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Catalytic Activity of ADAM8, ADAM15, and MDC-L (ADAM28) on Synthetic Peptide Substrates and in Ectodomain Cleavage of CD23*[S]

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The ADAM family of disintegrin metalloproteases plays important roles in "ectodomain shedding," the process by which biologically active, soluble forms of cytokines, growth factors, and their receptors are released from membrane-bound precursors. Whereas ADAM8, ADAM15, and MDC-L (ADAM28) are expressed in specific cell types and tissues, their *in vivo* functions and substrates are not known. By screening a library of synthetic peptides as potential substrates, we show that soluble recombinant forms of these enzymes have similar proteolytic substrate specificity, clearly distinct from that of ADAM17 (TNFα-converting enzyme). A number of tumor necrosis factor (TNF) family proteins and CD23 were screened as potential substrates for ectodomain cleavage. We found that ADAM8, ADAM15, and MDC-L, but not ADAM17, catalyzed ectodomain shedding of CD23, the low affinity IgE receptor. ADAM8-dependent, soluble CD23 release required proteolytically active ADAM8, and a physical association of ADAM8 was observed with the membrane-bound form of CD23. The ADAM8-dependent release of sCD23 and the endogenous release from B cell lines could be similarly inhibited by a hydroxamic acid, metalloprotease inhibitor compound. We conclude that ADAM8 could contribute to ectodomain shedding of CD23 and may thus be a potential target for therapeutic intervention in allergy and inflammation.

The disintegrin metalloprotease (or ADAM)[1] family of cell surface and secreted proteolytic enzymes is known to play roles in sperm-egg binding and fusion, muscle cell fusion, neurogenesis, modulation of Notch receptor and ligand processing, and processing of the pro-inflammatory cytokine, TNFα. The TNFα-converting enzyme (TACE) or ADAM17, is currently being explored as a target for anti-inflammatory drugs (1, 2). The genes for ADAM8, ADAM15, and MDC-L have been cloned, and proteolytic activity has been demonstrated for the corresponding proteins (3–5), but the *in vivo* substrates for these enzymes are not known. They are likely to play roles in ectodomain shedding, the process by which biologically active, soluble forms of cytokines, growth factors, and their receptors are released from membrane-bound precursors.

ADAM8 is expressed mainly in cells of the immune system, particularly monocytes and granulocytes (6). Furthermore, its expression has been shown to be inducible by lipopolysaccharide and γ-interferon (7) and by TNFα in the central nervous system (8). ADAM8 was recently shown to be a novel osteoclast-stimulating factor, induced during osteoclast differentiation from monocytic precursors (9). ADAM8 is also highly expressed in eosinophils (from Incyte Genomics, Lifeseq® Gold clone tissue distribution), one of the most important effector cell types at the site of inflammation in allergic asthma. ADAM8 may therefore represent a therapeutic target for treatment of allergy and/or asthma.

ADAM15 is a membrane-bound disintegrin metalloprotease containing an RGD integrin-binding sequence, which may function in cell adhesion through binding to integrins $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ (10–12), and two proline-rich sequences, shown to interact with SH3 domains in endophilin-I and SH3PX1 (13). ADAM15 is found in human aortic smooth muscle and cultured umbilical vein endothelial cells (14). Although ADAM15 is not expressed in normal blood vessels *in vivo*, it has been detected in developing atherosclerotic lesions (14) and has also been shown to be up-regulated in osteoarthritic *versus* normal human cartilage (15). Thus, ADAM15 may play a role in atherosclerosis and/or cartilage degeneration.

Another member of the ADAM family, MDC-L or ADAM28, was shown to be specifically expressed by lymphocytes in two alternative forms, a membrane-bound form, MDC-Lm, and a secreted protein, MDC-Ls (16). The lymphocyte-specific expression of MDC-L suggests that it may have an important immunological function, but its *in vivo* substrate(s) are unknown. ADAM28 is also highly expressed in epididymis (17) and has recently been shown to catalyze limited cleavage of myelin basic protein (4).

