

The soluble form of a disintegrin and metalloprotease 33 promotes angiogenesis: Implications for airway remodeling in asthma

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Background: A disintegrin and metalloprotease (ADAM)–33 is a susceptibility gene for asthma and chronic obstructive pulmonary disease whose function remains unknown.

Objective: Because asthmatic bronchoalveolar lavage fluid contains high levels of soluble ADAM33 (sADAM33), which includes the catalytic domain, we postulated that its release from cell membranes might play functional roles in airway remodeling by promoting angiogenesis.

Methods: The proangiogenic activity of the highly purified catalytic domain of ADAM33 or a catalytically inactive mutant was studied *in vitro* (Matrigel assay), *ex vivo* (human embryonic/fetal lung explants) and *in vivo* (chorioallantoic membrane assay). The regulation of sADAM33 release from cells overexpressing full-length ADAM33 and its biological activity were characterized.

Results: We show that the purified catalytic domain of ADAM33, but not its inactive mutant, causes rapid induction of endothelial cell differentiation *in vitro*, and neovascularization *ex vivo* and *in vivo*. We also show that TGF- β_2 enhances sADAM33 release from cells overexpressing full-length ADAM33 and that this truncated form is biologically active.

Conclusion: The discovery that sADAM33 promotes angiogenesis defines it as a tissue remodeling gene with potential to affect airflow obstruction and lung function independently of

inflammation. As TGF- β_2 enhances sADAM33 release, environmental factors that cause epithelial damage may synergize with ADAM33 in asthma pathogenesis, resulting in a disease-related gain of function. This highlights the potential for interplay between genetic and environmental factors in this complex disease. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: ADAM33, asthma, angiogenesis, airway remodeling

A disintegrin and metalloprotease (ADAM)–33 is an asthma susceptibility gene whose polymorphic variation has been linked to asthma and bronchial hyperresponsiveness, but not to indices of allergic inflammation.¹ These findings have been replicated in several other studies, including a meta-analysis² that included all literature-reported studies, including those that have shown weak or no association. ADAM33 polymorphism also predicts impaired lung function in young children,³ suggesting that ADAM33 may contribute to the early-life origins of asthma. A similar association of ADAM33 with impaired lung function has also been reported in asthma,⁴ the general population,⁵ and chronic obstructive pulmonary disease (COPD).⁶

ADAM33 belongs to the a disintegrin and metalloprotease family of membrane-anchored glycoproteins that play diverse roles in cell surface remodeling, ectodomain shedding of growth factors and receptors, and mediating cell-cell and cell-matrix interactions.⁷ It is made up of a full-length molecule of 813 amino acids including 7 domains: pro-, metalloprotease, disintegrin, cysteine-rich, epidermal growth factor, transmembrane, and cytoplasmic, serving the functions of activation, proteolysis, adhesion, fusion, and signaling.¹ Since its discovery in 2002, ADAM33 has been extensively characterized at molecular and structural levels,^{8–12} but its biological role and its contribution to asthma pathogenesis have remained elusive.

Expression of ADAM33 mRNA and protein is restricted to mesenchymal cells, which include airway fibroblasts, myofibroblasts, and smooth muscle.^{1,13,14} In bronchial biopsies from normal and asthmatic airways, ADAM33 colocalizes to smooth muscle and fibroblastlike cells in the submucosa, supporting its link with bronchial hyperresponsiveness and airway remodeling, but no disease-related differences in total levels of expression were found.¹³ In human embryonic lung, ADAM33 is expressed in mesenchymal progenitor cells,¹³ suggesting a potential role in lung development. A number of alternatively spliced mRNA transcripts of ADAM33 have been detected in bronchial biopsies¹³ and mesenchymal cells,¹⁴ but again, no disease-related differences in splicing have been detected. These disease-derived findings

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Supported by the Medical Research Council UK; the Rayne Foundation; the British Lung Foundation; the Asthma, Allergy, and Inflammation Research Charity; the Roger Brooke Charitable Trust; and the Wellcome Trust. S.T.H. is supported by a Medical Research Council Clinical Professorship. I.P. was supported by a European Respiratory Society Short Term Traveling Fellowship and a European Academy of Allergy and Clinical Immunology Fellowship.

Disclosure of potential conflict of interest: G. Clough has received research support from the British Heart Foundation, Unilever, and Probe Scientific UK. N. Hanley has received research support from Novo Nordisk. D. I. Wilson has received research support from the British Heart Foundation and the Department of Health UK. The rest of the authors have declared that they have no conflict of interest.

Received for publication November 2, 2007; revised February 12, 2008; accepted for publication March 5, 2008.

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 0091-6749/\$34.00

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 doi:10.1016/j.jaci.2008.03.003

Abbreviations used

ADAM:	A disintegrin and metalloprotease
BALF:	Bronchoalveolar lavage fluid
CAM:	Chick embryo chorioallantoic membrane
CHO:	Chinese hamster ovary
COPD:	Chronic obstructive pulmonary disease
FGF:	Fibroblast growth factor
HEK:	Human embryonic kidney
HUVEC:	Human umbilical vein endothelial cell
SNP:	Single nucleotide polymorphism
sADAM33:	Soluble a disintegrin and metalloprotease 33
UK:	United Kingdom
VEGF:	Vascular endothelial growth factor

contrast with the suggestion that the disease associated intronic single nucleotide polymorphism (SNP) between exons B and C (BC+1), which has a marked repressive effect on ADAM33 expression in an *in vitro* reporter gene system, might affect pre-mRNA alternative splicing in asthma.¹⁵ Although high levels of ADAM33 and aberrant expression of ADAM33 in airway epithelium of patients with severe asthma have been suggested as a possible pathogenetic mechanism,¹⁶ we have not been able to reproduce these findings.¹⁷ ADAM33 knockout mice have also failed to yield any functional clues, showing normal growth and development and no abnormalities in a model of allergic airway sensitization.¹⁸ In contrast with these negative findings, the occurrence of high levels of a 55-kDa soluble form of ADAM33 (sADAM33) in bronchoalveolar lavage fluid (BALF) of subjects with asthma, detected using an antibody to the ADAM33 metalloprotease domain, and its correlation with disease severity and reduced lung function¹⁹ are one of the few pieces of nongenetic evidence showing a disease-related effect.

In children and adults, angiogenesis is believed to be an important aspect of asthma pathogenesis. Increased expression of angiogenic mediators and their receptors has been correlated with disease severity and accelerated lung function decline.²⁰⁻²² In the current study, we hypothesized that loss of the membrane anchor and regulatory cytoplasmic domain of ADAM33 results in a disease-related gain of function whereby the aberrant localization of the ADAM33 metalloprotease results in inappropriate cleavage of substrates to cause angiogenesis. *In vivo*, endothelial cells are supported by a vascular basement membrane that overlies layers of mesenchymal cells such as pericytes, fibroblasts, and smooth muscle cells. Because endothelial cells do not express ADAM33,¹ we postulated that their proximity to the mesenchymal cells that do express ADAM33^{1,13,14} would make them a target for sADAM33. Here, we describe the production and regulation of sADAM33 and its ability to promote angiogenesis.

