Catalytic Activity of Human ADAM33*

Jun Zou‡§, Feng Zhu‡, Jianjun Liu‡, Wenyan Wang‡, Rumin Zhang‡, Charles G. Garlisi‡, Yan-Hui Liu‡, Shihong Wang‡, Himanshu Shah‡, Yuntao Wan‡, and Shelby P. Umland‡

From the Departments of Allergy and Structural Chemistry, Schering-Plough Research Institute, Kenilworth, New Jersey 07033

ADAM33 (a disintegrin and metalloproteinase) is an asthma susceptibility gene recently identified through a genetic study of asthmatic families (van Eerdewegh et al. (2002) Nature 418, 426–430). In order to characterize the catalytic properties of ADAM33, the metalloproteinase domain of human ADAM33 was expressed in Dro sophila S2 cells and purified. The N-terminal sequence of the purified metalloproteinase was exclusively 204EARR, indicating utilization of one of three furin recognition sites. Of many synthetic peptides tested as potential substrates, four peptides derived from β-amyloid precursor protein (APP), Kit-ligand-1 (KL-1), tumor necrosis factor-related activation-induced cytokine, and insulin B chain were cleaved by ADAM33; mutation at the catalytic site, E346A, inactivated catalytic activity. Cleavage of APP occurred at His14 | Cln15, not at the α-secretase site and was inefficient (Kcat/Km (1.6 ± 0.3) x 106 M⁻¹ s⁻¹). Cleavage of a juxtamembrane KL-1 peptide occurred at a site used physiologically with a similar efficiency. Mutagenesis of KL-1 peptide substrate indicated that the P3, P2, P1, and P3’ residues were critical for activity. In a transfected cell-based sheddase assay, ADAM33 functioned as a negative regulator of APP shedding and mediated some constitutive shedding of KL-1, which was not regulated by phorbol 12-myristate 13-acetate activation. ADAM33 activity was sensitive to several hydroxamate inhibitors (IK682, K1 = 23 ± 7 nM) and to tissue inhibitors of metalloproteinase (TIMPs). Activity was inhibited moderately by TIMP-3 and TIMP-4 and weakly inhibited by TIMP-2 but not by TIMP-1, a profile distinct from other ADAMs. The identification of ADAM33 peptide substrates, cellular activity, and a distinct inhibitor profile provide the basis for further functional studies of ADAM33.

ADAM33 (a disintegrin and metalloproteinase) was identified as an asthma susceptibility gene by a genetic linkage and polymorphism study of asthmatic families (1). Determining the role of ADAM33 in the pathophysiology of asthma will require defining its function at several levels. ADAM33 belongs to a family of type I transmembrane metalloproteinases. These integral membrane glycoproteins play important physiological roles in fertilization, myogenesis, and neurogenesis due to their participation in cell-cell interactions and proteolytic release of cell surface membrane proteins such as cytokines, growth factors, and receptors (2, 3). The structure of the ADAM1 proteins is conserved and characterized by multiple domains (an N-terminal secretion signal sequence, pro- and catalytic domains, disintegrin, and cysteine-rich domains), which are usually followed by an epidermal growth factor repeat, a transmembrane, and a carboxyl-terminal cytoplasmic tail (3).

Of 34 ADAMs identified, about half of them, including ADAM33, were predicted to be active proteinases based on the presence of the HEXXHXXGXXH zinc binding motif and a glutamic acid in the catalytic domain (4, 5). However, only some of these ADAMs were demonstrated experimentally to possess catalytic activity, including ADAM8 (6, 7), ADAM9 (8), ADAM10 (9), ADAM12 (10), ADAM15 (11, 12), ADAM17 (13, 14), ADAM19 (15–17), ADAM28 (18), and ADAM33 (19). For only a few ADAMs, a physiological substrate has been identified (3). ADAM17/TNF-α-converting enzyme is the best studied member of the ADAM family. In addition to shedding soluble TNF-α after cleavage of the membrane-anchored precursor (13), ADAM17 also processes many other integral membrane proteins including the amyloid precursor protein (APP) (20), transforming growth factor-α (TGF-α), L-selectin, and TNF receptor II (21) as well as IL-1 receptor and TNF receptor I (22). ADAM10/Kazanian is involved in neuronal development through the cleavage of Notch, a type I transmembrane receptor that controls cell fate determination (23).

The cDNAs of ADAM33 have been cloned from humans and mice and are most closely related to ADAM19, ADAM12, and Xenopus ADAM13 (1, 24, 25). Overexpression of ADAM19 in L929 cells suggested that ADAM19 may participate in the shedding of β3-neuregulin, a member of the epidermal growth factor family (15). Also, purified ADAM19 metalloproteinase cleaved peptides corresponding to the known cleavage sites of TNF-α, TRANCE, and KL-1 (17). ADAM12 was reported to process heparin-binding epidermal growth factor in vivo (26) and to cleave insulin-like growth factor-binding protein-3 and -5 (27).

ADAMs are synthesized as latent proforms in the endoplasmic reticulum. This latency is the result of a complex formation via a thiol-zinc bond between a conserved cysteine in the prodomain and the essential catalytic Zn2+ in the metalloprotease domain (28, 29). The latent ADAMs can be activated by multiple means that dissociate the thiol-zinc bond in the complex.

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§ To whom correspondence should be addressed: Dept. of Allergy, Schering-Plough Research Institute, 2015 Galloping Hill Rd., Kenilworth, NJ 07033. Tel.: 908-740-6578; Fax: 908-740-7101; E-mail: jun.zou@spcorp.com.

The abbreviations used are: ADAM, a disintegrin and metalloproteinase; MMP, matrix metalloproteinase; KL-1, Kit-ligand-1; APP, amyloid precursor protein; TRANCE, TNF-related activation-induced cytokine; TNF-α, tumor necrosis factor-α; TGF-α, transforming growth factor-α; TIMP, tissue inhibitor of metalloproteinase; PNGase, peptide; N-glycosidase F; Ni2+–NTA, nickel-nitrilotriacetic acid; HPLC, high performance liquid chromatography; WT, wild type; PMA, phorbol 12-myristate 13-acetate; MALDI-TOF, matrix-assisted laser-desorption/ionization time-of-flight; pMT, metallothionein promoter; Dabcyl, 4-[(4-dimethylamino)phenyl]azo]benzoic acid; Edans, N-acetyl-N'-[(5-sulfo-l-naphthyl)ethyl]enediamine.