In the present study, we have characterized the proteolytic specificity of ADAM8, ADAM15. and MDC-L for both synthetic peptides and protein substrates. By screening a library of synthetic peptides, we show that soluble recombinant forms of these three enzymes have similar proteolytic substrate specificity, clearly distinct from that of ADAM17/TACE. We also show that ADAM8 is able to cleave membrane-bound CD23, the low affinity IgE receptor, in transfected cells and human macrophage cell lines. ADAM15 and MDC-L, but not ADAM17, were also able to cleave CD23. ADAM8-dependent, soluble CD23 release required proteolytically active ADAM8, and a physical association of ADAM8 was observed with the mem-

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[S] The on-line version of this article (available at http://www.jbc.org) contains Table 2.

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[1] The abbreviations used are: ADAM, a disintegrin and metalloproteinase; MDC, metalloprotease/disintegrin/cysteine-rich protein; MMP, matrix metalloproteinase; HBS, Hepes-buffered saline; TNF, tumor necrosis factor; FITC, fluorescein isothiocyanate; FRET, fluorescence resonance energy transfer; TACE, TNFα-converting enzyme; Dabcyl, 4-([4-(dimethylamino)phenyl]azo)benzoic acid; Aedans, *N*-acetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine.

brane-bound form of CD23. The proteolytic release of soluble CD23, by an unidentified metalloprotease activity, has been shown to cause up-regulation of IgE production and the induction of inflammatory cytokines (18). Because ADAM8 is expressed in the same cell types as CD23, we conclude that ADAM8 can contribute to ectodomain shedding of CD23, and thus is a potential target for intervention in allergy and inflammation.

EXPERIMENTAL PROCEDURES

PCR and cDNA Cloning—The pro-domain and protease domain of human ADAM 8 were amplified by PCR with *Pfu* polymerase and the primers 5A8, 5′-GGAATCCGCCATGCGCGGCCTCGGGCTC-3′, and 3FLAGA8, 5′-CGGGATCCTCTAGACTACCCCTTGTCATCGTCGTCCTTGTAGTCCCCGGCGAGGCACACCGACTGCGGCCGCTCCAAAAAGCTCTC-3′. The template used for the PCR was a clone from Incyte Genomics (from ovarian tumor tissue) containing the coding sequence for the complete pro-domain but only part of the protease domain of ADAM8. By using the oligonucleotides described above, the resulting PCR product contained the pro-domain and full-length protease domains and a C-terminal FLAG epitope to be used for immunodetection and purification. The cDNA was initially cloned into pZeroBlunt (Invitrogen), the sequence verified, and then sub-cloned into pFastBac1 (Invitrogen). Similar expression vectors were constructed for ADAM15, MDC-L (ADAM28), and ADAM17/TACE. ADAM17 was amplified from THP-1 cells first-strand cDNA, ADAM15 from human heart cDNA, and MDC-L from human thymus cDNA.

The cDNA for full-length ADAM8 was constructed as follows. The cDNA coding for the pro-domain and protease domain was ligated to a human EST clone (IMAGE 1271035, purchased from Human Genetics), encoding the disintegrin, cysteine-rich, membrane-spanning, and cytoplasmic domains of ADAM8. The full-length sequence was then cloned into pCDNA3(−) for expression in mammalian cells. Sequence verification of the resulting cDNA showed an in-frame deletion within the cytoplasmic domain when compared with the published sequences. Similar alternative splicing has been observed for the MDC-L (ADAM28) C-terminal domain (19). Site-directed mutagenesis was performed using the Quikchange site-directed mutagenesis kit (Stratagene) to produce a catalytically inactive ADAM8 (H604A and H608A). cDNA clones (Genestorms) for expression of human CD23, CD27L, FasL, CD40L, CD30L, TRAIL, and TNFα were purchased from Invitrogen.

