A. The expression of full-length mouse ADAM12 (amino acids 1–903), a truncated form of ADAM12 extending from the disintegrin to the cytoplasmic domain (amino acids 425–903, ADAM12(425–903), ADAM12(425–903)) and a similarly truncated form of ADAM9 (amino acids 417–845, ADAM9(417–845)) was carried out as described (45). The mutation L73P in the prodomain of ADAM12 has been identified to be responsible for the lack of proteolytic processing of ADAM12, as observed in our previous work (45–47). ADAM12(1–424,ΔC) (amino acids 425–845) contains a 58-amino acid truncation at the C terminus in addition to the N-terminal truncation (Fig. 1B). In the ADAM12(1–424)-Myc or -His and ADAM12(1–424,-C)-Myc or -His constructs, a Myc or His tag was introduced immediately after the proteolytic processing site to facilitate the separation of extracellular and transmembrane domains of ADAM12(1–414) (amino acids 425–727) and the cytoplasmic tail of ADAM9 (amino acids 719–845). ADAM9/12 contains the extracellular and transmembrane domains of ADAM9(1–416) (amino acids 417–718) and the cytoplasmic tail of ADAM12 (amino acids 728–903). The N-terminally truncated ADAM12 constructs were ligated to the GFP signal sequence and inserted into the pIRESPuro vector (CLONTECH, Palo Alto, CA) at the ClaI/NcoI sites. The identities of all the constructs were verified by DNA sequencing (Iowa State University, Ames, IA).

Cell Culture, Cell Transfections, and Immunoblotting—C2C12 and COS-7 cells were cultured in growth medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum) in the presence of 5% CO2 at 37 °C. LeVo cells were cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1.5 g/liter sodium bicarbonate. Transient transfections were performed with LipofectAMINE Plus (Invitrogen) according to the manufacturer’s instructions. One or two days after transfection, cellular proteins were extracted with buffer A (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM 4-(2-aminoethyl)benzyl fluoride hydrochloride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM 1,10-phenanthroline; 2 ml/100-mm plate). Samples were centrifuged for 30 min at 21,000 × g, and supernatants were either directly analyzed by Western blotting or enriched for glycoproteins prior to SDS-PAGE and immunoblotting. To enrich samples for glycoproteins, cell extracts (2 ml) were incubated with concanavalin A-agarose (20-μl bed volume) for 2 h at 4 °C, followed by washing the beads with extraction buffer and elution with 30 μl of SDS-PAGE sample buffer. The eluates were subjected to SDS-PAGE and Western blotting with either anti-ADAM12 antibody (0.3 μg/ml) or anti-ADAM9 antibody (1 μg/ml), followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody and detection with the SuperSignal West Pico kit (Pierce).

Pulse-Chase Labeling—COS-7 cells were transfected with plasmids encoding mouse ADAM12 or control vectors. One day after transfection, cells were preincubated for 1 h in methionine/cysteine-free growth medium. Then, new medium containing 200 μCi/ml Expre35S/32P protein labeling mixture (PerkinElmer Life Sciences) was added, and cells were washed for 20 min. After removing the labeling medium, cells were maintained in growth medium for various times and subjected to extraction and immunoprecipitation with anti-ADAM12 antibody. The immune complexes were analyzed by SDS-PAGE and autoradiography. In some experiments, brefeldin A (BFA; Calbiochem) was applied at a concentration of 20 μg/ml through the pulse and chase periods.

Pepsin N-Glycosidase F and Endo H Digestion—Wild-type and mutant forms of ADAM12 were purified by affinity chromatography based on the established interaction between the cytoplasmic tail of ADAM12 and the SH3 domain from the phosphotyrosinolysis 3-kinase p85α subunit (47). The cell extract (4 ml) from transfected COS-7 cells was passed through a glutathione-Sepharose column (0.2-ml bed volume) containing immobilized glutathione S-transferase-p85α SH3 domain fusion protein (0.4 mg). For peptide N-glycosidase F treatment, the column eluate (0.5 ml) was dialyzed against 50 mM Tris-HCl and 50 mM EDTA (pH 7.5). For Endo H treatment, dialysis was carried out against 50 mM sodium citrate (pH 5.5). The samples after dialysis were supplemented with 0.1% (w/v) SDS and 0.5% (v/v) β-mercaptoethanol. After boiling, octyl glucopyranoside was added to a final concentration of 0.5% (w/v), and the samples (0.2 ml) were incubated for 24 h at 37 °C with or without 5 μg of peptide N-glycosidase F (Glyco, Novato, CA) or 10 μl of Endo H (Roche Molecular Biochemicals). Glycosidase digestion products were analyzed by immunoblotting with anti-ADAM12 antibody.

