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Human ADAM33: protein maturation and localization

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Abstract

ADAM33 (a disintegrin and metalloprotease) was recently found to be a novel asthma susceptibility gene [18]. Domain-specific antibodies were used to study its expression and processing. When the pro-domain and catalytic domain were expressed by a stable-transfected cell line, the pro-domain was removed by cleavage within a putative furin cleavage site. The catalytic domain was active in an α_2-macroglobulin complex formation assay and mutation of the catalytic site glutamic acid (E346A) eliminated activity. In transient transfections using the full-length protein, a pro-form and mature form were detectable and alternate glycosylation was demonstrated at sites within the catalytic domain. ADAM33 was detected on the cell surface, with the majority of protein detected intracellularly. The E346A mutation had no significant effect on protein processing. Endogenous ADAM33 was detected in bronchus tissue, bronchial smooth muscle cells, and MRC-5 fibroblasts, consistent with a role in the pathophysiology of asthma.

Keywords: ADAM; Metalloproteases; Asthma susceptibility gene

There are more than 30 members of the ADAM (a disintegrin and metalloprotease) family of proteins with orthologues in several species. ADAMs are membrane-anchored metalloproteases that play key roles in cell-to-cell interactions and shedding of pro-protein ectodomains [1,2]. They are characterized by a pro-domain, metalloprotease, and disintegrin domains, a cysteine-rich region sometimes containing an EGF repeat, a transmembrane domain, and a cytoplasmic carboxyl-terminus.

All ADAMs have a metalloprotease domain although approximately half carry the zinc-binding catalytic-site consensus sequence (HEXXHXXGXXH) and are predicted to be catalytically active. Members of the family have been implicated in the processing of membrane-anchored cytokines, cytokine receptors, and adhesion molecules and the remodeling of extracellular matrix components. For instance, ADAM17 can cleave tumor necrosis factor α (TNF-α), TNF-related activation-induced cytokine (TRANCE), TNF receptors, transforming growth factor-α (TGF-α), L-selectin, and fractalkine as well as amyloid protein precursor (APP) and cellular prion protein [3–10].

ADAM-family members are synthesized as inactive pro-proteins with signal sequences and pro-domains that require processing to glycosylated mature and active forms. The pro-domain is critically important in protein folding and processing in the endoplasmic reticulum and Golgi apparatus [11]. The pro-domain also functions as an inhibitor of protease activity through a mechanism that is thought to involve the folding of the domain in proximity to the catalytic site [11]. For many of the ADAMs, cleavage of the pro-domain is facilitated by furin or furin-like proteases in the *trans*-Golgi and occurs at an RX(K/R)R consensus sequence located adjacent to the catalytic domain boundary [11–13]. This cleavage can occur autocatalytically [14]. Mature ADAMs are translocated to extracellular and/or intracellular membrane surfaces [12–15].

The cDNAs for human and mouse ADAM33 were recently reported and their amino acid sequences share 70% similarity [16,17]. Human ADAM33 is most closely related to human ADAM19, human ADAM12, and *Xenopus* ADAM13. At the mRNA level, ADAM33 is

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expressed ubiquitously with highest expression measured in brain, heart, kidney, lung, and testes. Based on sequence similarity, ADAM33 contains all of the domains that characterize this family of proteins including the zinc-binding catalytic-site consensus sequence.

Although no activity has been demonstrated for ADAM33, a recent report provides evidence that polymorphisms within this gene are significantly associated with asthma [18]. In the present study, we expressed several forms of the protein and demonstrated that the catalytic domain was active. We also showed that it was processed to a mature glycosylated form and could be detected on the cell surface. Finally, we detected ADAM33 in protein extracts from lung tissue, primary human bronchial smooth muscle cells, and MRC-5 cells, a fibroblast cell line.

Materials and methods

Reagents. CHO-K1, COS-7, and MRC-5 cell lines were obtained from the American Type Culture Collection (Rockville, MD), HEK-293-D22 cells were obtained from Canji (San Diego, CA), and human bronchial smooth muscle cells were obtained from Clonetics Cell Discovery Systems (BioWhittaker, Walkersville, MD). Human bronchus was obtained from Tissue Transformation Technologies (Edison, NJ) with the appropriate legal consent of the donor and/or the donor's next of kin. KB8301 was from PharMingen (BD Biosciences, San Diego, CA).

cDNA constructs. The full-length cDNA for ADAM33 was isolated from a primary cDNA clone [GenBank Accession No. AF466287; 18] and subcloned into pcDNA3.1(−) (Invitrogen, Carlsbad, CA). Using this plasmid, the active site glutamic acid was mutated to an alanine (E346A) using oligonucleotide primers and *PfuTurbo* DNA polymerase (QuickChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA).

Constructs containing the Kozac sequence, the leader sequence, the pro-domain, and the catalytic domain (amino acids 1–432) were made using PCR primers that added a 5′ *XhoI* site and a 3′ *Hin*dIII site adjacent to nucleotides encoding six histidines and a stop codon. The amplification product was ligated into pFastBac 1 (Invitrogen) and transferred to pcDNA3.1(−).