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ADAM33 cDNA template (GenBank accession number AF466287) were from Amersham Biosciences. Peptides from amyloid precursor linked immunosorbent assay using the Quantakine kit from R & D was detected in culture supernatants of transfected cells by enzyme-blotting. Human stem cell factor, also know as c-Kit ligand (KL-1), berg, Heidelberg, Germany) (38) and was used to detect APP by Western blotting. The W0 (Cyt2) and their use are described elsewhere (19), and they were used for Western blotting. The W0–pro-domain (Pro1), catalytic domain (ASP2), or cytoplasmic domain (Schering Plough, Kenilworth, NJ) (37) or Dr. T. McClanahan (DNAX, Applied Biosystems, Foster City, CA) or an ACT model 496 multiple chemistry was used on either an ABI model 431A Peptide Synthesizer (Advanced ChemTech, Louisville, KY). The molecular synthesizer (Applied Biosystems, Foster City, CA) or an ACT model 496 multiple chemistry was used on either an ABI model 431A Peptide Synthesizer (Advanced ChemTech, Louisville, KY). The molecular

**MATERIALS AND METHODS**

**Reagents—**Restriction enzymes, T4 ligase, and peptide-N-glycosidase F (PNGase) were purchased from New England Biolabs (Beverly, MA). All chemicals and insulin B chain peptide were from Sigma unless indicated otherwise. Human TIMPs were purchased from R & D Systems (Minneapolis, MN). All reagents and media for Drosophila S2 cell expression including anti-His, antibody conjugated with horseradish peroxidase were from Invitrogen unless indicated otherwise. A pCDNA3.1 plasmid (Invitrogen, San Diego, CA) containing full-length ADAM33 cDNA (WT) was ligated into the pcDNA3.1 plasmid (Invitrogen) unless indicated otherwise. A molar extinction coefficient, \( \epsilon_{280} \), was determined for both recombinant human ADAM33 pro- and catalytic domains (1). The following PCR primers were used, which added a KpnI site to the 5′-end of the gene fragment: primer 1, 5′-TTA CAT GTA TCA TAG GGT CCG CCT CAA GGA CAT ATC CCT GGG CAG-3′ and nucleotides encoding a His, tag plus a XhoI site at the 3′端; primer 2, 5′-ATC TGA

and this mechanism is referred as the “cysteine switch” of activation (30). Many ADAMs possess a furin recognition site (RXKR) between their pro- and catalytic domains (31, 32). A common mechanism to activate these zymogens is through dissociation of the pro-domain following cleavage of the pro-domain by furin or furin-like proteases in the trans-Golgi (3). ADAMs belong to the metzincin superfamily of metalloproteinasises, which also includes matrix metalloproteinasises (MMPs) and snake venom metalloproteinasises (3). The activation of MMPs is regulated by tissue inhibitors of metalloproteinasises (TIMPs) during tissue remodeling; to date, four zymom classical TIMPs have been identified, which all inhibit the MMPs, but only TIMP-1 and TIMP-3 inhibit some ADAM family members (33).

For instance, TIMP-3 was found to inhibit ADAM12 (27), ADAM17 (34), and ADAM19 (16), whereas TIMP-1 and TIMP-3 inhibited ADAM10 (35). The TIMPs are secreted proteins but may be found at the cell surface in association with membrane-bound proteins. Only TIMP-3 is located in the extracellular matrix via its C-terminal domain binding to heparan sulfate proteoglycan (36). The physiological significance of the inhibition of ADAMs by TIMPs is still unclear, but it has been suggested that characterization of the TIMP inhibition profile of the remaining ADAMs can aid in endogenous substrate identification (7).

In this study, we expressed and purified a soluble recombinant form of human ADAM33 catalytic protein and used it to test a number of potential candidate substrate peptides. The identified ADAM33-cleavable peptide substrates, in combination with the knowledge gained from the study of substrate mutagenesis, were used to characterize the substrate specificity and enzymatic activity of ADAM33 and its regulation by various synthetic and physiological inhibitors.

**Expression of ADAM33 in Drosophila S2 Cells—**The nucleotides of ADAM33 pro- and catalytic domains (bases 91–1227, corresponding to amino acids Leu31–Pro409) were amplified by PCR using a full-length ADAM33 cDNA template (GenBank accession number AF466287) (1). The following PCR primers were used, which added a KpnI site to the 5′-end of the gene fragment: primer 1, 5′-TTC ATC CAT GTA TCA TAG GGT CCG CCT CAA GGA CAT ATC CCT GGG CAG-3′ and nucleotides encoding a His, tag plus a XhoI site at the 3′ end; primer 2, 5′-ATC TGA TAT TTC GAC TGA ATG GTG ATG ATG ATG TCC TGA CCG GTC ATT GGA GAG GCA AGC GC-3′. To generate a mutant ADAM33 construct with a single amino acid mutation from glutamic acid to alanine (E346A), a full-length ADAM33 cDNA with an E346A mutation (19) was used as a template and amplified with the above PCR primer 1 and primer 3 (5′-A TCT GAT TCG ACC CGG ATT GGA GAG GCA AGC GC-3′). This set of primers generated the same 5′- and 3′-ends of the ADAM33 cDNA fragment as the above, whereas its 5′-end fused with V5-His, tag in frame in the vector, pmT/Bip/V5-His-C (Invitrogen). The PCR-amplified cDNAs of wild type (WT) and E346A mutant were digested with KpnI and XhoI restriction enzymes and ligated into the Drosophila cell expression vector, pmT/Bip/V5-His-C. Both the WT and the E346A mutant cDNA constructs were verified by sequencing, and their amino acid sequences were identical to that encoded by GenBank accession number AF466287 (WT), with the exception of the E346A mutation. Each of the plasmid constructs was co-transfected with pcOHYGRO into Drosophila S2 cells according to the manufacturer’s instructions, and stable transfected cells were selected in the growth medium containing 300 μg/ml hygromycin-B. The stable transfected Drosophila S2 cell line was maintained at 23 °C in DESS® medium containing 10% fetal bovine serum, 0.1% fluronic F-68, 300 μg/ml hygromycin-B. Expression of human ADAM33 protein was induced in the presence of 10 μM CdCl2 and 200 μM ZnCl2 in serum-free medium. The culture medium was harvested 6 days post-induction and cleared by centrifugation at 8000 rpm for 20 min.