METHODS**Recombinant cells expressing ADAM33 and purification of ADAM33 proteins**

Recombinant human embryonic kidney (HEK)-293 and Chinese hamster ovary (CHO) cells were generated by transfection with either full-length ADAM33 cDNA or ADAM33 signal, Pro-, and metalloprotease domains fused to a C-terminal Fc tag; empty pcDNA3 vector or Signal pIg plus (R&D Systems, Abingdon, United Kingdom [UK]) was used as control. *Drosophila* S2 cells were cotransfected by using the pCoHygro vector and pMT/Bip/V5-His vector (Invitrogen, Carlsbad, Calif) containing the ADAM33 Pro- and

metalloprotease domains (residues 31-409) fused to a V5 and 6xHis tag. The E346A mutant was prepared by site-directed mutagenesis. ADAM33 expression was confirmed by SDS-PAGE and Western blotting using antibodies to the Pro-, metalloprotease, and cytoplasmic domains of ADAM33 (Abcam plc, Cambridge, UK). Conditioned medium from S2 cells was harvested and the ADAM33 Pro-metalloprotease purified through a series of chromatographic steps involving lectin affinity chromatography, immobilized metal ion affinity chromatography, cation exchange, and gel filtration.

Concanavalin A-Sepharose 4B beads (Sigma, Poole, UK) were used to pull down sADAM33 from the supernatants of recombinant HEK293 cells and BALF obtained by fiberoptic bronchoscopy, as described in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org. The bound proteins were either lysed into 1x SDS sample buffer for SDS-PAGE and Western blotting or eluted with 0.5 mol/L methyl α -D-mannopyranoside for analysis of protease activity.

ADAM33 biochemical activity

A fluorescence resonance energy transfer peptide cleavage assay based on that described by Zou et al⁹ was used to assess the enzyme activity of ADAM33. Synthetic inhibitors of matrix metalloproteases were tested in this assay for their potency against the catalytic activity of ADAM33.

***In vitro* and *in vivo* angiogenesis assays**

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords collected after ethical approval and informed consent. Endothelial cells were plated into wells precoated with growth factor-reduced Matrigel (BD Biosciences, Oxford, UK) and incubated with recombinant cell culture supernatants (minus G418) or ADAM33 proteins for 18 hours. The 3-dimensional organization of the endothelial cells was recorded and topological parameters measured by computer-aided image analysis using QWin image analysis software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).

The chick embryo chorioallantoic membrane (CAM) assay was performed as previously described.²³ CAMs were treated after 8 days of culture with ADAM33 Pro-metalloprotease, ADAM Pro-metalloprotease E346A, or recombinant CHO cells contained in 1-mm³ sterilized gelatin sponges (Gel-foam; Upjohn Co, Kalamazoo, Mich). CAMs were examined daily until day 12 and photographed *in ovo*. Blood vessels entering the sponge within the focal plane of the CAM were counted by 2 observers in a double-blind fashion at $\times 50$ magnification.

Human embryonic/fetal lung explant cultures

Human embryonic lungs were harvested according to the Polkinghorne Committee guidelines and after ethical approval and informed consent from the donor. They were dissected into pieces 1 to 2 mm and cultured in transwells in Matrigel in the presence of 60 ng/well ADAM33 Pro-metalloprotease or ADAM33 Pro-metalloprotease E346A for 12 days, replenishing media and ADAM33 protein every second day. The tissue was harvested and processed for immunohistochemistry with the antibody EN-4 recognizing the vessel marker platelet endothelial cell adhesion molecule 1 (PECAM-1). All sections were examined by 3 blind investigators and expression-quantified by computer-assisted image analysis.

Statistical analysis

Parametric ANOVA, followed by the Tukey-Kramer multiple comparisons test, was used to compare groups in the *in vitro* and *in vivo* experiments. Non-parametric ANOVA followed by Kruskal-Wallis test was used to compare groups in the *ex vivo* experiments.

Online supplemental material

Further details of each protocol can be found in this article's [Online Repository](http://www.jacionline.org).

RESULTS

The ADAM33 metalloprotease domain is proangiogenic *in vitro*

Angiogenesis is a process that critically involves proteolysis.²⁴ Because sADAM33 is made up of several functional domains, we tested whether the metalloprotease domain specifically was able to promote angiogenesis. When supernatants derived from CHO cells transfected with cDNA encoding the ADAM33 Pro- and metalloprotease domains with an Fc tag were tested in the Matrigel assay, they promoted endothelial cell differentiation, as assessed by an increase in both the number of branching points (597 ± 40 vs 336 ± 30 ; $P < .05$; $n = 3$) and meshes (20 ± 3 vs 13 ± 0.5 ; $P < .05$; $n = 3$) per field compared with control supernatants from pcDNA3.1 mock-transfected cells. Supernatant from Signal pIg plus mock-transfected cells expressing an Ig Fc fragment gave comparable results to the supernatant from pcDNA3.1 mock-transfected cells. These findings suggested that the ADAM33 metalloprotease domain possessed angiogenic potential.

Because the CHO ADAM33 Pro-metalloprotease cells did not produce enough recombinant protein to allow their purification for further functional testing, we used an insect cell expression system to generate large quantities of enzymatically active ADAM33 Pro-metalloprotease and an inactive mutant E346A protein (ADAM33 Pro-metalloprotease E346A) targeting the catalytic residue in the active site. Isolation of the recombinant proteins was achieved by multistep chromatography yielding highly purified preparations with the anticipated enzymatic activities (see this article's Fig E1 in the Online Repository at www.jacionline.org). As was found with the crude supernatants from ADAM33-expressing cells, purified ADAM33 Pro-metalloprotease protein stimulated endothelial cell differentiation and formation of tubelike structures on Matrigel (Fig 1, A, ii vs i). This effect was similar to the effect of fibroblast growth factor (FGF)-2 (Fig 1, A, iv) but was not reproduced by ADAM33 Pro-metalloprotease E346A (Fig 1, A, iii). The effect of ADAM33 Pro-metalloprotease was dose-dependent, as determined by assessment of branch points and meshes using computer-aided image analysis (Fig 1, B). As ADAM33 Pro-metalloprotease E346A was inactive, it not only controlled for any effects of the V5 and 6xHis tags that had been engineered into the protein to aid purification but also demonstrated that the angiogenic activity of ADAM33 Pro-metalloprotease was unlikely to be a result of a copurified contaminant. Because these data suggested that the enzymatic activity of ADAM33 was required for the angiogenic effect, 4 synthetic carboxylate and hydroxamate compounds that are known inhibitors of metalloproteases²⁵ were tested for their ability to inhibit ADAM33 Pro-metalloprotease. These were found to exhibit different potencies against ADAM33-mediated peptide cleavage activity, with (3R)-(+)-[2-(4-methoxybenzenesulfonyl)-1,2,3,4-tetrahydroisoquinoline-3-hydroxamate being the most potent (inhibitory concentration 50% [IC₅₀] of 330 nmol/L; Fig E1, D). When tested in the Matrigel assay, this inhibitor was also able to abrogate the proangiogenic effect of ADAM33 Pro-metalloprotease (Fig 1, C). Although this inhibitor is also known to inhibit matrix metalloprotease 8,²⁵ it did not affect the biological potency of FGF-2. This suggested that the inhibitor was selective for ADAM33 in our assays, although we cannot completely exclude the possibility that it affected another protease involved in the response to ADAM33.