For expression of the cytoplasmic domain in *Escherichia coli*, cDNA corresponding to amino acids 728–812 was amplified using PCR primers that added a 5′ *Nde*l site and ATG translation start codon and a 3′ *Xho*l site. The *Nde*l/*Xho*l fragment from an intermediate vector (pCR4-TOPO; Invitrogen) was purified and ligated into pET-43.1a(+) (Novagen, Madison, WI). Constructs were verified by restriction endonucleases digestion and DNA sequencing.

Transfections. One day before transfection, cells were cultured in 6- or 12-well plates at 3.0×10^5 or 1.5×10^5 cells/well, respectively, in complete media at 37 °C in 5% CO_2. Complete media contained 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine in F12K (Gibco–Invitrogen, Grand Island, NY) for CHO-K1 cells or in DMEM (Gibco) with 10 mM Hepes for HEK-293 cells. Washed cells were transfected with 0.8 μg (12-well plates) or 1.6 μg (6-well plates) plasmid DMA using Lipofectamine and Plus reagent (Invitrogen). After 5 h, cells were cultured in complete media containing 1× non-essential amino acids (Gibco) instead of serum.

For the generation of a stable-transfected cell line, CHO-K1 cells were transfected with ADAM33 (1–432) in pcDNA3.1(−) and grown with 0.5 mg/ml G418 (Gemini Bio-Products, Calabasas, CA). Limiting dilution culture was used to select and purify a cell line (3-1-C). Cells were maintained under serum-free conditions in a Cellmax artificial capillary cell culture system (Spectrum Laboratories, Rancho Dominguez, CA). Histidine-tagged ADAM33 protein (catalytic domain) was partially purified by chromatography on TALON metal affinity resin (Clontech, Palo Alto, CA) and dialyzed overnight into 25 mM Tris–HCl, pH 7.0, containing 10% glycerol. The amino-terminal sequence of protein immobilized on PVDF membranes (Bio-Rad Laboratories, Hercules, CA) was determined by automated Edman degradation.

α_2-Macroglobulin (α_2-M) assay. Partially purified ADAM33 was incubated with 350 μg/ml human α_2M (Calbiochem–Novabiochem, San Diego, CA) in 100 mM NaCl, 50 mM Tris (pH 7.4), 10 mM $CaCl_2$, and 0.02% sodium azide at 37 °C overnight [19,20]. Where indicated, reactions contained 5 mM of 1,10-phenanthroline, 50 μM KB8301, or a protease inhibitor cocktail [15] containing 2 μg/ml leupeptin, 0.4 μM benzamidine, 10 μg/ml soybean trypsin inhibitor, and 0.5 mM iodoacetamide. Reactions were terminated by boiling in SDS sample buffer.

Antibody production and Western blotting. Protein corresponding to the cytoplasmic domain (amino acids 728–812) of ADAM33 containing a histidine tag was expressed in *E. coli* and purified by affinity chromatography on Ni-NTA His•Bind resin (Novagen). The resulting protein was further purified by SDS–PAGE and electroelution followed by dialysis into PBS.

Peptides containing amino-terminal cysteines were synthesized (Zymed Laboratories, South San Francisco, CA) corresponding to regions within the pro-domain (Pro1—amino acids 44–61; NH_2-(C)VLDGQPWRTVSLEEPVSK-COOH), catalytic domain (ASP2—amino acids 303–319; NH_2-RAFQGATVGLAPVEGMC-COOH), and cytoplasmic domain (Cyt2—amino acids 777–790; NH_2-(C)DPENSHEPSSHPEK-COOH) of ADAM33 [18]. The peptides were chosen from regions with favorably predicted antigenicity, hydrophilicity, surface probability, and flexibility (MacVector software; Eastman Kodak, Rochester, NY), and low similarity to other ADAMs.

E. coli-derived protein and synthesized peptides were covalently conjugated to keyhole limpet hemocyanin, mixed with adjuvant, and injected into pairs of NZW rabbits (Zymed and Covance Research Products, Denver, PA). Antisera were purified with affinity columns prepared from unconjugated peptides. Serum from rabbits injected with *E. coli*-derived cytoplasmic domain (SP621) was used without purification.

Cell extracts were prepared from transfected cells and cell lines (approximately 6.7×10^5 cells/ml lysis buffer) or tissue samples (1 g tissue/10 ml lysis buffer) as described [13]. Lysis buffer contained 1% NP-40, 10 mM of 1,10-phenanthroline, and 1× Complete Protease Inhibitor Cocktail (Roche Applied Science; Indianapolis, IN) in Tris-buffered saline (TBS; 20 mM Tris–HCl, 500 mM NaCl, pH 7.5). Where indicated, protein content was determined using the micro bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL). Lysates were used directly or glycoproteins were enriched by selection with Con A–Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) as described [13]. Samples were heat-denatured with SDS, reduced with DTT, and separated by electrophoresis. Molecular mass was estimated by comparison to Kaleidoscope pre-stained standards (Bio-Rad). Proteins were transferred to PVDF membranes by electroblotting and were blocked with 5% ECL blocking reagent (Amersham) in TBS containing 0.1% Tween 20 (TBS-T). Membranes were incubated with antibodies (1–2 μg/ml affinity-purified antibodies, 1:10,000 non-purified sera, 1:5000 horseradish peroxidase conjugated anti-histidine antibody (Invitrogen)), and, if required, with goat anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:25,000). Detection was with ECL chemiluminescence reagents (Amersham). Protein bands were quantitated by digital scanning of developed films and analysis with Quantity One software (Bio-Rad). Multiple film exposures were acquired to assure bands did not saturate film.