**Purification and Characterization of Recombinant Human ADAM33—**Conditioned medium was mixed with an equal volume of S-buffer A (25 mM HEPES, pH 6.8, 50 mM NaCl, and 10% glycerol) and then applied to an SP-Sepharose Fast Flow column equilibrated with S-Buffer A, using the AKTA FPLC system (Amersham Biosciences). Bound protein on the column was washed with S-buffer A and 0.1 M NaCl, followed by elution with a linear gradient of NaCl (0.1–0.5 M). Fractions containing ADAM33 were identified by SDS-PAGE, Western blotting (19), or enzyme activity assays. Fractions containing ADAM33 were combined, adjusted to 20 mM imidazole, and loaded onto a Ni2+-NTA column equilibrated with Ni2+-NTA buffer A (25 mM HEPES, pH 7.9, 20 mM imidazole, 500 mM NaCl, and 10% glycerol). The bound protein was washed with Ni2+-NTA buffer A and 0.5 M NaCl, followed by elution with Ni2+-NTA buffer B (25 mM HEPES, pH 7.9, 500 mM imidazole, and 10% glycerol). Fractions containing ADAM33 were combined and concentrated by stirred ultrafiltration cell (Millipore Corp., Bedford, MA) before loading onto a Superdex-75 column equilibrated with G-buffer (25 mM HEPES, pH 7.5, 50 mM imidazole, 150 mM NaCl, and 10% glycerol). Protein was eluted with 1.5× column volumes of G-buffer. Fractions containing ADAM33 were identified by enzyme activity assays and SDS-PAGE. Protein purification was measured by Bradford assay (Bio-Rad), and ADAM33 catalytic domain (ADAM33cat) concentration was estimated by UV absorption using a molar extinction coefficient, \( \epsilon_{280} \), of 26 780 M⁻¹·cm⁻¹, which was calculated by Genetics Computer Group (GCC) software (University of Wisconsin, Madison, WI). The molecular weight was estimated by SDS-PAGE and the isoelectric points of recombinant ADAM33 pro- and catalytic domains (pl = 7.9 and 8.3, respectively).

The N-terminal amino acid sequence of ADAM33 protein was determined as follows. The protein was separated on a 14% Tris-glycine gel (Novex, San Diego, CA), transferred to polyvinylidene fluoride membrane, and analyzed by automated Edman degradation (39). For mass spectrometry sequence identification, Coomassie Blue-stained protein bands were excised from SDS-PAGE gel and digested with trypsin (Promega, Madison, WI) as described (40). Tryptic peptides were then subject to mass spectrometric analysis.

Deglycosylation of ADAM33 by PNGase F (New England Biolabs, Beverly, MA) was done according to the manufacturer’s instructions. Briefly, purified ADAM33 protein was denatured in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) for 10 min at 100 °C prior to deglycosylation with PNGase F for 2 h at 37 °C. The sample was then analyzed by SDS-PAGE.

**Peptide Synthesis—**Peptides were synthesized in house or purchased from commercial suppliers (San Jose, CA). Fmoc (N-9-fluorenylmethoxycarbonyl) chemistry was used on either an ABI model 431A Peptide Synthesizer (Applied Biosystems, Foster City, CA) or an ACT model 496 multiple synthesizer (Advanced ChemTech, Louisville, KY). The molecular masses of purified peptides were confirmed by electrospray ionization mass spectrometry.

**Protein Cleavage Assay—**The protein substrate (50 μM) was incubated with or without ADAM33 (0.1–0.5 μM) in assay buffer (20 mM HEPES, pH 7.5, 0.5 M NaCl, 0.2 mg/ml bovine serum albumin) for 2 h at room temperature in the presence or absence of various inhibitors (5 mM 1,10-phenanthroline or a protease inhibitor mixture (2 μg/ml leu-
peptin, 0.4 μM benzamidine, 10 μg/ml soybean trypsin inhibitor, and 0.5 mM iodoacetamide) (17). The reaction was quenched by adding 10% trifluoroacetic acid to a final concentration of 1%, and samples were analyzed on an Agilent model 1100 high performance liquid chromatograph with C8 column (4.6-mm inner diameter × 50-mm length). Solvents were as follows: A, 0.1% trifluoroacetic acid in water; B, 0.09% trifluoroacetic acid in acetonitrile. A linear gradient from 2 to 42% B was run over 7 min at 1.5 ml/min, and the eluate was monitored at 214 nm. The percentage of peptide cleavage was calculated using the peak area of the cleaved products divided by the sum of the peak areas of both the products and the remaining substrate. The cleavage sites of the peptide were identified by either electrospray or matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

**Kinetic Studies**—Kinetic constants of peptide substrates, APP, and KL-1, were obtained by using the HPLC assay described above and analysis as reported previously (8, 41, 42). In brief, ADAM33cat (80 nM) conditioned cell medium at 6 days after Cd²⁺ induction; lane 2, eluate by a gradient of 300–500 mM NaCl from SP-Sepharose column; lane 3, eluate from Ni²⁺-NTA column by 250 mM imidazole; lane 4, initial peak eluted from Sephadex-75 column; lane 5, second peak eluted from Sephadex-75 column (catalytic domain alone). The 14% reducing gel was stained with Coomassie Blue. The SeeBlue Plus2 protein standard was used as size marker (Invitrogen).

**Western analysis of eluate (sample from Fig. 1B, lane 4) from Sephadex-75 with specific antibodies against the pro-domain (Pro), the catalytic domain (Cat), or His₆ tag, respectively.**

**C**, Western analysis of eluate (sample from Fig. 1B, lane 4) from Sephadex-75 with specific antibodies against the pro-domain (Pro), the catalytic domain (Cat), or His₆ tag, respectively.

**D**, deglycosylation by PNGase of purified ADAM33 catalytic protein. Lane 1, untreated; lane 2, PNGase F for 2 h at 37 °C; lane 3, PNGase F alone, as control. The 14% reducing SDS-PAGE gel was stained with Coomassie Blue. The BenchMark™ protein ladder (Invitrogen) was used as a size marker.
**Inhibitor Titration of ADAM33cat to Determine Active Enzyme Concentration**—A fluorogenic substrate, R(Dabcyl)LPPVAASSLRNDKDQKL209, was used to measure ADAM33 enzyme activity. The concentration of ADAM33cat was determined to be 780 μM, using a molar extinction coefficient of 26,780 M−1 cm−1 at 280 nm. The enzyme was titrated with a serial dilution of inhibitor K6682 (0–2 μM) in the presence of substrate (30 μM) in assay buffer (20 mM HEPES, pH 7.5, 0.5 mM CaCl2, and 0.1 mM MgCl2) at 37 °C. The velocity was measured at excitation/emission wavelength 340/505 nm with a GEMINI fluorescence reader (Molecular Devices, Sunnyvale, CA) for 5 min. The initial velocities were plotted as a function of inhibitor concentration.

Transfected cells (in quadruplicate wells per experimental group) were cotransfected with plasmids (pcDNA3.1) containing full-length ADAM33, ADAM33E/A, APP, or KL-1. After 5 h, the transfected cells were maintained in culture for an additional 24 h. The plasmids used for transfection were titrated with a serial dilution of inhibitor IK682 (0–25 μM) based on peptide KL-1, was synthesized and used to measure ADAM33 enzyme activity. The concentration of ADAM33cat was determined to be 780 μM, using a molar extinction coefficient of 26,780 M−1 cm−1 at 280 nm. The enzyme was titrated with a serial dilution of inhibitor K6682 (0–2 μM) in the presence of substrate (30 μM) in assay buffer (20 mM HEPES, pH 7.5, 0.5 mM CaCl2, and 0.1 mM MgCl2) at 37 °C. The velocity was measured at excitation/emission wavelength 340/505 nm with a GEMINI fluorescence reader (Molecular Devices, Sunnyvale, CA) for 5 min. The initial velocities were plotted as a function of inhibitor concentration.