Cell-surface Biotinylation—One day after transfection, COS-7 cells were washed with Dulbecco’s phosphate-buffered saline and incubated in room temperature with 0.5 mg/ml N-hydroxysuccinimideactivated imidobiotin (Pierce). After washing with growth medium and Dulbecco’s phosphate-buffered saline, surface-labeled cells were extracted with buffer A, followed by immunoprecipitation with anti-ADAM12 antibody. The immunoprecipitates were resolved by SDS-PAGE on an 8% polyacrylamide gel, transferred to a nitrocellulose membrane, probed with horseradish peroxidase-conjugated streptavidin, and visualized with the SuperSignal West Pico kit.
Establishment of ADAM12 Antisense mRNA Clones—The sequence complementary to nucleotides 2937–2966 in the 3′ untranslated region of mouse ADAM12 mRNA was cloned into the pZeoU1 expression vector (a gift from Dr. H. Dietz, Johns Hopkins University) (48), which incorporates three critical features: (i) it uses U1 small nuclear RNA as a vehicle for presentation of the antisense targeting sequence; (ii) it includes an autocatalytic ribozyme that cleaves its target in a site-specific manner; and (iii) due to the 5′-cap hypermethylation and lack of polyadenylation of the transcript, it allows the antisense mRNA to be enriched within the nuclear compartment, where the target mRNA can be rapidly eliminated. C2C12 cells were transfected with the pZeoU1ADAM12 vector; individual clones of stable transfectants were established by growing cells in the presence of Zeocin, an antibiotic selection marker. ADAM12 expression was examined in 12 different clones by subjecting the glycoprotein-enriched fractions of cellular proteins to Western blotting with anti-ADAM12 antibodies. ConcanaValin A chromatography, which produced an ∼100-fold enrichment of the samples in cellular glycoproteins prior to immunoblotting, was critical in this experiment, as the antibody did not detect endogenous ADAM12 in total cell lysates.

Immunofluorescence Microscopy—C2C12 or COS-7 cells were plated on glass coverslips and transfected with expression vectors. Two days after transfection, cells were fixed with 3.7% paraformaldehyde in Dulbecco’s phosphate-buffered saline and permeabilized with 0.1% Triton X-100 in Dulbecco’s phosphate-buffered saline. For the studies on the immunolocalization of ADAM12, ADAM12(Δ1–424), and ADAM9/12, C2C12 or COS-7 cells were incubated for 1 h with anti-ADAM12 antibody (1:500 dilution) and anti-KDEL antibody (1:200 dilution) or anti-TGN38 antibody (1:50 dilution) and then for 30 min with rhodamine-conjugated anti-rabbit IgG antibody and fluorescein isothiocyanate-conjugated anti-mouse IgG antibody. For visualization of Myc- and His6-tagged ADAM12, transfected C2C12 cells were co-stained with anti-Myc and anti-pentahistidine antibodies, followed by incubation with rhodamine-conjugated anti-rabbit IgG antibody and fluorescein isothiocyanate-conjugated anti-mouse IgG antibody. The coverslips were washed extensively, mounted onto slides with 10% (w/v) Mowiol 4-88 (Calbiochem) in 25% glycerol, and viewed on a Zeiss laser scanning confocal microscope.

RESULTS
ADAM12 contains the sequence RHKR at the boundary between the prodomain and the metalloprotease, which corresponds to the consensus cleavage site (R/K)R of furin, a member of the subtilisin-like family of endoproteases (49). The subtilisin-like proteases are ubiquitously expressed, are localized predominantly in the TGN or secretory granules, and function as mammalian proprotein convertases (49, 50). To examine whether ADAM12, similarly to several other ADAM proteins, undergoes a proteolytic cleavage between the pro- and metalloprotease domains, C2C12 myoblasts or COS-7 cells were transfected with an expression vector encoding full-length ADAM12. Analysis of total cell lysates (Fig. 2A) or cellular glycoproteins (Fig. 2B) by Western blotting with an antibody raised against a peptide from the cytoplasmic domain of ADAM12 revealed the presence of two major forms of ADAM12 of ∼120 and ∼90 kDa, respectively. (Several protein bands observed on immunoblots of total cell lysates were absent in the eluates from the concanaValin A column; and therefore, they represented nonspecific antibody binding rather than endogenous ADAM12.) The ∼120-kDa form corresponded to full-length ADAM12. The ∼90-kDa form was larger than ADAM12(Δ1–424), i.e. ADAM12 lacking the pro- and metalloprotease domains and containing an exogenous secretion signal.
Processing of ADAM12