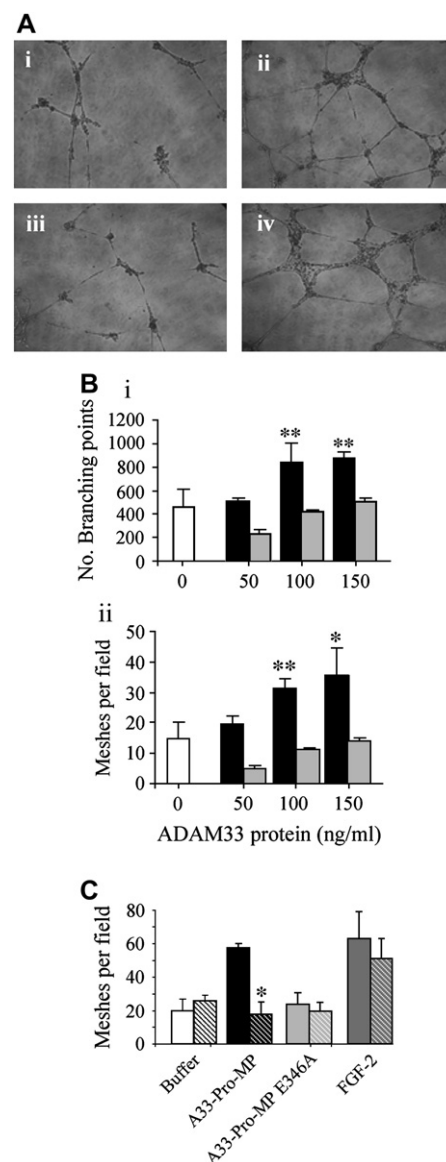


FIG 1. ADAM33 promotes angiogenesis *in vitro*. HUVEC differentiation was tested with buffer (A, i, B, C, white bars), ADAM33 Pro-metalloprotease (MP) (A, ii, B, C, black bars), ADAM33 Pro-MP E346A (A, iii, B, C, light gray bars), or FGF-2 (A, iv, C, dark gray bars). In C, the effect of (3R)-(+)-[2-(4-methoxybenzenesulfonyl)-1,2,3,4-tetrahydroisoquinoline-3-hydroxamate (5 μ Mol/L, hatched bars) was tested. Topological parameters of capillary structure (branch points and meshes) were quantified by computer-aided image analysis (B, C). Data are means $\pm$ SDs; $n = 3$; * $P < .05$; ** $P < .01$.

The ADAM33 metalloprotease cleaves CD23¹¹ and a repertoire of peptides derived from membrane-anchored proteins⁸; however, the rate of cleavage of these peptide substrates is low, suggesting that the physiological substrate for ADAM33 has yet to be identified. Because other ADAM proteins cleave growth factors from the cell surface and degrade the extracellular matrix (ECM)-releasing sequestered growth factors such as members of the FGF and vascular endothelial growth factor (VEGF) families that promote angiogenesis,²⁴ we investigated whether this could explain the proangiogenic activity of ADAM33. However, there was no significant inhibition of ADAM33 Pro-metalloprotease-mediated angiogenesis in the presence of neutralizing antibodies against either

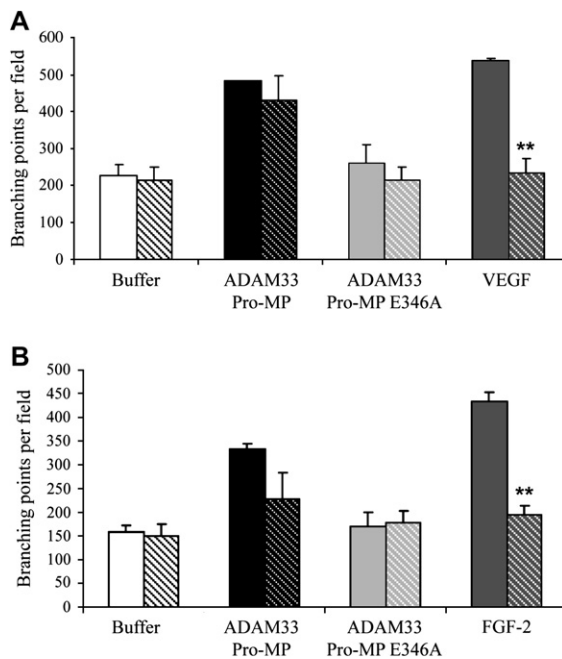


FIG 2. The pro-angiogenic effect of ADAM33 is not mediated by FGF-2 or VEGF. HUVEC differentiation was tested in the presence of buffer (white bars), ADAM33 Pro-metalloprotease (MP) (150 ng/mL; black bars), ADAM33 Pro-MP E346A (150 ng/mL; gray bars), VEGF (10 ng/mL; A, dark gray bars) or FGF-2 (30 ng/mL; B, dark gray bars) with control immunoglobulins (plain bars) or with neutralizing antibodies to VEGF receptor 2 (A, hatched bars) or FGF-2 (B, hatched bars). Data are means $\pm$ SDs; n = 3; **P < .01.

VEGF receptor 2 (Fig 2, A) or FGF-2 (Fig 2, B). In these experiments, the antibodies significantly reduced endothelial cell differentiation induced by either VEGF or FGF-2, respectively.