**RESULTS**

**Expression and Purification of Recombinant Human ADAM33**—To study the catalytic activity of ADAM33, a cDNA construct of human ADAM33 pro- and catalytic domains, corresponding to amino acids 31–409, was made to express a soluble form of catalytic protein from pro-ADAM33. The pro-domain (amino acids 1–30) was included in the expression construct to allow for proper protein folding and processing (50), and a His6 tag was placed on the C terminus of catalytic domain to aid purification and thiol redox isoelectric focusing, and equivalent protein was separated by electrophoresis through 10% SDS-polyacrylamide gels (Bio-Rad). Molecular mass was estimated by comparison with Kaleidoscope (Bio-Rad) or BenchMark (Invitrogen) prestained standards included on every gel. Proteins were transferred to polyvinylidene fluoride membranes by electroblotting. For immunodetection, membranes were blocked with 5% ECL blocking solution (Pierce, Rockford, IL) or sheep anti-mouse IgG conjugated to horseradish peroxidase (1:25,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or sheep anti-mouse IgG conjugated to horseradish peroxidase (1:25,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or sheep anti-mouse IgG conjugated to horseradish peroxidase (1:25,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or sheep anti-mouse IgG conjugated to horseradish peroxidase (1:25,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or sheep anti-mouse IgG conjugated to horseradish peroxidase (1:25,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or sheep anti-mouse IgG conjugated to horseradish peroxidase (1:25,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

**Peptides not cleaved by ADAM33**

| Peptide | Full name | Sequence and cleavage site | Reference |
|---------|-----------|----------------------------|-----------|
| α1-PI   | α1-PI     | [LEAPM]SIPPEVK             | 41        |
| ACE     | Angiotensin-converting enzyme | TPNSAR | SEGPLPDSGR | 53        |
| ACE     | Angiotensin-converting enzyme | AQQAR | VQQWL | 51        |
| ACE     | Angiotensin-converting enzyme | EAGQR | LATAM | 51        |
| ACE     | Angiotensin-converting enzyme | PNSAR | SEGPL | 51        |
| AR(C)   | Amphiregulin | ERCEK | SMKTHS | 54        |
| AR(N)   | Amphiregulin | SVRVEQ | VKVPFP | 54        |
| BTC(C)  | B-cellulin | RSPETN | LLLC | 54        |
| BTC(N)  | B-cellulin | RPDYLF | LGDRG | 54        |
| CD40L   | KENSFM | QRGQAQ | 35        |
| EGF(C)  | Epidermal growth factor | KWSELR | HAGHQ | 54        |
| EGF(N)  | Epidermal growth factor | HHYSVB | NSDSEC | 54        |
| EPR(C)  | Epiregulin | NFRVAQ | VSHTRC | 54        |
| EPR(N)  | Epiregulin | ESSKPMDIAT | 41        |
| Fibrinogen peptide 1 | Fibrinogen | FHIITKLTVSTKG | 41        |
| Fibrinogen peptide 2 | Fibrinogen | SGKDKEKGTE | 41        |
| Fibrinogen peptide 3 | Fibrinogen | FHHTEKLVTSKG | 41        |
| Fibrinect | Fibrinect | FHHTEKLVTSKG | 41        |
| Folate R | Folate receptor | EEEVA | R | 7        |
| HB-EGF  | Heparin-binding EGF-like growth factor | HGLSL | PVENRL | 41        |
| HRGβ1 linker | Neuregulin β1 | PNEFTGDRCQNYVMA53YIEFMEA41EYKRVLT | 15        |
| Syndecan-1 | | | | |
| Syndecan-4 | | | | |
| IGFBP-1 | Insulin-like growth factor-binding protein-1 | | | |
| IL-1Rc | Interleukin-1 receptor c | TVKESV | STFSWG | 7        |
| IL-6R | Interleukin-6 receptor | ANATSIVQ | DSSSVP | 8        |
| L-selectin | | | | |
| L-selectin | | | | |
| MBP | Myelin basic protein | | | |
| Notch | | | | |
| P55-TNFFR | Tumor necrosis factor-a receptor, p55 | LQIEN | VKGTED | 8        |
| P75-TNFFR | Tumor necrosis factor-a receptor, p75 | SMAPOA | VHLPQP | 8        |
| Pre-TNFα | Pre-tumor necrosis factor-α | | | |
| TGF-α(C) | Transforming growth factor | | | |
| TGF-α(N) | Transforming growth factor | | | |
| Vitronectin | Vitronectin | | | |

θ C, C-terminal; N, N-terminal.
To facilitate efficient secretion from \textit{Drosophila} cells, the native signal sequence of ADAM33 was replaced by the \textit{Drosophila} BiP protein signal sequence in the expression vector, pMT/BiP. The expression of the ADAM33 gene was under the control of the inducible metallothionein promoter (pMT). To investigate the requirements for catalytic activity, a second construct was made with a single amino acid mutation (E346A) at the catalytic site of ADAM33, which is known to abolish the catalytic activity of Zn$^{2+}$/H$_{11001}$-dependent metalloproteases (8) and ADAM33 (19).

Purification of soluble ADAM33 protein from \textit{Drosophila} S2 cell conditioned medium was done with multiple sequential chromatography steps that included ion exchange, Ni$^{2+}$-NTA affinity, and gel filtration. SDS-PAGE analysis at each chromatography stage is shown in Fig. 1B. Three prominent protein bands were observed during chromatography: a doublet at \(~30\) kDa and a faster moving band at \(~25\) kDa. All three bands were significantly enriched by cation exchange chromatography (lane 2, SP-Sepharose elution) and were further purified after the affinity column (lane 3, Ni$^{2+}$-NTA elution). The \(~30\)-kDa doublet bands were separated from the \(~25\)-kDa protein by gel filtration (lanes 4 and 5). N-terminal sequencing of the \(~30\)-kDa doublet bands identified a single N-terminal sequence as EARR, confirming that the first of three potential furin recognition sites was used for cleavage of the pro-domain in \textit{Drosophila} cells, as observed for human ADAM33 expressed in mammalian cells (19). The \(~25\)-kDa band was also sequenced, and the N terminus sequence was determined to be RSPWPGVP\_LQG, in which the first eight amino acids corresponded to the vector DNA sequence (5'-end to the cloning site KpnI); the last three amino acids (LQG) matched the sequence of the pro-domain starting at Leu31. The \(~25\)-kDa protein was further digested by trypsin, followed by mass spectrometry analysis of the digested peptide fragments. The results confirmed the \(~25\)-kDa band as the pro-domain of ADAM33. In addition, Western blot analysis of the Sephadex-75 eluate proteins (Fig. 1B, lane 4) showed that the doublet of \(~30\) kDa was recognized by antibodies to the ADAM33 catalytic domain (Fig. 1C, lane 2) or the carboxyl-terminal His$_6$ tag (lane 3), whereas the \(~25\)-kDa lower band was recognized by antibody specific to the ADAM33 pro-domain (Fig. 1C, lane 1). Thus, the ADAM33 pro-domain remained associated with the catalytic domain after furin cleavage. The association of pro- and catalytic domains was not due to disulfide bond formation, because the