Antibodies described above were tested for reactivity with recombinantly expressed forms of ADAM33 and compared with reactivity of pre-immune serum (data not shown). Anti-Pro1 but not pre-immune sera detected only forms of ADAM33 that were not processed (i.e. contained the pro-domain) in transfected cells but not in control cells. Anti-Cyt2 and anti-SP621 but not pre-immune sera detected non-processed and processed full-length ADAM33 but not versions of ADAM33 that did not contain the cytoplasmic domain. Anti-ASP2 but not pre-immune sera detected all forms of ADAM33 that contained the catalytic domain. All antibodies did not detect recombinant mouse ADAM33, the most closely related protease of the ADAM family [17].

Protein deglycosylation. Partially purified protein or proteins in cell lysates were denatured by the addition of 0.5% SDS, 1% β-mercaptoethanol for 10 min at 100 °C prior to digestion with peptide: *N*-glycosidase F (PNGase F; New England BioLabs, Beverly, MA) as indicated. Denatured or non-denatured samples were deglycosylated in reactions supplemented with 50 mM sodium citrate (pH 5.5) for endoglycosidase H_f (Endo H; New England BioLabs), 50 mM sodium citrate (pH 6.0) for acetylneuraminyl hydrolase (neuraminidase, sialidase; New England BioLabs), 50 mM sodium phosphate (pH 7.5), and 1% NP-40 for PNGase F or with 50 mM sodium phosphate (pH 6.0) for *O*-glycosidase DS (Bio-Rad). Samples digested with PNGase F were further treated with *O*-glycosidase DS, β-*N*-acetylhexosaminidase (HEXase I; Bio-Rad), β-galactosidase (GALase III, Bio-Rad), and sialidase II (NANase II; Bio-Rad) in 50 mM sodium phosphate (pH 7.5). Reaction mixtures were incubated for 2 h (*O*-glycosidase DS, HEXase I, GALase III, and NANase II) or overnight (Endo H, neuraminidase, PNGase F) at 37 °C with and without the addition of enzyme.

Cell surface biotinylation or trypsin treatment. Washed cells were treated with 0.5 mg/ml sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin; Pierce Chemical, Rockford, IL) in PBS for 45 min at 4 °C and then washed with 0.1 M glycine in TBS before lysis [14]. Biotinylated proteins were selected with streptavidin–Sepharose (Pharmacia). Control aliquots of 10-fold less extract by weight were selected by adherence to Con A–Sepharose. For trypsinization, cells were treated with 500 μg/ml bovine pancreas trypsin for 30 min at 4 °C [14]. Following a wash with 500 μg/ml soybean trypsin inhibitor in PBS, the cells were collected and lysed as described above.

Results

Catalytic activity of the metalloprotease domain

Because ADAM33 substrates are currently unknown, it was not possible to test full-length, membrane-bound protein for protease activity in cell-based cleavage assays. However, it was possible to demonstrate activity using a soluble form of the enzyme in combination with α_2M, a substrate known to be cleaved by most proteases [19,20].

A cell line was developed that expressed a soluble form of the ADAM33 catalytic domain with a carboxyl-terminus at aspartic acid 432 followed by a six-histidine tag for detection and purification. The plasmid used to transfect these cells also contained the native signal sequence as well as the pro-domain to allow proper folding and processing. These domains were cleaved from the mature catalytic domain by the cell prior to secretion into cell culture media. Western blot analysis of partially purified protein indicated that the catalytic domain existed in two forms of similar molecular mass when visualized with the ASP2 antibody (Fig. 1A, lanes 1 and 3). The two forms were visible upon Coomassie blue staining (Fig. 2A). Both anti-ASP2-reactive bands migrated predominantly as a single band following deglycosylation of non-denatured protein with PNGase F (Fig. 1A, lane 4), suggesting that the difference in mobility was due to differential glycosylation. Incomplete digestion occurred when PNGase F was used following protein denaturation (Fig. 1A, lane 2). A small amount of pro-domain co-purified with catalytic domain and was visualized with the Pro1 antibody (Fig. 1B). The prodomain existed in predominantly one form with diffuse migration that could be seen in some experiments as one predominant band and a less prevalent, faster migrating band (Fig. 1B, lanes 1 and 3 and data not shown). Deglycosylation of non-denatured and denatured proteins with PNGase F resulted in a reduction in molecular mass and the appearance of two distinct forms of the pro-domain (Fig. 1B, lanes 2 and 4).

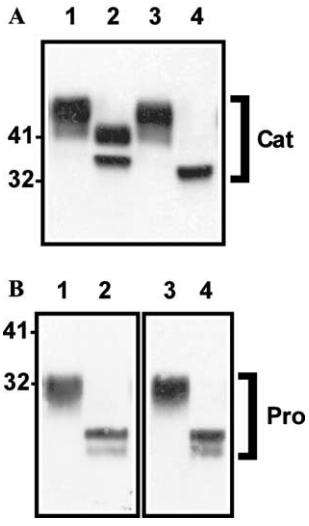

Fig. 1. Expression of a soluble form of the catalytic domain of ADAM33. Partially purified protein from a cell line (3-1-C) stably transfected with the pro-domain-catalytic domain (Pro-Cat) of ADAM33 was analyzed by Western blotting with antibodies recognizing the catalytic domain (ASP2; A) or the pro-domain (Pro1; B). Aliquots of protein were denatured (lanes 1 and 2) or not denatured (lanes 3 and 4) prior to incubation with (lanes 2 and 4) or without (lanes 1 and 3) PNGase F overnight. Numbers indicate molecular mass in kilodalton.