Fig. 2. Proteolytic processing of full-length ADAM12. C2C12 myoblasts or COS-7 cells were transfected with an expression vector encoding full-length mouse ADAM12 (lanes 1), the L73P mutant of ADAM12 (lanes 2), ADAM12Δ(Δ1–424) (without the pro- and metalloprotease domains and with the Ig secretory signal; lanes 3) or with vector only (lanes 4). A, 48 h after transfection, total cell lysates from transfected C2C12 cells were subjected to SDS-PAGE and Western blotting with an antibody raised against the cytoplasmic tail of ADAM12. B, cell extracts from transfected C2C12 (left panel) or COS-7 (right panel) cells were loaded onto concanavalin A-agarose columns, and the column eluates were analyzed by Western blotting using the same antibody as described for A. Arrows indicate the positions of full-length (FL) ADAM12, a proteolytically processed form of ADAM12, and ADAM12Δ(Δ1–424). Molecular mass markers (in kilodaltons) are shown on the left.

(Fig. 2, lanes 3; see also Fig. 1A), indicating that the ~90-kDa form most likely represented ADAM12 without the prodomain. (The predicted molecular mass of the prodomain is ~20 kDa, and two potential N-linked glycosylation sites are present.)

Remarkably, substitution of Leu73 in the prodomain with proline resulted in complete inhibition of ADAM12 processing, as only the 120-kDa form was detected both in C2C12 and COS-7 cells transfected with the ADAM12(L73P) construct (Fig. 2, lanes 2). Leu73 resides in a region in the prodomain that is predicted to form an α-helix (amino acids 68–89). Analysis of the ADAM12 amino acid sequence with a secondary structure prediction software (GOR IV)2 indicated that the probability of Leu73 to participate in an α-helix was ~70%. Substitution of Leu73 with a helix-breaking proline decreased this probability to ~30%; and therefore, it might have affected the proper folding of ADAM12. Because misfolded proteins are routinely retained in the ER and later degraded (51–53), the L73P mutation of ADAM12 most likely prevented the export of ADAM12 from the ER and its further processing in post-ER compartments (see also Figs. 4, 7, and 8).

To evaluate the proteolytic processing of mouse ADAM12 in the secretory pathway, ADAM12-transfected COS-7 cells were pulse-labeled with [35S]methionine/cysteine, followed by incubation for various periods of time in 35S-free medium. Cell lysates from 35S-labeled cells were then subjected to immunoprecipitation with anti-ADAM12 antibody, SDS-PAGE, and autoradiography. As shown in Fig. 3A (lanes 2 and 3), within 1 h after metabolic labeling, only the ~120-kDa form of ADAM12 was immunoprecipitated. The ~90-kDa form was detected after a 3-h chase period (lane 4). With the increasing chase time, the amount of 35S-labeled ADAM12 protein in the immunoprecipitate gradually decreased (lanes 5 and 6). These results suggested that the ~90-kDa form of ADAM12 was generated from the ~120-kDa form of ADAM12 and that the proteolytic event occurred ~3 h after the initiation of ADAM12 synthesis.

To identify the intracellular compartment where ADAM12 was processed, COS-7 cells were transfected with a vector encoding ADAM12, pulse-labeled with [35S]methionine/cysteine, and incubated in the presence of BFA. BFA inhibits transport through the secretory pathway by redistributing proteins from the cis-, medial-, and trans-Golgi cisternae, but not from the trans-Golgi network, into the ER (54). Fig. 3B demonstrates that BFA treatment resulted in the immunoprecipitation of only unprocessed ADAM12 after a 3-h chase (lane 3), whereas without BFA, the processed form could be easily detected (lane 2). These results suggested that the proteolytic processing of ADAM12 occurred in the trans-Golgi network. Because furin is a ubiquitous proprotein convertase that resides in the trans-Golgi network, it has been postulated that it might be directly involved in the proteolytic processing of ADAM12. However, we have observed that expression of ADAM12 in LoVo cells, a furin-deficient cell line, resulted in a similar processing of ADAM12 as in C2C12 and COS-7 cells (data not shown), which strongly suggested that furin was not responsible for the cleavage of ADAM12.