ADAM33 stimulates blood vessel sprouts in CAMs

We next tested the effect of ADAM33 on angiogenesis *in vivo* using the CAM assay. Purified ADAM33 Pro-metalloprotease protein induced a vigorous angiogenic response that was characterized by the presence of allantoic vessels spreading radially toward the sponge in a spoked wheel pattern, similar to that observed with VEGF (Fig 3, A). The response was dose-dependent (Fig 3, B), with maximal stimulation at 15 ng/sponge, and was significantly greater than the mutant ADAM33 Pro-metalloprotease E346A (Fig 3, C). To assess the contribution of mammalian cell-derived ADAM33 on angiogenesis, ADAM33 Pro-metalloprotease-transfected CHO cells or mock-transfected cells were also tested. As shown in Fig 3, C, both cell types were able to induce a vascular reaction compared to buffer alone; however, the ADAM33 Pro-metalloprotease CHO cells were significantly more potent than the mock-transfected CHO cells.

ADAM33 promotes new blood vessel formation in human embryonic lung explants

To determine whether ADAM33 could have a direct effect on neovascularization in the lung, we tested the recombinant ADAM33 Pro-metalloprotease and ADAM33 Pro-metalloprotease E346A in human embryonic lung tissue cultured in Matrigel. After 12 days of culture, no significant changes in vascularity were observed in explants treated with mutant ADAM33 Pro-metalloprotease E346A (17.3 ± 5.3 vessels/mm²) compared with the

buffer control (17.6 ± 3.5 vessels/mm²; Fig 4). However, an increase in the number of new vessels in the mesenchymal compartment surrounding the bronchial tubular structures was observed in explants treated with ADAM33 Pro-metalloprotease protein (24.9 ± 7.9 vessels/mm²; P < .05). The fact that ADAM33 Pro-metalloprotease was able to affect angiogenesis in embryonic lung tissue is consistent with it acting as an asthma susceptibility gene in early life,³ perhaps as early as *in utero*. However, because lung development and physiological angiogenesis are not affected in ADAM33^{-/-} mice,¹⁸ it seems unlikely that ADAM33 is involved in the normal physiological processes of pulmonary vessel development.

TGF- β 2 regulates shedding of active sADAM33 from cells expressing full-length ADAM33

Alternative splicing has been predicted to give rise to a secreted variant of ADAM33,¹⁴ but mRNA encoding this variant is very low and no disease-related differences have been detected so far.^{13,14} Therefore, we considered whether the sADAM33 that has been detected in BALF using an antibody to the catalytic domain might be generated by shedding from the cell surface, similar to the shedding observed in the closely related molecule, *Xenopus* ADAM13.²⁶ Clonal cell lines of HEK293 cells transfected with cDNA encoding full-length ADAM33 were selected for their ability to process the 120-kDa ADAM33 pro-protein to yield a mature 100-kDa form that was detected with an antibody to the cytoplasmic domain of ADAM33. This antibody also detected a third band at 45 kDa in lysates of ADAM33-expressing cells that was absent in mock-transfected cells, suggesting the potential for shedding of the extracellular domain. We confirmed this by Western blotting using an antibody to the metalloprotease domain applied to glycoproteins pulled down from culture supernatants (Fig 5, A, i). This detected a 55-kDa band in media from ADAM33, but not control, cells starting after 48 hours of culture. Western blot analysis confirmed that sADAM33 produced by recombinant HEK cells was similar to that found in asthmatic BALF (see Table E1 in the Online Repository at www.jacionline.org): both recombinant and natural forms had comparable molecular weights and were detected by an antibody against the metalloprotease domain, but not the Pro- domain. This suggests that 55-kDa sADAM33 in BALF and culture media is the processed (ie, catalytically active) ectodomain of ADAM33 (predicted mass is 52 kDa) with some posttranslational glycosylation.

Although asthma has a strong genetic predisposition, environmental factors are important triggers of the disease. We have previously proposed that activation of the epithelial mesenchymal trophic unit is an essential step in asthma pathogenesis.²⁷ In this paradigm, TGF- β 2 is produced during epithelial repair²⁸ and is responsible for propagation of signals from the damaged epithelium to the underlying mesenchyme. Consistent with this model, spontaneous shedding of sADAM33 from recombinant HEK cells was enhanced by TGF- β 2 in a time-dependent and dose-dependent fashion (Fig 5, A, i and ii). This suggests that an interplay between epithelial-derived TGF- β and underlying mesenchymal cells that express ADAM33 may contribute to increased production of sADAM33 in asthma.

We also examined whether native sADAM33 was biochemically and functionally active. Using fluorescence resonance energy transfer peptide cleavage assays, sADAM33 was shown to be catalytically active. The rate of cleavage of peptide by

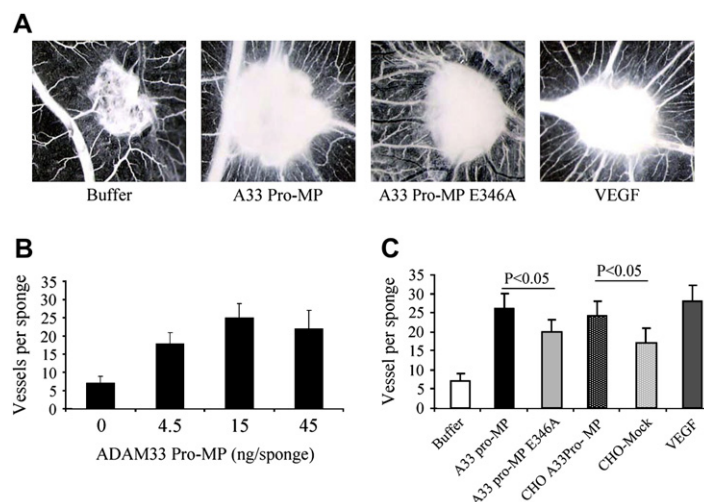


FIG 3. ADAM33 promotes angiogenesis *in vivo*. **A**, Photomicrographs of CAMs, treated with buffer, ADAM33 Pro-metalloprotease (MP) (45 ng/sponge); ADAM Pro-MP E346A (45 ng/sponge); or VEGF (500 ng/sponge). **B**, Dose-response for the angiogenic effect of ADAM33 Pro-MP, and **C**, comparison of the effects of ADAM33 Pro-MP (15 ng/sponge) with ADAM33 Pro-MP E346A (15 ng/sponge), ADAM33 Pro-MP-expressing CHO cells, mock-transfected cells (each with 40,000 cells/sponge), or VEGF (500 ng/sponge). Data are means $\pm$ SDs from 8 CAMs per condition, from 2 independent experiments.