Following partial purification of the soluble catalytic domain protein (Fig. 2A), the amino-terminal sequence was determined to be EARRT. This is within a slightly longer sequence that contains three overlapping furin cleavage consensus sequences (RGRREARRTR), suggesting that furin is responsible for pro-domain removal.

To test the activity of the soluble catalytic domain of ADAM33, partially purified protein was used in the

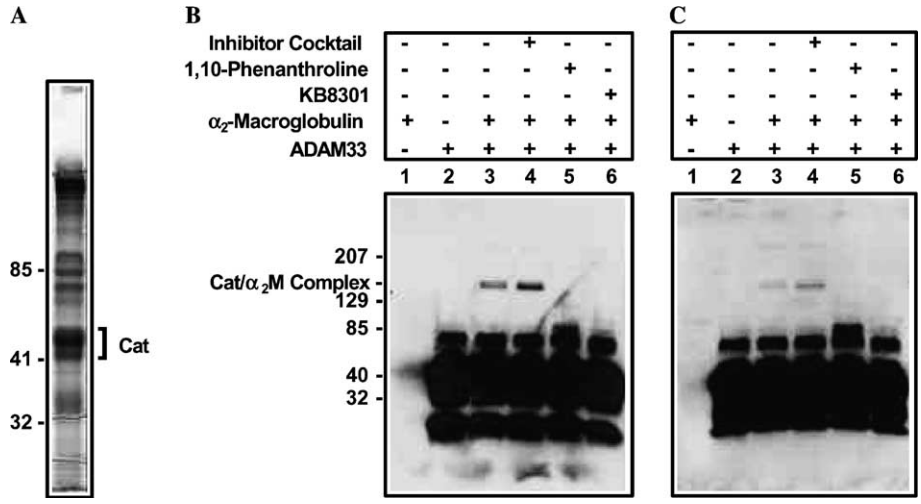

Fig. 2. Protease activity of the catalytic domain of ADAM33. (A) The catalytic domain (Cat) of ADAM33 expressed by 3-1-C cells was purified by affinity chromatography to approximately 20% purity when assessed by SDS–PAGE with Coomassie blue staining. (B and C) Partially purified catalytic domain was used in the α_2M complex formation assay to demonstrate enzymatic activity. Protease inhibitors (1,10-phenanthroline, KB8301 or inhibitor cocktail containing leupeptin, benzamidine, soybean trypsin inhibitor, and iodoacetamide) were included in reactions as indicated. Complexes were visualized by Western blotting using an anti-histidine (B) or anti-ASP2 (C) antibody.

α_2M complex formation assay [19,20]. When α_2M is cleaved it forms a covalent complex with the enzyme, resulting in a change in its electrophoretic mobility upon SDS–PAGE. The catalytic domain of ADAM33 formed a complex with α_2M (Figs. 2B and C, lane 3) that was inhibited by addition of the non-selective metalloproteinase inhibitors 1,10-phenanthroline or KB8301 (Figs. 2B and C, lanes 5 and 6) but not by a protease inhibitor cocktail containing leupeptin, benzamidine, soybean trypsin inhibitor, and iodoacetamide (Figs. 2B and C, lane 4). Similar results were observed when complex formation was assessed with anti-His (Fig. 2B) or ADAM33-specific anti-ASP2 antibodies (Fig. 2C). Therefore, the observed complex contained ADAM33 protein and was not due to contaminating protease activity. Catalytic domain expressed by baculovirus-infected insect cells also was active and the corresponding E346A mutant catalytic domain was inactive in this assay (data not shown).

Western blot analysis of transiently expressed ADAM33

In order to study the processing of full-length ADAM33, three cell lines (human HEK-293, monkey COS-7, and hamster CHO-K1) were transiently transfected with expression plasmids. HEK-293 cells transfected with wild-type ADAM33 expressed ~100 and ~123 kDa proteins that were reactive with anti-Cyt2 and anti-SP621 antibodies (Figs. 3B and C, lane 2). In addition, the ~123 kDa but not the ~100 kDa protein was detected by the Pro1 antibody (Fig. 3A, lane 2). These data are consistent with processing of a ~123 kDa pro-form of ADAM33 to a ~100 kDa mature form. The pro-form was detected 6 h after cells were transfected and processing to the mature form was detected after 48 h (Fig. 3D). There was little difference between 48 and 72 h in the expression levels of both forms of the protein but cell viability deteriorated at the latter time. Similar results were observed with ADAM33 containing the E346A mutation (Figs. 3A–C, lane 3 and Fig. 3E). Therefore, all transfection experiments were assessed at 48 h. At this time, 79% ± 7% of wild-type ADAM33 was of the pro-form and 21% ± 7% was of the mature form in HEK-293 cells ($n = 3$). Although not statistically significant, we consistently observed more processing of the E346A mutant to the mature form compared to that observed with the wild-type protein (71% ± 10% pro-form, 29% ± 10% mature form, $n = 3$; see Fig. 3C, lanes 2 and 3).