To further define the subcellular localization of individual proteolytic forms of ADAM12 and ADAM12 mutants, we assessed their sensitivity to two glycosidases: peptide N-glycosidase F and Endo H. The sensitivity to peptide N-glycosidase F, an enzyme that removes all types of N-linked oligosaccharides

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2 Available at abs.cit.nih.gov.
from glycoproteins, is an indication of N-linked protein glycosylation. Endo H selectively removes high mannose-type oligosaccharides from glycoproteins, but it does not cleave complex glycans. Because the conversion of high mannose oligosaccharides into complex glycans occurs in the medial-Golgi compartment, resistance to Endo H is an indication that a glycoprotein has reached this compartment (55). First, wild-type or mutant ADAM12 was purified from transfected COS-7 cells by affinity chromatography based on the established interaction between the ADAM12 cytoplasmic tail and the phosphatidylinositol 3-kinase subunit of phosphatidylinositol 3-kinase (47). Column eluates without any further treatment (lanes 1, 4, and 7) or treated with Endo H (lanes 2, 5, and 8) or peptide N-glycanase F (PNGase F, lanes 3, 6, and 9) were analyzed by SDS-PAGE and Western blotting with anti-ADAM12 antibody.

Deglycosylation of the recombinant forms of ADAM12. COS-7 cells were transfected with an expression vector encoding ADAM12 (lanes 1–3), ADAM12(L73P) (lanes 4–6), or ADAM12Δ1–424 (lanes 7–9). Forty-eight hours after transfection, recombinant forms of ADAM12 were partially purified by affinity chromatography on glutathione-Sepharose columns containing immobilized glutathione S-transferase that was fused to the SH3 domain of the p85α subunit of phosphatidylinositol 3-kinase (47). Column eluates without any further treatment (lanes 1, 4, and 7) or treated with Endo H (lanes 2, 5, and 8) or peptide N-glycanase F (PNGase F, lanes 3, 6, and 9) were analyzed by SDS-PAGE and Western blotting with anti-ADAM12 antibody.

To determine which form of ADAM12 resides at the cell surface, COS-7 cells transfected with an ADAM12-containing vector were incubated for 30 min with N-hydroxysulfosuccinimidobiotin, a membrane-impermeable biotinylation reagent. ADAM12 was then immunoprecipitated with anti-ADAM12 antibody, and the immunoprecipitates were resolved by SDS-PAGE and analyzed by blotting with horseradish peroxidase (HRP)-conjugated streptavidin. This result implied that the ~120-kDa form was located inside the cell and that the ~90-kDa form was predominantly located at the cell surface.

We next addressed the question of the intracellular processing of endogenous ADAM12 in C2C12 cells. To identify unambiguously endogenous ADAM12 by Western blotting, (i) we took advantage of the fact that ADAM12 binds to concanavalin A (Fig. 2), and (ii) we used C2C12 cells with decreased expression of endogenous ADAM12 using an antisense mRNA approach as negative controls. Concordavinal A chromatography, which allowed an ~100-fold enrichment of the samples in cellular glycoproteins prior to immunoblotting, was critical in this experiment, as the antibody did not detect endogenous ADAM12 in total cell lysates (Fig. 2). After purification on a concanavalin A column, we observed two distinct protein bands with mobilities similar to those observed in ADAM12-transfected cells (compare Fig. 6A with Fig. 2; notice that ~100-fold more cells were used for the experiment in Fig. 6A than for that in Fig. 2). The two proteins of ~120 and ~90 kDa were observed in normal C2C12 cells both before and after differentiation (lanes 1 and 2, respectively) and in cells stably transfected with a vector used for expression of ADAM12 antisense mRNA (lane 3). In contrast, the ~120- and ~90-kDa protein bands were below the detection limit in 10 out of 12 antisense clones examined, two of which are shown in lanes 4 and 5. Based on these observations, the ~120- and ~90-kDa proteins were attributed to endogenous ADAM12. Cell-surface biotinylation further documented that the 90-kDa form of endogenous ADAM12 was present at the cell surface and that the 120-kDa form was located intracellularly and was not available for biotinylation (Fig. 6B). Altogether, these results suggested that the proteolytic processing and transport to the cell surface of endogenous ADAM12 were similar to those observed for ectopically expressed ADAM12 (compared with Figs. 2 and 5).