| Peptide substrate | Sequence | ADAM8 | ADAM9 | ADAM10 | ADAM17 | ADAM19 | ADAM33 |
|-------------------|----------|-------|-------|--------|--------|--------|--------|
| KL-1              | A|L|S|H|I|L | ADAM9p | ADAM10p | ADAM17p | ADAM19p | ADAM33p |
| APP               | A|S|L|I|L | ADAM9p | ADAM10p | ADAM17p | ADAM19p | ADAM33p |
| TRANCE            | Y|E|V|H|I | ADAM9p | ADAM10p | ADAM17p | ADAM19p | ADAM33p |
| Insulin B chain   | H|Y|L | ADAM9p | ADAM10p | ADAM17p | ADAM19p | ADAM33p |

Note that all of the substrate sequences are from human peptides except insulin B chain, which is from bovine. A downward arrow denotes the cleavage site. NC, no cleavage.

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\begin{array}{|c|c|c|c|c|c|c|}
\hline
\text{Peptide substrate} & \text{Sequence} & \text{ADAM8} & \text{ADAM9} & \text{ADAM10} & \text{ADAM17} & \text{ADAM19} & \text{ADAM33} \\
\hline
\text{KL-1} & A | L & S & H & I & L & \text{ADAM9p} & \text{ADAM10p} & \text{ADAM17p} & \text{ADAM19p} & \text{ADAM33p} \\
\text{APP} & A | S & L & I & L & \text{ADAM9p} & \text{ADAM10p} & \text{ADAM17p} & \text{ADAM19p} & \text{ADAM33p} \\
\text{TRANCE} & Y | E | V | H | I & \text{ADAM9p} & \text{ADAM10p} & \text{ADAM17p} & \text{ADAM19p} & \text{ADAM33p} \\
\text{Insulin B chain} & H | Y | L & \text{ADAM9p} & \text{ADAM10p} & \text{ADAM17p} & \text{ADAM19p} & \text{ADAM33p} \\
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The catalytic activity of ADAM33 toward peptide substrates—

### Protease activity of Drosophila cell-expressed ADAM33 catalytic protein

The protease activity of Drosophila cell-expressed ADAM33 catalytic protein was demonstrated by an α, macrocogulbin assay (data not shown), which confirmed activity of mammalian cell-expressed ADAM33 in the same assay (19). Here we further explored the protease activity of ADAM33cat with peptide substrates. Many membrane-anchored proteins are shed from the cell membrane by metalloproteases (51). To identify peptide substrates of ADAM33cat, peptides corresponding to cleavage sites of proteins known to be cleaved by ADAMs and MMPs were tested for cleavage by ADAM33 (7, 8, 11, 41, 51). Table I lists peptides that showed little or no cleavage by ADAM33.

The difference between the apparent molecular masses of the catalytic domain (~30 kDa) and pro-domain (~25 kDa) by reducing SDS-PAGE and their predicted masses of 23.5 and 20 kDa, respectively, was most likely due to protein glycosylation. Sequence analysis predicted two potential N-linked glycosylation sites in the catalytic domain (Asn[109] and Asn[145]) and in the pro-domain (Asn[109] and Asn[145]). Deglycosylation by peptide:N-glycosidase F (PNGase) resulted in reduction of the catalytic domain doublet of ~30 kDa to a single band (~25 kDa) (Fig. 1D). Similarly, deglycosylation of the pro-domain by PNGase also reduced the apparent molecular mass of the pro-domain from ~25 to ~20 kDa (data not shown).

### Catalytic Activity of ADAM33 toward Peptide Substrates

Catalytic activity of ADAM33 toward peptide substrates was assessed by HPLC analysis of tryptic digests of pro-domain (100 ng) incubated at 37°C for 2 h with ADAM33 (200 nM). Digests were analyzed by HPLC (Fig. 1E). The cleavage was inhibited by the Zn²⁺ chelator, 1,10-phenanthroline (Fig. 2C), and the catalytically inactive mutant ADAM33E346A was unable to cleave APP28 (Fig. 2D). 

Four peptides from the following proteins were found to be cleaved by ADAM33: KL-1 (51), APP (55), TRANCE (56), and insulin B chain (17). A representative mass spectrometry analysis of APP cleavage is shown in Fig. 2. The 28-amino acid APP peptide (APP28) ran as a single peak with monoisotopic mass (m/z) of 3261.432 on mass spectrometry (Fig. 2A). A significant amount of APP28 was cleaved by incubation with ADAM33, resulting in two products with masses (m/z) of 1698.726 and 1581.827 (the third peak of mass 1603.882 is the sodium adduct (Na⁺) of a derivative). The cleavage was inhibited by the Zn²⁺ chelator, 1,10-phenanthroline (Fig. 2C), and the catalytically inactive mutant ADAM33E346A was unable to cleave APP28 (Fig. 2D), indicating that the cleavage was mediated by ADAM33cat and required the glutamate residue.

The ADAM33 cleavage site(s) of each peptide (KL-1, APP, TRANCE, and insulin B chain) are summarized in Table II. The cleavage of KL-1 peptide is shown in Fig. 3A, and the relative cleavage efficiencies of the peptides were determined by HPLC. Reactions, including 200 nM active ADAM33cat and 100 μM peptide, resulted in 34% of KL-1, 15% of APP, 6% of TRANCE, and insulin B chain within 2 h. The site of cleavage by ADAM33 was compared with that of other ADAMs (Table II). ADAM33 cleaved the APP peptide at His(1)Gln, as did...
ADAM8 and ADAM9. In contrast, the α-secretases ADAM17 and ADAM10 cleaved at Lys-I-Leu. The site of cleavage of KL-1 by ADAM33 was the same as that of ADAM8, ADAM17, and ADAM19 (Ala-Ser), which is one of two sites used physiologically in the shedding of the soluble KL-1 protein (51). In contrast, ADAM9 cleavage of KL-1 peptide occurred at multiple sites (Ser-Ser, Ala-Ser, and Ala-Ala). Although ADAM33, ADAM19, ADAM9, and ADAM17 could cleave insulin B chain, the cleavage sites differed (Ala-Leu by ADAM33 and ADAM19; Tyr-Leu by ADAM9 and ADAM17). In addition, ADAM33 cleaved human TRANCE at His-Ile, which corresponds to the ADAM19 cleavage site (Arg-Phe) of mouse TRANCE, a much less efficient substrate for human ADAM33 (data not shown).