Similar results were obtained when CHO-K1 or COS-7 cells were transfected with each construct (data not shown). Overall protein expression was highest in HEK-293 cells with less expression in COS-7 cells and the least amount of expression in CHO-K1 cells. In contrast to the level of expression, more of the mature form of the protein was formed in HEK-293 and CHO-K1 cells and very little to no processing was observed in COS-7 cells. Therefore, HEK-293 or CHO-K1 cells were used in the experiments described below.

Deglycosylation of ADAM33

Both the pro-form and mature form of ADAM33 from transfected HEK-293 cells were susceptible to cleavage by PNGase F (Fig. 4A, lane 4). In contrast, all forms of ADAM33 were resistant to *O*-glycosidase DS (Fig. 4A, lane 8), suggesting that all polysaccharides were asparagine-linked. The pro-form and possibly one

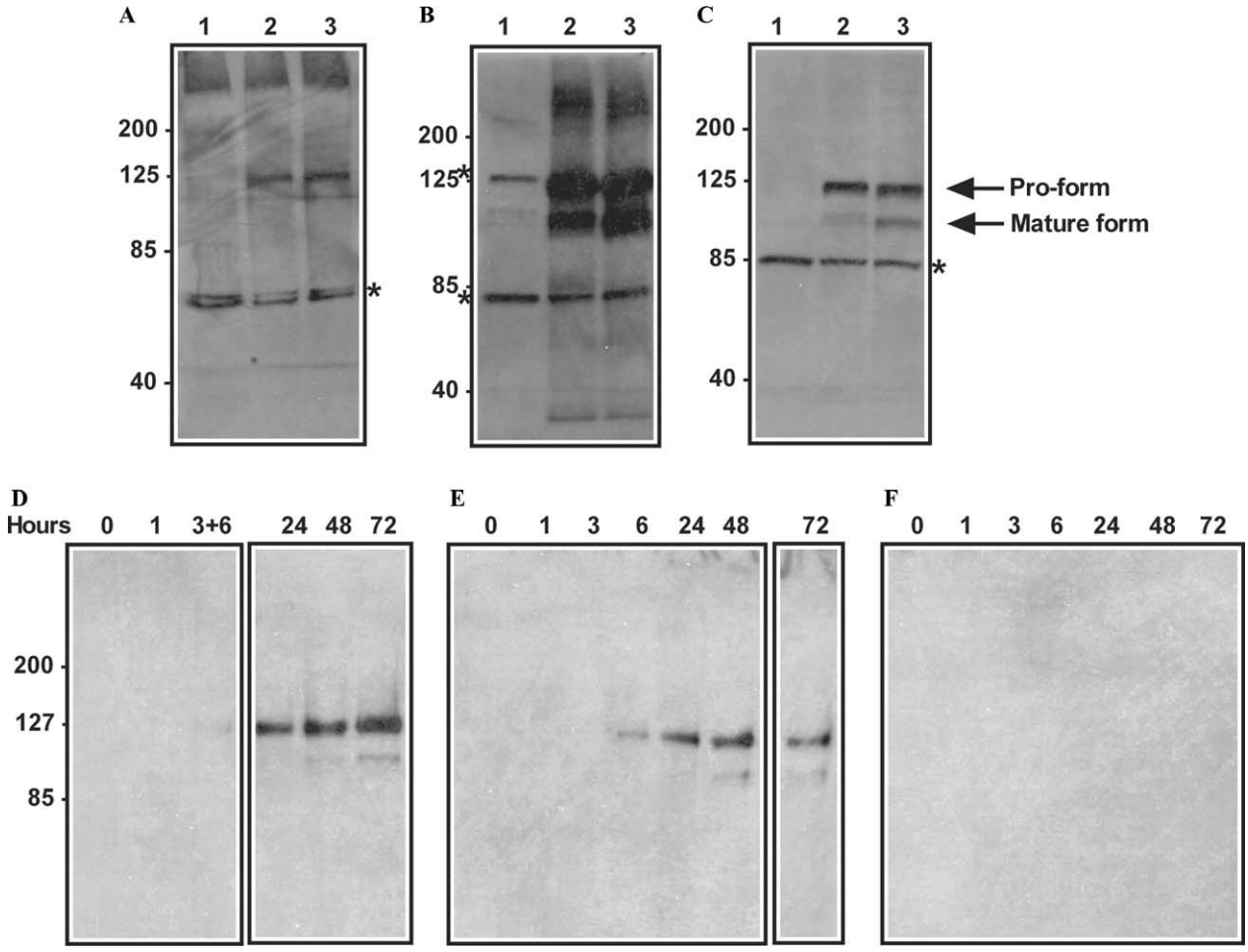

Fig. 3. Expression of wild-type and E346A mutant ADAM33. Plasmids carrying full-length wild-type ADAM33 (A–C, lane 2, D), E346A mutant ADAM33 (A–C, lane 3, E) or no insert control (A–C, lane 1, F) were transfected into HEK-293 cells. Lysates prepared 48 h later were analyzed by Western blotting with antibodies that recognized the pro-domain (Pro1, A) or cytoplasmic domain (Cyt2, B; SP621, C). The pro-forms and mature forms are indicated with arrows. For time courses (D–F), equal amounts by weight of each extract were adhered to Con A–Sepharose prior to Western blot analysis with anti-Cyt2 antibodies. When Con A–Sepharose-adherence was not used, antibodies produced non-specific bands at $\leqslant$85 kDa (A–C) and >125 kDa (B) as indicated by asterisks. The relative amounts of ADAM33 pro-form:mature form were 78%:22% (C, lane 2), 61%:39% (C, lane 3), 87%:13% (D, 48 h), 78%:22% (D, 72 h), 82%:18% (E, 48 h), and 87%:13% (E, 72 h).