To obtain a direct insight into the intracellular localization of ADAM12, we analyzed transfected COS-7 cells by immunofluorescence microscopy. As shown in Fig. 7 (A, D, J, and M), full-length ADAM12 and ADAM12Δ1–424 showed a similar cellular localization, suggesting that the removal of the pro-and metalloprotease domains of ADAM12 did not have a major effect on ADAM12Δ1–424 trafficking. Importantly, in addition to their location at the intracellular compartments, both ADAM12 and ADAM12Δ1–424 were clearly detected at the plasma membrane. Co-staining of the cells with anti-ADAM12 antibody and either a TGN-specific antibody (anti-TGN38) or an ER-specific antibody (anti-KDEL) further demonstrated their localization.
that ADAM12 and ADAM12(Δ1–424) inside COS-7 cells resided mostly in the ER (Fig. 7, A–C and J–L) and partially in the trans-Golgi network (D–F and M–O). In contrast, the L73P mutant was retained entirely in the ER, and no immunostaining was detected at the cell surface (Fig. 7, G–I). Interestingly, overexpression of all ADAM12 constructs in COS-7 cells typically led to a rearrangement of the ER network into an unusual tubular pattern (Fig. 7, B, H, and K). Although the molecular mechanism underlying the effect of ADAM12 on the structure of the ER is not clear, the results presented in Fig. 7 were consistent with those in Fig. 2 and showed that the lack of proteolytic processing of the L73P mutant, predicted to occur in the trans-Golgi network (Figs. 3 and 4), was a direct consequence of the retention of ADAM12(L73P) in the ER.

We next investigated the intracellular localization of the recombinant ADAM12 proteins in C2C12 cells, a cell line in which endogenous ADAM12 is expressed and in which it is assumed to play a physiological role during myogenesis (29, 31, 34). As shown in Fig. 8 (A–C and G–I), in C2C12 cells, as in COS-7 cells, ADAM12 and ADAM12(Δ1–424) were clearly detected in post-ER compartments and at the plasma membrane, but ADAM12(L73P) mutant staining was confined to the ER. All three ADAM12 constructs caused a rearrangement of the ER network, although this effect in C2C12 cells was less dramatic than in COS-7 cells. Together with the findings in Fig. 7, these data indicated that wild-type ADAM12 and the truncated ADAM12(Δ1–424) construct, when expressed in either a heterologous (COS-7) or a homologous (C2C12) system, passed through the secretory pathway to the plasma membrane. The misfolded ADAM12(L73P) mutant was, however, completely trapped in the ER.

It was previously shown that human ADAM12 fused to GFP is mainly retained in the trans-Golgi network and that the retention signal is located in the transmembrane and cytoplasmic domains (42). Moreover, deletion of the C-terminal 59 amino acids is sufficient for the release of ADAM12 from the trans-Golgi network to the cell surface (42). Because the cytoplasmic tails of human and mouse ADAM12 share a high degree of homology (Fig. 1B), we investigated whether the cytoplasmic domain of mouse ADAM12 also controls its transport to the plasma membrane.