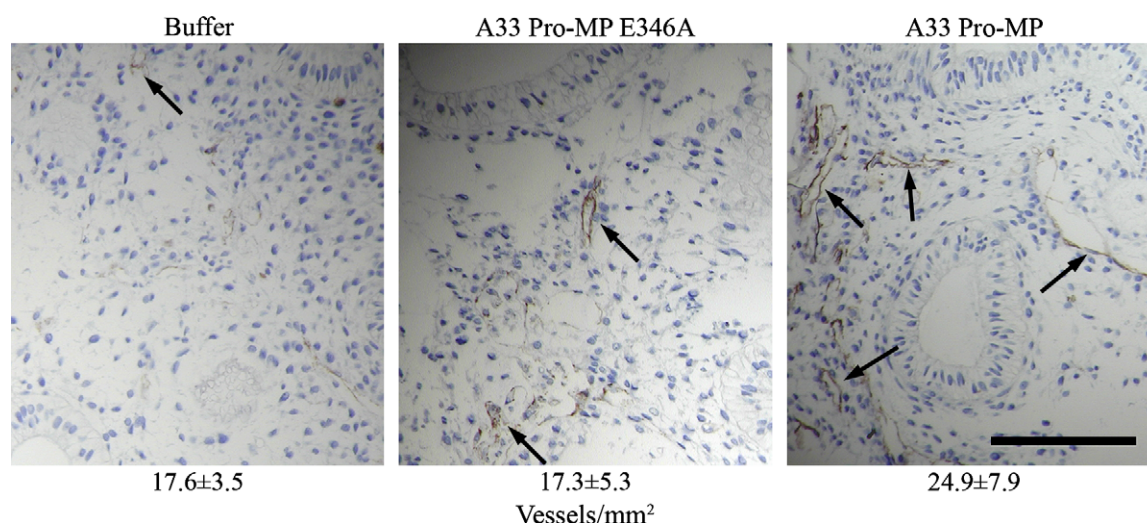


FIG 4. ADAM33 promotes angiogenesis in lung tissue. Human embryonic lung pieces (8 wk postconception) were cultured with buffer alone, 60 ng/well ADAM33 Pro-metalloprotease (MP) or ADAM33 Pro-MP for 12 days. The lungs were fixed and stained for the endothelium marker, PECAM-1, using immunohistochemistry. The arrows indicate the presence of vessels showing both positive brown immunostaining and an identifiable lumen. Scale bar = 100 μ m.

sADAM33 was 85 relative fluorescence units/min, whereas no activity was observed in the mock control sample. This is consistent with processing and removal of the Pro-domain, which we could not detect by Western blotting of the 55-kDa sADAM33. As shown in Fig 5, B, the supernatant derived from ADAM33-expressing cells was also biologically active in increasing endothelial cell differentiation into capillary-like structures in Matrigel compared with the supernatants from the mock-transfected cells.

DISCUSSION

Although several factors are involved in airway vascular remodeling,²⁹ the ability of sADAM33 to promote angiogenesis

is consistent with the increased vascularity observed in asthmatic airways and its correlation with disease severity.²¹ Although not as well studied, there is evidence that the vascular area of the airway is also significantly increased in COPD and correlates with the degree of airflow obstruction.²² Angiogenesis is fundamental for supporting many aspects of tissue inflammation and remodelling.³⁰ During angiogenesis, blood vessels enlarge and increase in number, enhancing the production of inflammatory mediators, augmenting and sustaining cellular infiltration, and contributing to edema through microvascular leakage. The new vessels can also supply nutrients and oxygen to the inflamed tissue, facilitating proliferation of structural cells such as smooth muscle cells, fibroblasts, and myofibroblasts. Thus,

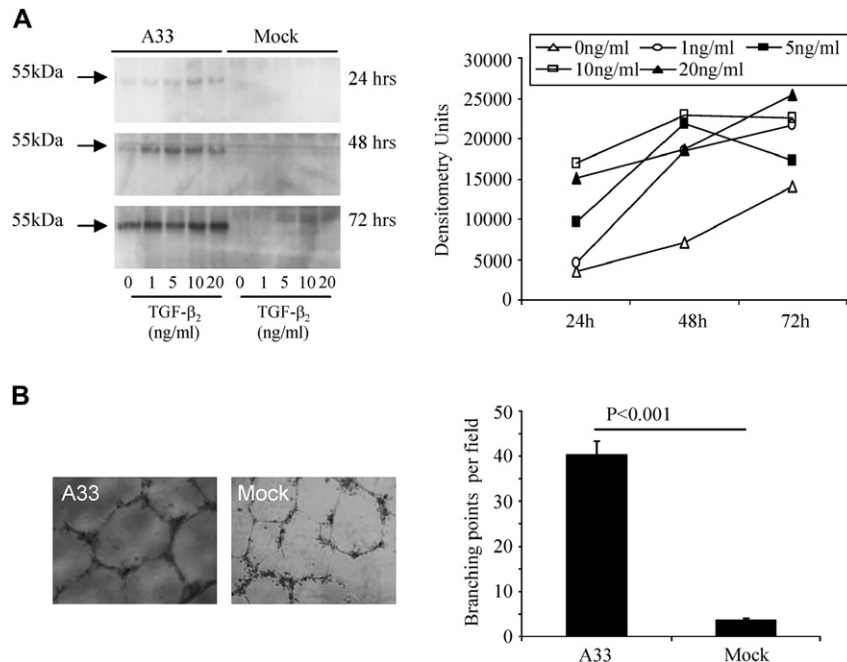


FIG 5. Characterization of sADAM33. **A**, Culture supernatants from control or TGF- β_2 -treated HEK293-expressing full-length recombinant ADAM33 (A33) or empty vector (Mock) were analyzed for sADAM33 by Western blotting (left panel) and the 55-kDa sADAM33 band quantified by densitometry (right panel); data are representative of 3 individual experiments. **B and C**, Culture supernatants from recombinant ADAM33 or mock-transfected cells were tested for their ability to promote HUVEC differentiation *in vitro* (left panel) and branching points quantified (right panel). Data are means $\pm$ SDs ($n = 3$ experiments).

increased angiogenesis has the potential to contribute to abnormal airway function including fixed airflow obstruction and consequent accelerated lung function decline through direct and indirect mechanisms.

Proteolysis has been recognized as one of most important activities during angiogenesis. Members of the matrix metalloprotease, ADAM and ADAMTS (a disintegrin and metalloprotease domain with thrombospondin motifs) families, as well as cysteine and serine proteases, have been implicated in this regulation.²⁴ These enzymes mediate their effect by degradation of the endothelial basement membrane and extracellular matrix proteins; release of angiogenic factors; processing of cytokines, growth factors, and growth factor receptors; and the production of endogenous inhibitors. Although it is likely that the substrate for ADAM33 falls into one of these categories, we have shown that its effect is not mediated by release of VEGF or FGF-2. Even though both of these factors are strong angiogenic mediators, it is noteworthy that the potency of sADAM33 in the CAM assay exceeded that of VEGF, whereas it was quantitatively less potent than either FGF-2 or VEGF in the endothelial tube formation assay. Although many factors can influence the outcomes of the individual assays (eg, human vs chicken receptors), it is possible that the more complex CAM assay contained better substrates for sADAM33. It is also important to note that most of our studies were performed with the purified metalloprotease domain without the more proximal disintegrin and cysteine-rich domains. Because these domains are involved in substrate presentation for other ADAMs,^{31,32} the activity of the purified catalytic domain may be an underestimate of the true activity of shed ectodomain of ADAM33 present in BALF.