form of the mature metalloprotease were susceptible to Endo H treatment (Fig. 4A, compare lane 6 to lane 5), suggesting that some forms of the protein carry high mannose or hybrid oligosaccharides. Some of the mature forms appeared susceptible to neuraminidase cleavage (Fig. 4A, compare lane 2 to lane 1), consistent with the presence of sialic acid containing carbohydrates. It was not possible to assess the sialic acid content of the pro-form because polyacrylamide gels could not discriminate the relatively small change in mobility caused by the cleavage of sialic acid from a protein of this size.

The partially purified catalytic domain expressed by CHO cells also was detected as two forms that were reduced to a single form following digestion with PNGase F (Figs. 1A and 4B, lane 6). Treatment of PNGase F-deglycosylated protein with *O*-glycosidase DS, HEXase I, NANase II, and GALase III did not change the mobility of the protein (Fig. 4B, compare lane 6 to lane 8), suggesting that all carbohydrates were asparagine-linked. Treatment with sialidase resulted in a minor change in mobility (Fig. 4B, compare lane 2 to lane 1), suggesting the presence of sialic acid-containing oligosaccharides. The partially purified catalytic domain was not susceptible to cleavage by Endo H (Fig. 4B, compare lane 4 to lane 3).

Cellular localization of ADAM33

To determine if ADAM33 was present on the outer surface of the cell membrane, HEK-293 cells were transfected and then treated with a cell-impermeable biotinylating reagent, NHS-LC-biotin. Following cell lysis, all extracts were normalized for total protein content per volume. Both the pro-form and mature from of ADAM33 were labeled with biotin and could be

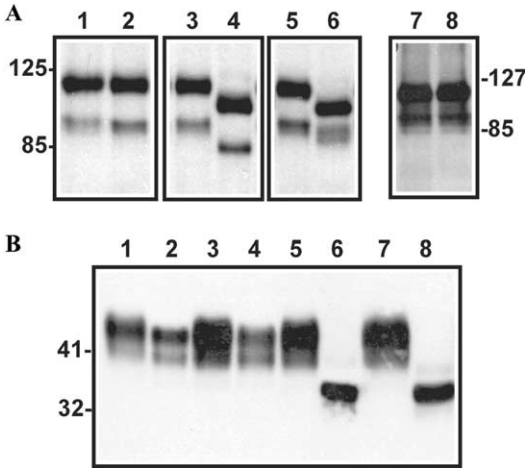

Fig. 4. Deglycosylation of ADAM33. Non-denatured cell extract from HEK-293 cells transfected with full-length ADAM33 containing an E346A mutation (A) or partially purified catalytic domain from 3-1-C CHO cells (B) was incubated with sialidase (lane 2), PNGase F (A lane 4, B lane 6), Endo H (A lane 6, B lane 4), or *O*-glycosidase (A lane 8). Partially purified catalytic domain treated with PNGase F was further treated with *O*-glycosidase, HEXase I, NANase II, and GALase III (B lane 8). Control samples were incubated in identical conditions without glycolytic enzymes (lanes 1, 3, 5, and 7). Reactions were analyzed by Western blotting using the anti-Cyt2 antibody (A) or anti-ASP2 antibody (B). E346A mutant protein was analyzed and shown because it was processed to the mature form more efficiently than the wild-type (see Figs. 3B, lane 3 and 3C, lane 3). Similar results were observed when extract from HEK-293 cells transfected with wild-type full-length ADAM33 was analyzed. The same cell extract was used for A lanes 1–6 and for Figs. 3A–C, lane 3.

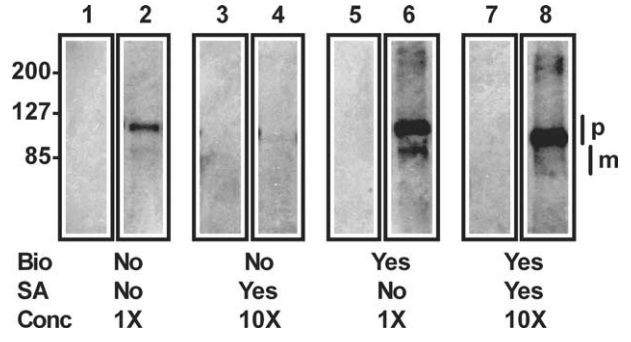

Fig. 5. Cell-surface biotinylation of HEK-293 cells transfected with ADAM33. Forty-eight hours after transfection with wild-type full-length ADAM33 (lanes 2, 4, 6, and 8) or no insert control plasmid (lanes 1, 3, 5, and 7), cells were incubated with (lanes 5–8) and without (lanes 1–4) NHS-LC-biotin. Biotinylated proteins in total cell extracts were selected with streptavidin–Sepharose (lanes 3, 4, 7, and 8). For comparison purposes, protein was selected from 10-fold less extract with Con A–Sepharose (lanes 1, 2, 5, and 6). Samples were analyzed by Western blotting using the anti-Cyt2 antibody. The relative amounts of ADAM33 pro-form:mature form were 76%:24% (lane 2), 84%:16% (lane 6), and 87%:13% (lane 8). Bio, biotinylated; SA, streptavidin selected; Conc, concentration; p, ADAM33 pro-form; and m, ADAM33 mature form.