We first examined the localization of ADAM9(Δ1–416), another member of the ADAM family that contains an N-terminal truncation of the pro- and metalloprotease domains equivalent to that present in ADAM12(Δ1–424). We then engineered two chimeric proteins (ADAM12/9 and ADAM9/12) in which the cytoplasmic and extracellular domains of ADAM12(Δ1–424) were replaced with the corresponding domains of ADAM9(Δ1–416). As shown in Fig. 9 (A and B), ADAM9(Δ1–416) and ADAM12/9 were localized predominantly in the post-ER compartments and the plasma membrane. By contrast, ADAM9/12, similarly to ADAM12(Δ1–424), was concentrated mostly in the ER, with a smaller fraction of the protein located at the plasma membrane. This suggested that the cytoplasmic domain of mouse ADAM12, similarly to that of human ADAM12, played an inhibitory role in the protein transport to the cell surface.
We next examined whether deletion of the C-terminal 58 amino acids of mouse ADAM12 (corresponding to the C-terminal 59 amino acid region of human ADAM12) (Fig. 1B) would facilitate its transport to the plasma membrane. C2C12 cells were cotransfected with two different forms of ADAM12: ADAM12-(Δ1–424) and ADAM12(Δ1–424,ΔC), i.e. ADAM12(Δ1–424) containing a C-terminal 58-amino acid truncation. Because the site recognized by our anti-ADAM12 antibody (amino acids 774–791) (45) is located upstream from the C-terminal truncation and is present in both ADAM12(Δ1–424) and ADAM12(Δ1–424,ΔC), to discriminate between the two forms of ADAM12, one construct contains a C-terminal His6 tag, and the other carries a C-terminal Myc tag. Two different proteins (with and without the C-terminal 58 amino acids) expressed in the same cell were visualized by co-staining transfected cells with anti-His and anti-Myc antibodies and analysis by confocal microscopy. To differentiate between the effects produced by the C-terminal truncation of ADAM12 and by the addition of the His6 or Myc tag, reciprocal experiments in which the tags were switched between the two constructs were included. As shown in Fig. 9 (D–I), the presence or absence of the intact C terminus of ADAM12 did not have any influence on the localization of the recombinant proteins. Regardless of the deletion of the C terminus, a large portion of both ADAM12(Δ1–424)-His (Fig. 9H) and ADAM12(Δ1–424,ΔC)-His (Fig. 9E) was localized in the ER. Surprisingly, the Myc-tagged proteins did not colocalize with the His-tagged proteins (Fig. 9, F and I). Both ADAM12(Δ1–424)-Myc and ADAM12(Δ1–424,ΔC)-Myc were essentially absent from the ER, and they were localized mostly at the cell surface (Fig. 9, D and G). The expression of all the proteins under investigation in Fig. 9 (A–I) was further verified by immunoblotting of the transfected cell lysates with anti-ADAM12 or anti-ADAM9 antibody (Fig. 9J). Taken together, our results suggested that the exit from the ER constituted a rate-limiting step for the transport of ADAM12 to the plasma membrane and that the cytoplasmic domain of ADAM12 did impede the export from the ER. However, the sequence responsible for delayed transport of mouse ADAM12 to the cell surface, unlike in its human counterpart, does not reside within the 58-amino acid C terminus of the protein.

DISCUSSION

In this report, we have shown that mouse metalloprotease disintegrin ADAM12 ectopically expressed in C2C12 or COS-7 cells or endogenous ADAM12 in C2C12 cells is subject to intracellular processing, giving rise to a major proteolytic form of ∼90 kDa. Because this form was detected with an antibody raised against a peptide located ∼12 kDa upstream from the C terminus of ADAM12, it might have represented ADAM12 containing truncations either at the N or C terminus. However, because the processed form was able to interact with the SH3 domain from the p85α subunit of phosphatidylinositol 3-kinase (Fig. 4) and because all SH3 domain-binding sites are located within the 12-kDa C-terminal region of ADAM12 (47), we conclude that the ∼90-kDa form represented ADAM12 with N-terminal rather than C-terminal truncations.

The ∼90-kDa form most likely corresponded to ADAM12 without the prodomain, and it represented catalytically active ADAM12 with a metalloprotease activity (56, 57). The time course of the appearance of the ∼90-kDa form in a pulse-chase labeling experiment and its inhibition by BFA as well as the resistance of the ∼90-kDa form to Endo H treatment suggested that this form was generated during the passage of the ADAM12 precursor through the trans-Golgi network. Our results further suggested that furin, a ubiquitous proprotein convertase that resides in the trans-Golgi network, was dispensable for the cleavage of ADAM12. The identity of the protease that is primarily responsible for the removal of the prodomain of ADAM12 is currently not known.

We have found that the L73P mutant form of ADAM12 was localized exclusively in the ER compartment (Figs. 4, 7, and 8) and that it was not proteolytically processed (Fig. 2). A lack of proteolytic processing was observed previously for an ADAM9 mutant in which a cysteine in the prodomain (critical for the regulation of metalloprotease activity by the cysteine-switch mechanism) was replaced with alanine (38) and for an ADAM19 mutant in which His346 and His350 (both essential for the metalloprotease activity) were substituted with alanines (25). Because misfolded proteins are routinely retained in the ER and later subjected to ER-associated degradation (51–53), these observations may simply reflect a fact that many different amino acid substitutions may interfere with the folding process of ADAMs. In fact, the L73P mutation in ADAM12 was predicted to inhibit the formation of a major α-helix in the prodomain and to have a substantial effect on ADAM12 folding, which indeed resulted in complete retention of ADAM12 in the ER and inhibition of its processing.