Our results showing TGF- β -mediated ectodomain shedding of ADAM33 led us to propose that loss of its membrane anchor allows ADAM33 to gain the capacity to contribute to pathological neovascularization. Because the proangiogenic effect of sADAM33 is dependent on its catalytic activity, our data further suggest that therapeutic interventions that are able to target specifically the ADAM33 metalloprotease may be effective disease modifiers that can affect the natural history of the disease. ADAM15, a close relative of ADAM33, has also been shown to promote angiogenesis.²⁴ However, unlike ADAM15, ADAM33 is not expressed by endothelial cells,¹ even though it can act on them to promote angiogenesis. In this respect, the gain of function achieved by sADAM33 may enable it to mimic the proangiogenic properties of endothelium-derived ADAM15. This gain of function may explain why there was no obvious angiogenic phenotype in *ADAM33*^{-/-} mice (ie, loss of function), even after antigen sensitization and airway challenge.¹⁸

Identification of a function for ADAM33 and the involvement of TGF- β in ectodomain shedding of ADAM33 should pave the way for identifying how polymorphic variation in the *ADAM33* gene is linked to asthma and COPD pathogenesis. Although there are a large number of SNPs in *ADAM33*, 4 SNPs (S1, S2, T1, and T2) encoding amino acid substitutions in the transmembrane and cytoplasmic tail of ADAM33 have been associated with asthma.¹ It is conceivable that these SNPs may potentiate trafficking of ADAM33 to the cell surface and/or modulate its ability to be shed from the cell surface in a TGF- β -dependent manner. Alternatively, the *ADAM33* locus that is associated with reduced lung function in young children lies at the 5' end of the gene within the regions encoding the Pro- and metalloprotease domains,³ which may directly affect catalytic activity.

In conclusion, we have shown that a truncated, soluble form of ADAM33 containing the catalytic domain causes rapid induction of endothelial cell differentiation *in vitro*, and angiogenesis both *ex vivo* and *in vivo*. By demonstrating a role of ADAM33 in angiogenesis, our study is the first to demonstrate that ADAM33 acts as a remodeling gene that can act independently of airway inflammation. The ability of TGF- β_2 to augment sADAM33 release further suggests that environmental factors that cause epithelial damage may synergize with ADAM33 in the pathogenesis of asthma and COPD, highlighting the potential for interplay between genetic and environmental factors in this complex disease. The importance of angiogenesis in many physiological and pathological processes broadens the range of diseases in which ADAM33 could be implicated, the most obvious being cancer and atherosclerosis.

Clinical implications: The proangiogenic potential of the soluble form of ADAM33 suggests that therapeutic interventions that are able to target specifically the ADAM33 metalloprotease may be effective disease modifiers.

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METHODS

Recombinant cells expressing ADAM33

Recombinant HEK293 cells were generated by lipid (Effectene; Qiagen, Crawley, UK) transfection with the pcDNA3 vector containing full-length ADAM33 cDNA or empty vector as control. Recombinant CHO cells were also generated using the pcDNA3.1 vector that contained the ADAM33 signal, Pro- and metalloprotease domains fused to a C-terminal Fc tag. Stable transfectants were selected by using G418 and cell lines (HEK293 cells) generated by 2 rounds of cloning by limiting dilution. HEK293 cells were routinely cultured in Dulbecco's modified Eagle medium supplemented with 10% vol/vol heat-inactivated FBS and antibiotics, whereas CHO cells were in Ultraculture (Lonza Biologics plc, Slough, UK). Conditioned media prepared for use in angiogenesis assays were generated without the addition of G418. ADAM33 expression was confirmed by SDS-PAGE and Western blotting using antibodies to the Pro-, metalloprotease, and cytoplasmic domains of ADAM33 (Abcam plc, Cambridge, UK).

Drosophila S2 cells were maintained in Schneider's S2 medium supplemented with 10% FBS and 10 U/mL penicillin and 10 µg/mL streptomycin, or *Drosophila* Serum Free medium (all from Invitrogen, Paisley, UK) supplemented with 10 U/mL penicillin and 10 µg/mL streptomycin. Cells were cultured at 27°C and were cotransfected by using the pCoHygro vector and pMT/Bip/V5-His vector containing the ADAM33 Pro- and metalloprotease domains (residues 31-409) fused to a V5 and 6xHis tag. Transfected S2 cells were seeded at 4×10^6 cells/mL in *Drosophila* Serum Free medium supplemented with 500 µmol/L copper sulfate, which induced expression of ADAM33 Pro-metalloprotease-His or ADAM33 Pro-metalloprotease E346A-His proteins. Conditioned medium was collected by centrifugation after 5 days.

Expression and purification of the recombinant human ADAM33 Pro-metalloprotease

Conditioned medium from S2 cells expressing recombinant ADAM33 Pro- and metalloprotease domains (Pro-metalloprotease) fused to a C terminal 6xHis tag was harvested and the ADAM33 purified through a series of 5 chromatographic steps. Steps i to iv involved loading the sample onto a column, washing away the unbound proteins using the binding buffer, and elution of the unbound proteins. The ADAM33 containing fractions after each step were identified by SDS-PAGE and dialyzed into the binding buffer of the relevant column of the proceeding step before continuing. The column type, binding buffer, and elution details of each step are as follows. Step i, 20 mL Concanavalin A-Sepharose 4B column (Sigma, Poole, UK), binding buffer: 20 mmol/L TrisHCl pH 7.4, 0.5 mol/L NaCl. Proteins were eluted using a 1-step elution with 0.5 mol/L methyl α-D mannopyranoside (Sigma). Step ii, His Trap column (Amersham Biosciences, Chalfont St Giles, UK), binding buffer 25 mmol/L HEPES pH 7.9, 0.5 mol/L NaCl, 5 mmol/L imidazole, 5 mmol/L CaCl₂, 10% glycerol. Proteins were eluted by using a gradient of imidazole (5-250 mmol/L over a period of 50 minutes). Step iii, SP Sepharose Fast Flow column (Amersham Biosciences), binding buffer 25 mmol/L HEPES pH 7.0, 50 mmol/L NaCl, 20 mmol/L imidazole, 5 mmol/L CaCl₂, 10% glycerol. Proteins were eluted using a gradient of NaCl (50-500 mmol/L over a period of 20 minutes). Step iv, Same as step ii with the exception of the elution step. Elution was carried as a 1-step elution, which was performed to concentrate the sample. Step v, Superose 12 column (Amersham Biosciences), binding/elution buffer 25 mmol/L HEPES pH 7.5, 150 mmol/L NaCl, 50 mmol/L imidazole, 5 mmol/L CaCl₂, 10% glycerol.