detected by anti-Cyt2 antibody after binding to streptavidin–Sepharose (Fig. 5, compare lane 8 to lane 4). The amount of biotinylated ADAM33 that was captured by streptavidin–Sepharose from 200 μl of cell extract was approximately equivalent to the amount of total (non-biotinylated) ADAM33 in 20 μl extract (Fig. 5, compare lane 8 to lane 6). This result demonstrated that at least 10% of the ADAM33 detected in transfections was located on the cell surface. Similar results were observed with ADAM33 containing the E346A mutation (data not shown). The relative amounts of ADAM33 pro-form and mature form on the plasma membrane (84% and 16%, respectively) did not differ from the relative amounts within the whole cell (87% and 13%, respectively).

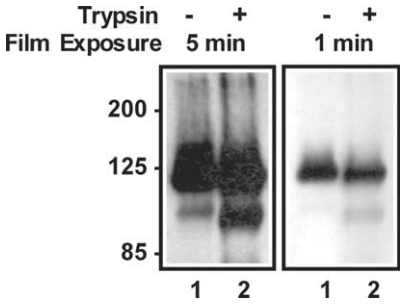

Fig. 6. Cell-surface trypsinization of CHO-K1 cells transfected with ADAM33. Forty-eight hours after transfection with wild-type full-length ADAM33, cells were incubated with (lane 2) and without (lane 1) bovine pancreas trypsin followed by washing with soybean trypsin inhibitor. Following adherance to Con A–Sepharose, samples were analyzed by Western blotting with the anti-Cyt2 antibody. Film exposures of 5 and 1 min are shown to better visualize the migration of each band. The relative amounts of ADAM33 pro-form:mature form were quantitated using the 1 min exposure and were 93%:7% (lane 1) and 78%:22% (lane 2).

To further distinguish intracellular ADAM33 from that expressed on the outer cell surface, transfected CHO-K1 cells were treated with trypsin. Trypsin cleavage of the mature form and pro-form of ADAM33 was likely to generate the same protein fragment when detected with the anti-Cyt2 antibody. Trypsin treatment of ADAM33-transfected cells resulted in an increase in mobility of the mature form of the metalloprotease (Fig. 6). In addition, the amount of the trypsin-cleaved protein fragment increased compared to the amount of untreated mature ADAM33. This increase in trypsin-cleaved protein was likely contributed by cleavage of cell surface associated pro-form. Quantitation of the bands showed that the pro-form decreased by 15% after trypsin cleavage, consistent with the relative amount of pro-form detected on the plasma membrane by biotin labeling.

Expression of native ADAM33 in human cell lines and tissue

ADAM33 has been identified as an asthma susceptibility gene [18] and its mRNA has been detected in lung tissue, bronchial smooth muscle cells, and lung fibro-

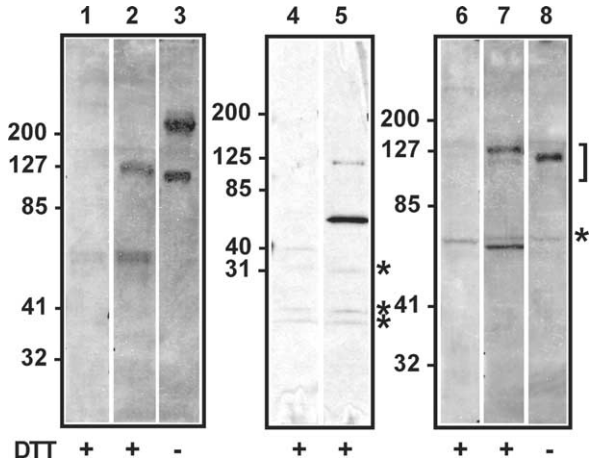

Fig. 7. Detection of native ADAM33 in tissue and cell extracts. Protein extracts from human bronchus tissue (lanes 1–3), cultured human bronchial smooth muscle cells (lanes 4 and 5), and human MRC-5 fibroblast cells (lanes 6–8) were selected with Con A–Sepharose and analyzed by Western blotting. PVDF membranes were probed with anti-Cyt2 antibody (lanes 2, 3, 5, 7, and 8) or pre-immune serum (lanes 1, 4, and 6). Prior to SDS–PAGE, extracts were boiled in gel loading buffer with (lanes 1, 2, and 4–7) or without (lanes 3 and 8) DTT. ADAM33 protein is indicated by a bracket and non-specific bands are indicated by asterisks.