Our results may help to explain a controversy in the literature regarding the cellular localization and processing of ADAM12. Although endogenous ADAM12 has been reported to be proteolytically processed and it has been found mainly in the plasma membrane (29, 43), exogenous mouse ADAM12 has been claimed either to reside in the plasma membrane (44) or to be entirely retained in the ER, where it lacks any processing (43). In contrast, human ADAM12 expressed in COS-7 cells as a GFP fusion protein is confined exclusively to the trans-Golgi network and is subject to proteolytic processing (42). Based on the results obtained in this study, we argue that properly folded and GFP-free mouse ADAM12 is not restricted to the ER or Golgi network and that a sizable pool of the protein is present in the plasma membrane.

Studies of the intracellular trafficking of human ADAM12-GFP fusion protein have recently suggested that the C-terminal 59-amino acid region of human ADAM12 (corresponding to the C-terminal 58-amino acid region of mouse ADAM12) may be directly responsible for the retention of the recombinant protein within COS-7 cells and the lack of its cell-surface lo-
This region of ADAM2, which is highly conserved between human and mouse proteins (Fig. 1B) and contains, in addition to the SH3 domain-binding motifs, a major binding site for α-actinin-1, may be involved in the interaction between ADAM2 and the actin cytoskeleton (46). As shown in this work, replacement of the entire cytoplasmic domain of ADAM2 (176 amino acids) with the cytoplasmic domain of ADAM9 resulted in enhanced transport of the chimeric protein to the cell surface (Fig. 9), which was consistent with the postulated role of the cytoplasmic domain in the control of intracellular trafficking of ADAM2. However, deletion of the C-terminal 58-amino acid region of ADAM2 did not affect the cellular localization of the recombinant protein (Fig. 9), suggesting that this region was not directly involved in the intracellular trafficking of ADAM2. We therefore postulate that a protein motif controlling the transport of ADAM2 to the cell surface is located outside the C-terminal 58-amino acid region.

Overexpression of ADAM2 in COS-7 cells and, to a lesser extent, in C2C12 cells led to a striking rearrangement of the ER network into an unusual tubular pattern (Figs. 7 and 8), which was very reminiscent of the ER rearrangement in COS-7 cells induced by overexpression of the cytoskeleton-binding membrane protein CLIMP-63 (58, 59). In the case of CLIMP-63, this ER rearrangement is a direct consequence of the interactions between the cytoplasmic domain of CLIMP-63 with microtubules along the altered ER membranes (58). The reason why ADAM2 was able to induce a similar ER rearrangement is not clear, and the possibility that ADAM2 may interact with microtubules needs to be explored.

Recent studies have shown that a diacidic sorting signal ((D/E)X(D/E)) is required for efficient ER export (60, 61). This motif, which is found in the cytoplasmic tail of a number of transmembrane proteins, is not present in ADAM2, but is present in the Myc tag (EQKLISEEDL) that was fused to the C terminus of several ADAM2 constructs used in our studies. This may account for the considerable acceleration of the exit of the Myc-tagged ADAM2 protein constructs from the ER and their efficient transport to the cell surface (Fig. 9), and it...
further supports the observation that the cytoplasmic domain of ADAM12 constitutes a rate-limiting factor in the export of ADAM12 from the ER. Surprisingly, the cytoplasmic tail of ADAM9, similar to the cytoplasmic domain of ADAM12, does not contain the (D/E)X(D/E) motif, and its role in enhancing the export of the ADAM12/9 chimera from the ER is not clear.

Collectively, our results indicate that mouse ADAM12 ectopically expressed in C2C12 or COS-7 cells or endogenous ADAM12 in C2C12 cells is transported through the secretory pathway to the plasma membrane, with the exit from the ER constituting a rate-limiting step of the transport. Catalytically active ADAM12, i.e., lacking the prodomain and containing the metalloprotease domain, resides mainly at the cell surface, where it may have a role in proteolytic processing of other proteins that are synthesized as precursors and that need to be cleaved to generate biologically active forms. Moreover, because the cell-surface form of ADAM12 contains also an intact cell adhesion domain (62, 63), this form is fully capable of mediating cell adhesion-activated signaling.

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