Kinetic constants \(k_{cat}, K_m\), and \(k_{cat}/K_m\) for KL-1 and APP were determined. The initial cleavage velocities of the peptides were measured under conditions of low turnover (<15%) so that the substrate concentration did not change significantly. The \(k_{cat}, K_m\), and \(k_{cat}/K_m\) values of KL-1 were determined to be 0.7 ± 0.1 s\(^{-1}\), 3.0 ± 0.1 mM, and \((2.6 ± 0.5) \times 10^2\) M\(^{-1}\) s\(^{-1}\) \((n = 3)\), respectively (Fig. 3B). For APP, the \(k_{cat}, K_m\), and \(k_{cat}/K_m\) values were 0.2 ± 0.1 s\(^{-1}\), 1.0 ± 0.1 mM, and \((1.6 ± 0.3) \times 10^2\) M\(^{-1}\) s\(^{-1}\) \((n = 2)\), respectively.

A fluorogenic substrate was generated by labeling the KL-1 peptide with Dabcyl/Edans, and the substrate was used to determine the active ADAM33 enzyme concentration by active site titration with the hydroxamate-based inhibitor IK682 (57) (Fig. 3C). The titration analysis revealed that the concentration of active ADAM33 was 53 ± 5% \((n = 4)\) of the total enzyme concentration. The concentration of active ADAM33 enzyme was used to calculate kinetic constants.

**Alanine/Glycine-scanning Mutagenesis of KL-1 Peptide**—The substrate specificity of ADAM33 was investigated using alanine/glycine-scanning mutagenesis of the peptide substrate KL-1. Each of the 12 individual amino acid residues of the peptide was substituted by alanine (or glycine for alanine in the original sequence) to probe the relative contributions of the residues to cleavage efficiency. The 12 alanine/glycine-scanning peptides were compared with the WT KL-1 peptide. As shown in Fig. 4, the side chains of the original residues at P3, P2, and P1' sites proved most critical, being intolerant of small truncations of the aliphatic side chains (Val→Ala, Ala→Gly, Leu→Ala). The residues at P6, P2', and P1' contributed marginally to the cleavage efficiency, whereas the side chains of P5, P4, and P1' residues were dispensable. A slight improve-
Catalytic Activity of Human ADAM33

The substrate KL-1 peptide (LPPVAA | SSLRND) was substituted by Ala at each of the individual residues except at P2 and P1 positions, where Gly instead of Ala was used. The 12 dodecamers (50 μM) were incubated with ADAM33 (80 nM) for 2 h at room temperature, followed by HPLC analysis. The relative cleavage activity of ADAM33 for each mutant peptides was obtained by comparison with WT KL-1 peptide (n = 2).

Inhibition of ADAM33 Activity—The cleavage of the KL-1 peptide by ADAM33 was used to evaluate the effect of different classes of protease inhibitors. Inhibition constants of several hydroxamate compounds, known to inhibit either ADAM17 (IK682 (57) and the Immunex compound 1 (45)) or MMPs (Marimastat (58)), were determined (Fig. 5). The compound IK682 was the most potent inhibitor of ADAM33 activity, with a K_i value of 23 ± 7 nM (n = 2). Marimastat was a less potent inhibitor, with a K_i value of 160 ± 40 nM (n = 2). The Immunex compound 1 was the least potent, with a K_i value of 2800 ± 150 nM (n = 2).

Human TIMP-1, -2, -3, and -4, endogenous inhibitors of MMPs and ADAMs (33), were tested for inhibition of ADAM33 activity (Table III). TIMP-3 (K_i = 60 ± 20 nM, n = 2) and TIMP-4 (K_i = 220 ± 20 nM, n = 2) demonstrated significant ADAM33-inhibitory activity, whereas there was no inhibition by TIMP-1 at 2 μM and weak inhibition by TIMP-2 (K_i = 1400 ± 300 nM, n = 2). By comparison, human ADAM17 activity was inhibited by TIMP-2 (K_i = 11 ± 0.9 nM, n = 2), TIMP-3 (K_i = 1.1 ± 0.5 nM, n = 3), and TIMP-4 (K_i = 92 ± 25 nM, n = 3) and weakly inhibited by TIMP-1 (K_i = 760 ± 160 nM, n = 2) (Table III).

Identification of a protein as an endogenous substrate of an ADAM requires several confirmatory steps (2). Toward that end, we examined the ability of recombinant full-length ADAM33 to exhibit sheddase activity in an HEK293 cell-based cotransfection system. APP and KL-1 were evaluated, because peptides derived from the membrane-proximal region of each were cleaved significantly by ADAM33 in the enzyme activity assay (Table II, Figs. 2 and 3). Following cotransfection of full-length ADAM33 or full-length ADAM33E/A, the inactive mutant, and KL-1, secreted KL-1 was measured by enzyme-linked immunosorbent assay and compared with control transfections (pcDNA3.1(−) plasmid + KL-1). The combined results of four independent cotransfection experiments quantitating secreted KL-1 are shown in Fig. 6A. Relative to control (pcDNA3.1(−) + KL-1), the amount of secreted KL-1 was higher (138 ± 54%) in the presence of ADAM33. A similar increase in secreted KL-1 relative to control was observed with PMA stimulation (126 ± 53%). In contrast, secreted KL-1 was somewhat less in cultures cotransfected with KL-1 and the inactive mutant ADAM33E/A, in the absence of PMA (75 ± 22%) or the presence of PMA (75 ± 19%) as compared with control. Western blotting analysis of transfected cell lysates indicated an equivalent expression level of ADAM33 and ADAM33E/A (data not shown; see Fig. 6C). This indicates that ectopically expressed full-length KL-1 is cleaved to a small extent by ADAM33 and that this is not regulated by PMA stimulation.