sADAM33 pull down with concanavalin A beads

Concanavalin A-Sepharose 4B beads (Sigma) were used to pull down sADAM33 from the supernatants of recombinant HEK293 clones or BALF. The supernatants (2 mL) or BALF samples (1 mL) were adjusted to contain 20 mmol/L TrisHCl pH 7.4, 0.5 mol/L NaCl, and protease inhibitors (Roche Applied Science, Burgess Hill, UK) before addition of activated concanavalin A beads (20 µL beads per mL sample). After binding overnight at 4°C, the beads were washed twice in equilibration buffer (20 mmol/L TrisHCl pH 7.4, 0.5 mol/L NaCl) and then twice in 20 mmol/L TrisHCl pH 7.4 to remove excess salt. The beads were then lysed into 1x SDS sample buffer for SDS-PAGE

and Western blotting with antibodies against the ADAM33 Pro- or metalloprotease domains (Abcam plc), or the protein was eluted from the beads by using 0.5 mol/L methyl α-D-mannopyranoside in 20 mmol/L Tris HCl, 0.5 mol/L NaCl, pH 7.4 for analysis of protease activity using the fluorescence resonance energy transfer (FRET) peptide cleavage assay.

FRET peptide cleavage assay

A FRET peptide cleavage assay based on that described by Zou et al^{E1} was used to assess the enzyme activity of ADAM33. All assay measurements were made in real time by using the Bio-Rad iCycler (Bio-Rad Laboratories, Herts, UK) with a 490-nm filter, allowing determination of initial rates. The ADAM33 substrate peptide was based on a modification of amyloid precursor protein known to be cleaved by ADAM33.^{E1} Typically 20-µL reactions containing 4.4 µmol/L FRET peptide (DABCYL-YRVAFQKLAE[FAM]K-NH₂) (kindly synthesized by Dr JJ Diaz-Mochón, University of Edinburgh) were incubated with sADAM33 (pulled down from HEK cell medium by using ConA beads), purified ADAM33 Pro-metalloprotease-His, or ADAM33 Pro-metalloprotease(E346A)-His, at 37°C in assay buffer containing 20 mmol/L HEPES pH 7.0, 0.5 mol/L NaCl, 10 mmol/L CaCl₂, 10 µmol/L ZnCl₂, and 0.2 mg/mL BSA. Synthetic inhibitors of matrix metalloproteases (Merk Chemicals Ltd, Nottingham, UK), were tested for their potency against the catalytic activity of ADAM33. The inhibitors were serially diluted to cover a concentration range from 1.6 nmol/L to 5 µmol/L.

In vitro angiogenesis assay

Human umbilical cords were collected after ethical approval from Southampton and South West Hampshire Research Ethics Committee and informed consent from the donor. HUVECs were isolated from human umbilical cord vein. The cells were grown in gelatin-coated flasks and maintained in M199 media supplemented with 10% FBS, 10% newborn calf serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 30 µg/mL endothelial cell growth supplement, and 0.1 mg/mL heparin (both from Sigma, Poole, UK). All the experiments were conducted with HUVECs from passage 2 to 7.

Ninety-six-well plates were precoated with 50 µL growth factor reduced Matrigel solution (BD Biosciences, Oxford, UK), and the gel was allowed to solidify at 37°C for 1 hour. Endothelial cells were then plated at a density of 8×10^3 cells/well and further incubated for 18 hours with supernatants (minus G418) obtained from either full-length ADAM33-transfected HEK293 cells or ADAM33 Pro-metalloprotease-transfected CHO cells. Supernatants from mock-transfected HEK293 cells or CHO cells were used as negative controls. In another set of experiments, endothelial cells were stimulated with the recombinant active ADAM33 Pro-metalloprotease domain or its inactive mutant at different concentrations. Buffer alone and recombinant human FGF-2 (30 ng/mL) or VEGF (10 ng/mL; R&D Systems, Abingdon, UK) were used as negative and positive controls, respectively. Where required, antibodies against VEGF receptor 2 or FGF-2 (R&D Systems) were used according to the manufacturer's recommendations and were compared with responses in the presence of isotype control immunoglobulins. The 3-dimensional organization was examined under an inverted, phase contrast microscope, and the topological parameters of the capillary mesh (mesh areas, vessel length, and mesh branching points) were measured by computer aided image analysis^{E2} using QWin image analysis software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). Inhibitors were tested alone or together with either ADAM33 Pro-metalloprotease or ADAM33 Pro-metalloprotease E346A or FGF-2.

In vivo angiogenesis assays

The contribution of ADAM33 to angiogenesis *in vivo* was investigated using the CAM assay.^{E3} Briefly, fertilized White Leghorn chicken eggs (30/group) were incubated under constant humidity at 37°C. On the third day of incubation, a square window was opened in the shell after removal of 2 to 3 mL albumen to detach the developing CAM from the shell. The window was sealed with a glass of the same dimension, and eggs were returned to the incubator. CAMs were treated after 8 days of culture with either different concentrations of ADAM33 Pro-metalloprotease or ADAM Pro-metalloprotease E346A (4.5-45 ng/sponge) dissolved in 3 µL serum-free medium and adsorbed

on 1-mm³ sterilized gelatin sponges (Gelfoam; Upjohn Co, Kalamazoo, Mich). CHO cells expressing ADAM33 Pro-metalloprotease and mock-transfected cells were applied to sponges, using 4×10^4 cells per sponge. Sponges containing buffer alone and VEGF (500 ng/embryo) were used as negative and positive controls, respectively. CAMs were examined daily until day 12 and photographed *in ovo* with a stereomicroscope SR equipped with a Zeiss Camera System MC63 (Carl Zeiss S.p.A., Lombardia, Italy). Two observers in a double-blind fashion at $\times 50$ magnification counted blood vessels entering the sponge within the focal plane of the CAM.

Human embryonic/fetal lung explant cultures and immunohistochemical analysis

Human embryonic lungs were harvested according to the Polkinghorne Committee guidelines and after ethical approval from the Southampton and South West Hampshire Joint local research ethics committee and informed consent from the donor. Human embryonic lungs, development age 7 to 11 weeks, were dissected into pieces of tissue of 1 to 2 mm and cultured in transwells in Matrigel in the presence of 60 ng/well ADAM33 Pro-metalloprotease or ADAM33 Pro-metalloprotease E346A for 12 days, replenishing the media and ADAM33 protein every second day. The tissue was then harvested and processed into glycol methacrylate resin for immunohistochemistry, as previously described.^{E4} Semithin 2- μ m sections were cut and immunostained using a streptavidin-biotin-peroxidase detection system with aminoethylcarbazole as a chromogen. To investigate the expression of blood vessels, serial sections were stained by using the mAb EN-4 (dilution 1/200; Bradshaw Biologicals, Loughborough, UK) that detects the vessel marker PECAM-1. All sections were examined simultaneously by 3 blind investigators and expression-quantified by computer-assisted image analysis.