blasts but not in leukocytes or bronchial epithelium [17,18]. Therefore, we assessed the expression of ADAM33 protein in human lung tissue, primary bronchial smooth muscle cells, and a fibroblast cell line (MRC-5). An anti-Cyt2-reactive protein of ~120 kDa was detected in each sample when separated under reducing conditions (Fig. 7; lane 2, bronchus, ~121 kDa; lane 5, bronchial smooth muscle cells, ~115 kDa; and lane 7, MRC-5 cells, ~124 kDa). When samples where examined under non-reducing conditions, the ~120 kDa protein exhibited faster migration (Fig. 7, lanes 3 and 8). This change in mobility under non-reducing conditions is characteristically observed with the ADAM family of metalloproteases and is believed to be due to their high cysteine content [13]. A larger reactive protein (>200 kDa; Fig. 7, lane 3) was observed in bronchus tissue extracts when examined under non-reducing conditions, possibly due to the presence of a complex of ADAM33 with itself or some other protein. Also, a smaller protein was observed under reducing conditions that might have been due to the cleavage of ADAM33 proximal to the transmembrane region (Fig. 7, lane 5, bronchial smooth muscle cells, ~58 kDa; lane 7, MRC-5 cells, ~65 kDa). A similar observation was made for ADAM15 [13].

Discussion

ADAM33 contains five potential sites for asparagine-linked (N-linked) oligosaccharide modification; two sites within the pro-domain, two sites within the catalytic domain, and one site within the disintegrin domain [17 and computer analysis not shown]. Analysis of full-length ADAM33 for oligosaccharide content indicated that the immature protein consisted of predominantly high-mannose or hybrid oligosaccharides. As the protein matured, some but perhaps not all of the oligosaccharides were processed into complex forms and became resistant to Endo H cleavage. Upon removal of all asparagine-linked oligosaccharides with PNGase F, the mature full-length protein migrated on SDS–PAGE as a single band. The same pattern, that is, multiple forms of the mature protein, was also observed when the partially purified catalytic domain was examined, suggesting that alternative processing occurred on one or both of the asparagine-linked sites within this domain. The glycosylation patterns of the mature full-length protein and the partially purified catalytic domain differed in their susceptibility to Endo H digestion; a form of the full-length protein was susceptible and the expressed catalytic domain was resistant to cleavage by Endo H. Because the full-length protein contains a potential asparagine-linked oligosaccharide site in the disintegrin domain in addition to those contained within the expressed catalytic domain, it is possible that this site contained high mannose or hybrid oligosaccharides and accounted for the susceptibility to Endo H digestion.

Although ADAM33 contains a furin cleavage site between the pro-domain and catalytic domain [17,18], it was possible that pro-domain removal required proteolytic activity of ADAM33 as does ADAM28 processing [14]. To test this possibility, HEK-293 cells were transfected with a mutant form of ADAM33 in which the glutamic acid in the catalytic site consensus sequence "HEIGH" was changed to alanine (E346A). In other metalloproteases this mutation is known to result in the complete loss of proteolytic activity [21] and ADAM33 E346A was inactive in the α2M assay. The same pattern of protein expression was observed with the E346A mutant as with wild-type ADAM33, suggesting that processing was not due to autocatalysis and likely occurs by a furin-dependent mechanism.

In every instance in which full-length ADAM33 protein was detected, the immature, pro-form of the protein predominated over the processed, mature form by approximately 4-fold. Also, approximately 10% of ADAM33 protein was detected on the plasma membrane, including both non-processed and mature proteins. In contrast, when only the pro-domain with the catalytic domain was recombinantly expressed, a majority of the truncated form of the protein was processed to the catalytic domain and secreted. This suggests a regulatory role in processing for regions carboxy-terminal to the catalytic domain.

Glycosylated protein becomes Endo H resistant after traversing the *medial*-Golgi compartment where

asparagine-linked sugars are converted to complex oligosaccharides. Subsequent processing can occur by the action of furin convertases in the *trans*-Golgi compartment [22]. A majority of the ADAM33 protein in transfected cells was sensitive to Endo H digestion and was not processed to a mature form. Therefore, it is likely that most of the protein was localized in the endoplasmic reticulum and the proximal Golgi. However, a small amount of protein was able to traverse the Golgi, was converted into mature ADAM33, and was detectable on the cell surface. In addition, a small amount of immature protein also was detected on the cell surface. It is possible that the Golgi acts as a trafficking checkpoint and allows full processing of ADAM33 only after appropriate stimulation. This has been suggested for ADAM12 with the cytoplasmic or transmembrane regions playing important roles in retention within the *trans*-Golgi network [23,24]. The observation that a majority of the pro-domain-catalytic domain recombinant protein was processed and secreted is consistent with this concept.

The expression of native ADAM33 in tissue and cell lines was similar to that seen when protein was expressed recombinantly. It is likely that the endogenous ~120 kDa protein observed in tissue and cell extracts represented unprocessed pro-enzyme based on our transfection results in which the recombinant pro-form was ~123 kDa and was always expressed at higher levels than the mature form. It is possible and likely that processing to the mature form does occur for the native protein, but the amount of mature ADAM33 was below the level of detection of our antibody. Nonetheless, ADAM33 could be detected in lung tissue as well as in smooth muscle cells and fibroblasts. Together with mRNA analysis [17,18], these results clearly demonstrate that ADAM33 is present in tissue and cells important in the asthmatic response and provide experimental support to the claim that this gene is associated with asthma [18]. It has been speculated that ADAM33 might affect airway disease by playing a role in growth factor or cytokine shedding and subsequent airway remodeling [25]. Further studies are required to identify the functional roles polymorphic forms of ADAM33 might play in normal homeostasis and in the asthmatic response.

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