Similar studies were done with APP. Soluble, secreted APP was detected in the culture medium of transfected cells by Western blotting using the W0–2 antibody (Fig. 6B), which detects secreted APP cleaved at the α-secretase site (38). In the same cultures, the expression of ADAM33, ADAM33E/A (Fig. 6C), and APP (data not shown) was monitored in the transfected cell lysates. Soluble APP was detected in the culture medium only when full-length APP was ectopically expressed (Fig. 6B, lanes 3, 5, 9, and 13), and its expression was greater in PMA-stimulated cultures (lanes 4, 6, 10, and 14). Interestingly, when APP was cotransfected with ADAM33, lower levels of soluble APP were detected than in control cultures (Fig. 6B, lane 9 versus lane 3). Although PMA also increased the level of secreted APP when cotransfected with ADAM33, the level detected was also less than PMA-stimulated controls (Fig. 6B, lane 10 versus lane 4). In contrast, the level of APP in the culture medium of cells transfected with ADAM33E/A and APP was similar to that of controls in the absence (Fig. 6B, lane 13 versus lane 3) and presence of PMA (Fig. 6B, lane 14 versus lane 4). The expression levels of ADAM33 and ADAM33E/A were similar in cell lysates of APP-cotransfected cultures (Fig. 6C, lane 9 versus lane 13) and after PMA stimulation (Fig. 6C, lane 10 versus lane 14), although each was expressed at a lower level than when expressed alone (Fig. 6C, lane 9 versus lane 7, lane 10 versus lane 8, lane 13 versus lane 11, and lane 14 versus...
A comparable level of APP expression was observed in unstimulated control, ADAM33, and ADAM33E/A cotransfected samples and also among the same samples in the presence of PMA (data not shown). These findings were reproduced in another independent transfection experiment, and identical results were observed with mouse ADAM33 or mouse ADAM33E/A with APP cotransfection (data not shown). Collectively, this suggests that ADAM33 is not an H9251-secretase and does not directly act on APP.

DISCUSSION

In this study, we report the first biochemical characterization of the catalytic activity of human ADAM33. Our strategy was to express and purify recombinant ADAM33 catalytic protein and evaluate candidate peptides as substrates. The ADAM33-cleavable peptide substrates identified were used to examine the substrate specificity and to characterize ADAM33 enzymatic activity and its inhibition by various synthetic compounds and physiological regulators. Last, the catalytic activity of recombinant full-length ADAM33 was assessed via cotransfection with potential substrates in HEK293 cells.

The expression system that we chose for the production of the soluble recombinant human ADAM33 catalytic domain protein was Drosophila S2 cells, because efficient conversion of the pro-form to the mature form of ADAM33 was consistently observed. In contrast, when using the baculovirus insect cell expression system, only a small portion of recombinant ADAM33 protein was secreted and processed to the mature form, whereas the majority of the protein was trapped inside the cell as a pro-form (data not shown). The site of pro-domain cleavage was highly conserved. Among the three cell expression systems tested (baculovirus-infected insect cells, Drosophila S2 cells, and mammalian cells (19)), mature ADAM33 protein shared an identical N terminus, EARR, suggesting that only the first of three potential furin cleavage sites was utilized by furin or a furin-like enzyme.

Interestingly, the pro-domain remained strongly associated with the catalytic domain after endoproteolytic cleavage and during several purification steps. A similar phenomenon was reported for ADAM12-S (27), ADAM15 (12), and ADAM17 (50). It is known that many MMPs and ADAMs are synthesized in a latent form that is maintained by the formation of a thiol-zinc bond between a conserved cysteine in the pro-domain and the catalytic Zn\(^{2+}\) in the metalloprotease domain (28, 29). Activation of the ADAM zymogens can be achieved by endoproteolytic cleavage of the pro-domain (59) and/or by oxidation and dissociation of the cysteine thiol-zinc linkage, referred to as the cysteine switch mechanism (30). Full-length ADAM17 zymogen can also be activated by alkylation of the pro-domain thiol with aminophenyl mercuric acetate (50). However, it is unlikely that the association of the cleaved pro- and catalytic domains of ADAM33 observed here is due to the cysteine switch mechanism for the following reasons. First, the association of pro- and

| Human TIMP inhibition dissociation constants, \(K_i\) | TIMP-1 | TIMP-2 | TIMP-3 | TIMP-4 |
|---|---|---|---|---|
| ADAM33\(^{b}\)| - | 1400 ± 300 | 60 ± 20 | 220 ± 20 |
| ADAM17\(^{\#}\)| 760 ± 160 | 11 ± 0.9 | 1.1 ± 0.5 | 92 ± 25 |

\(^{a}\) no inhibition; values are mean ± S.D.  
\(^{b}\) The assay was performed with 60 nM human ADAM33cat and 100 \(\mu\)M KL-1 substrate (\(n = 2\)).  
\(^{\#}\) The assay was performed with 1 nM human ADAM17 and 25 \(\mu\)M TNF-\(\alpha\) substrate (TIMP-1 and -2: \(n = 2\); TIMP-3 and -4: \(n = 3\)).
catalytic domains of ADAM33 was not due to the cysteine thiol-zinc linkage, because dissociation was not observed by treatment with aminophenyl mercuric acetate. Second, the purified pro- and catalytic domain complex was enzymatically active, evident by its cleavage of peptide substrates with an efficiency similar to the catalytic domain only (data not shown). Last, mouse ADAM33 (24, 25) lacks the cysteine residue corresponding to the potential cysteine switch (Cys<sup>179</sup>) of human ADAM33 (1) and other ADAMs (32), and thus, ADAM33 activity may be regulated by other mechanisms (60).
The identification of synthetic peptide substrates and their cleavage sites by ADAM33 allowed us to look into the substrate specificity of this enzyme. Of the 40 synthetic peptides tested, which were derived from the known cleavage sites of substrates of MMP and ADAMs, four were cleaved by ADAM33. From alignment of this limited set of peptide substrates (Table II), no consensus cleavage site was obvious for ADAM33. However, the cleavage site was not random. For example, small residues with uncharged side chains such as valine/serine (P3) or leucine/alanine (P3') dominated at the P3 and P3' positions, whereas the P1 position was either histidine or alanine. Indeed, a single amino acid substitution at positions P3, P2, P1, or P3' of the KL-1 peptide reduced ADAM33 activity by more than 90%, suggesting limited flexibility of amino acid residues in these positions. The residue preference in these key positions might reflect restrictions in shape and size of the binding pocket in the active site, and further elucidation of this will require the crystal structure of the active site. By alignment of eight peptides cleaved by ADAM8, Fourie et al. (11) demonstrated that this enzyme mainly cleaves between hydrophilic or polar amino acids, although general conclusions about site specificity from such a small collection of peptide substrates could not be made. Our study of ADAM33 and others of ADAM9 (8), ADAM17 (52), and ADAM28 (18) support the general notion that ADAMs have distinct cleavage preferences in vitro, although clear consensus cleavage sites for each enzyme have not been identified. The substrate specificity in vitro is probably influenced by additional factors such as secondary structure and proximity to the membrane (5).