Fiberoptic bronchoscopy

After ethical approval by the Southampton and South West Hampshire Local Research Ethics Committees and written informed consent, subjects underwent fiberoptic bronchoscopy under local anesthesia as previously described.^{E1} BALF was obtained by wedging the bronchoscope into a segmental bronchus and introducing six 20-mL aliquots of 0.9% saline, prewarmed to 37°C. Gentle suction was used to collect the fluid into a 100-mL plastic trap. The lavage fluid was centrifuged at 400g for 10 minutes at 4°C and the supernatant aliquoted and stored at -80°C until assayed for sADAM33. BALF was obtained from 2 normal subjects and 2 subjects with severe asthma, whose details are provided in Table E1.

Statistical analysis

Data are expressed as means $\pm$ SDs. Parametric ANOVA, followed by the Tukey-Kramer multiple comparisons test, was used to compare groups in the *in vitro* and *in vivo* experiments. Nonparametric ANOVA followed by the Kruskal-Wallis test was used to compare groups in the *ex vivo* experiments. A probability of $<.05$ was considered statistically significant.

RESULTS

Purification and catalytic activity of the recombinant ADAM33 Pro-metalloprotease

Because the CHO ADAM33 Pro-metalloprotease cells did not produce enough recombinant protein to allow its purification for further functional testing, we used an insect cell expression system to generate large quantities of the ADAM33 Pro-metalloprotease and a mutant E346A protein (ADAM33 Pro-metalloprotease E346A) targeting the catalytic residue in the active site. Isolation of the recombinant proteins was achieved by multistep chromatography yielding highly purified preparations composed of the pro- and catalytic domains, which copurified under native conditions. However, SDS gel electrophoresis and mass spectrometry revealed that this association was noncovalent, because 2 individual proteins with the expected masses of the Pro- and metalloprotease domains were detected (Fig E1, A and B). This ability of the Pro- and metalloprotease domains to remain associated after cleavage has also been reported for ADAM12.^{E5} A third, minor band was found to be an alternative glycoform of ADAM33 Pro-metalloprotease, as determined by enzymatic deglycosylation (data not shown). As shown in Fig E1, C, ADAM33 Pro-metalloprotease was catalytically active, even though the Pro-domain remained noncovalently associated; in contrast, the E346A mutant was completely inactive. Synthetic carboxylate and hydroxamate compounds, which are known inhibitors of metalloproteases,^{E6} exhibited different potencies against ADAM33-mediated peptide cleavage activity, the most potent being (3R)-(+)-[2-(4-methoxybenzenesulfonyl)-1,2,3,4-tetrahydroisoquinoline-3-hydroxamate with an IC₅₀ of 330 nmol/L (Fig E1, D).

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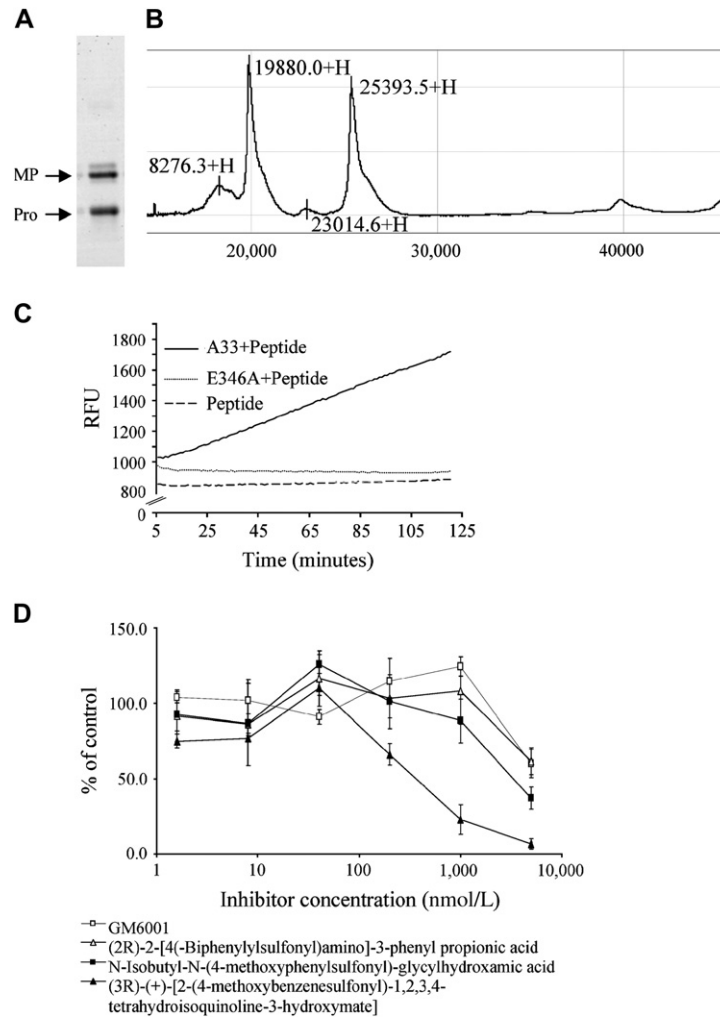


FIG E1. Purification and catalytic activity of the recombinant ADAM33 Pro-metalloprotease (MP). **A and B,** Purified ADAM33 was analyzed by SDS-PAGE and staining with EZBlue (Sigma, Poole, UK) or by mass spectrographic analysis. The bands representing the Pro- and metalloprotease domains are indicated. **C,** The proteolytic activity of ADAM33 Pro-MP and ADAM33 Pro-MP E346A was determined in FRET peptide cleavage assays. Values are expressed as relative fluorescence units (RFUs). **D,** The potencies of synthetic MP inhibitors against ADAM33 activity in FRET peptide cleavage assays. Data are means $\pm$ SDs (n = 3).

TABLE E1. Subject characteristics for BALF study

	Control	Asthma
No. of subjects	2	2
Sex (female/male)	2/0	1/1
Age (y), mean (range)	39 (19-59)	30 (17-43)
FEV ₁ , % predicted (range)	106 (79-134)	60 (46-71)
Atopy (yes/no)	1/1	1/1
Inhaled corticosteroids (beclomethasone dipropionate equivalent), µg/d (range)	0	3400 (2800,4000)
Long-acting β-agonists (yes/no)	0	2/0