Mohan et al. (52) reported that the substrate selectivity of ADAM17 can be defined by comparing specificity constants ($k_{cat}/K_m$ values) for each of its synthetic substrates. They reported that the $k_{cat}/K_m$ value for TNF-α (1.7 × 10^5 M^−1 s^−1) was at least 100–1000-fold higher than that of eight other peptide substrates (APP, HER4, KL-1, TRANCE, TNFR-II, TNFR-I, interleukin-6 receptor, and NOTCH). The cleavage of APP and TNF-α by ADAM9 was also very efficient, with $k_{cat}/K_m$ values of 2 × 10^5 and 6 × 10^5 M^−1 s^−1, respectively (8). As a comparison, we measured the specificity constants for the two synthetic peptide substrates of ADAM33, KL-1 and APP ($k_{cat}/K_m$; KL-1, 2.6 ± 0.5) × 10^5 M^−1 s^−1 and APP, (1.6 ± 0.3) × 10^2 M^−1 s^−1, respectively). This indicates that the KL-1 peptide is a better ADAM33 substrate than the APP peptide and that neither the KL-1 nor APP peptide is an optimal substrate for ADAM33. Thus, this indicates that a more efficient substrate for ADAM33 has yet to be identified.

The identification of a synthetic peptide substrate for ADAM33 made it possible to measure the specific activity of this enzyme and allowed us to test its sensitivity to various inhibitors. ADAM33 activity was inhibited by the hydroxamate-based inhibitors, IK682, Marimastat, and the Immunex compound 1. For each of these, ADAM33 was less sensitive to inhibition than ADAM17 (8, 45, 57), and for Marimastat, ADAM33 was less sensitive to inhibition than MMP-1, -3, -9, and -13 but more sensitive than ADAM9 (8). This inhibitor profile of ADAM33 suggests that the binding pocket of the ADAM33 active site shares some similarities with but is also distinct from that of other metalloproteinases. Therefore, it may be possible to develop small, selective synthetic inhibitors of ADAM33.

TIMPs are a group of natural protein inhibitors of MMPs. The balance between TIMPs and MMPs is critical during tissue remodeling (33). Recently, it was found that some members of the ADAM family could also be inhibited by TIMP-3 and TIMP-1 (summarized in Table IV). For example, ADAM10 was inhibited by TIMP-1 and TIMP-3 but not TIMP-2 (35); ADAM12 was inhibited by TIMP-3 but not TIMP-1. In contrast, ADAM9 was not inhibited by TIMP-1, -2, and -3 (7), whereas ADAM8 was not inhibited by any of the four TIMPs (6). Mouse ADAM17 catalytic domain was reported to be inhibited by human TIMP-3 but not by human TIMP-1 and -2 and mouse TIMP-4 (34). In this study, we found that the activity of human ADAM17 catalytic domain versus a TNFα peptide substrate was also inhibited by human TIMP-2 and -4 in addition to human TIMP-3 and was weakly inhibited by human TIMP-1 (Table III). The discrepancy of ADAM17 inhibition by TIMP-2 and TIMP-4 between that reported by Amour et al. (34) and our study is probably due to differences in the species of ADAM17 and TIMPs tested and/or assay conditions. It is also worth noting that in many of the studies cited above (ADAM-8, ADAM-9, ADAM-10, and ADAM-12), TIMP activity was assessed by its ability to inhibit the degradation of myelin basic protein or insulin-like growth factor-binding protein-3 and -5. Additional studies with peptide substrates for each ADAM are warranted. With a peptide substrate, we observed that ADAM33 activity was inhibited by TIMP-3 and -4 and slightly by TIMP-2 but not TIMP-1. The physiological relevance of these observations and the ability of TIMPs to inhibit ADAM33 in cells remains to be determined.

Identification of a protein as a physiological substrate of an ADAM or MMP requires several confirmatory steps (2). Toward that end, we examined the ability of recombinant full-length ADAM33 to exhibit sheddase activity in an HEK293 cell-based cotransfection system. APP and KL-1 were evaluated, because peptides derived from the membrane-proximal region of each were cleaved significantly by ADAM33 in the enzyme activity assay. In addition, both APP and KL-1 (61) are expressed in cells and tissues in which ADAM33 is also expressed (data not shown) (47), and thus each represented a reasonable candidate physiological substrate. In contrast, although a juxtamembrane peptide of TRANCE was also cleaved in the peptide enzyme assay, TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival (62), is highly and ubiquitously expressed in hematopoietic cells (63), where ADAM33 is notably absent (24, 47); hence, TRANCE is unlikely to be a physiological substrate given the evidence that ADAMs cleave in cis only (22).

Our studies of cell-based cleavage of KL-1 and APP revealed interesting activities of ADAM33. Consistent with the observation that the ADAM33 cleavage site of the juxtamembrane KL-1 peptide is the site used physiologically (17, 51) and the inefficient cleavage observed in cell-free enzyme assay, ADAM33 also mediated weak constitutive shedding of KL-1 in a transfected cell system, which was not regulated by protein kinase C activation via PMA stimulation. Moreover, the
ADAM33/EA mutant was not inert but rather reduced the amount of KL-1 shed relative to control, suggesting dominant-negative effects. However, given that we were unable to detect KL-1 in the cell lysates by Western blot, the reduction in KL-1 shedding with cotransfected ADAM33/EA could be due to decreased expression instead of a dominant negative effect. Additional levels of confirmation (2) remain to be done to interpret these findings, including the use of embryonic fibroblasts from ADAM33 knock-out mice to determine whether ADAM33 is required for constitutive or activated KL-1 shedding or whether it can function as a sheddase only in cells where both are highly expressed.

In contrast to the increased cell-based shedding of KL-1 by ADAM33 observed in transfected cells, decreased soluble APP was observed in ADAM33 cotransfected cultures and, in contrast to KL-1, no effect was seen with the inactive mutant of ADAM33. These findings indicate that ADAM33 may be a negative regulator of APP shedding in a cellular environment. Interestingly, ADAM19, a closely related homolog of ADAM33 (24, 25), also serves as a negative regulator of KL-1 shedding in different cell types (17). This suggests the possibility that a mechanism, such as inactivation of a sheddase, shared by both ADAM33 and ADAM19 underlies this phenomenon.

In summary, we have demonstrated proteolytic activity of purified human ADAM33 catalytic protein using four synthetic peptide substrates, KL-1, APP, TRANCE, and insulin B chain. The cleavage site of KL-1 by ADAM33 is identical to the known sheddase cleavage site, and ADAM33 does act as a sheddase of KL-1 in a cell-based transfection assay. Additional confirmatory studies must be done to understand the physiological significance of this. In contrast, the cleavage site of APP by ADAM33 is different from the α-secretase cleavage site, indicating that ADAM33 is not an α-secretase. The inhibitor profile of the synthetic hydroxamate-based compounds and the natural inhibitor TIMPs provides a useful set of tools for further characterization of the biological role of ADAM33 in cellular events. Last, the kinetic analysis of ADAM33 substrates indicates that more efficient substrates have yet to be identified.

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