Antibodies—M2-anti-FLAG mouse monoclonal antibody and M2-anti-FLAG-agarose, for purification of FLAG-tagged soluble ADAM proteins, were obtained from Sigma. Polyclonal antiserum against full-length ADAM8 protein was generated by immunizing rabbits with peptides corresponding to the C-terminal sequence, CPIQRKQGAGAPTAP, conjugated to activated keyhole limpet hemocyanin via the N-terminal cysteine. The resulting antiserum detected a number of specific bands in ADAM8-transfected HEK293 cells that were not detected by pre-immune sera or by the specific antisera in mock-transfected cells (see Fig. 5, *A* and *C*). Mouse monoclonal anti-V5 antibody and FITC anti-V5 were purchased from Invitrogen. FITC anti-human CD23 and anti-actin mouse monoclonal antibodies were obtained from BD Biosciences and Roche Applied Science, respectively.

Expression and Purification of Recombinant Soluble Human ADAM Proteins from the Medium of Sf9 Cells—Recombinant baculovirus for expression of the prodomain and protease domain of ADAM8 was generated from the pFastBac1 construct using the Bac-to-Bac system (Invitrogen). Sf9 cells were infected with virus, and the medium was collected after 72 h and analyzed for expression of ADAM8 by SDS-PAGE, transfer to nitrocellulose, and immunoblotting with M2-αFLAG antibody. Media from the infected cells were concentrated 10-fold by ultrafiltration and exchanged to TBS by repeated addition and reconcentration. The supernatant was centrifuged at 15,000 × *g*, filtered through a 0.45-μm filter to remove debris, and M2-αFLAG-agarose was added with mixing overnight at 4 °C. The resin was loaded into a column and washed with TBS, followed by elution of the bound material with 0.1 M glycine, pH 3.5, and immediate neutralization by addition of 12.5 μl/ml 2 M Tris-HCl, pH 8. Fractions from the purification were analyzed as above by Western blotting. Soluble ADAM17, ADAM15, and MDC-L proteins were generated similarly to ADAM8.

Detection and Assay of Proteolytic Activity of Recombinant ADAM8, ADAM15, MDC-L, and ADAM17—Forty nine different peptides were synthesized for testing protease activity. The peptides comprised the following: (i) a collection of substrates for other proteases and (ii) a number of sequences corresponding to membrane proximal cleavage sites of various proteins postulated to be released by metalloproteases (including those published by Roghani *et al.* (21) for ADAM9/MDC9). In order to use the principle of fluorescence resonance energy transfer, or FRET, for the assay, the peptides were labeled at the C terminus with Dabcyl and at the N terminus with Aedans. Cleavage of the peptides could be monitored by the increase in Aedans fluorescence at 460 nm (excitation 360 nm) as a result of the decrease in proximity of the Dabcyl quencher. The assay was performed by diluting the soluble ADAM protease domain in assay buffer, 10 mM Hepes, pH 7.5, containing 0.001% Brij-35. The reaction was initiated by the addition of substrate to a final concentration of 20 μM in an assay volume of 100 μl, for initial screening, and then different concentrations, as indicated in figure legends, for analysis of affinities. The assay was run for 20–60 min at room temperature. The cleavage site(s) for ADAM8 within four peptides were determined by liquid chromatography-mass spectrometry and matrix-assisted laser desorption ionization/time of flight.

Expression of Recombinant ADAM8, CD23, and TNF Family Proteins in Mammalian Cells—HEK293 cells were grown in Dulbecco's modified Eagle's medium, containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The cDNA constructs described above were used to transfect the HEK293 cells using Effectene® (Qiagen) as recommended by the manufacturer. Forty eight hours after transfection, the cells were placed under selection in 500 μg/ml G418 and/or 300 μg/ml Zeocin to create stable transfectants. The medium was collected, and the cells were lysed in phosphate-buffered saline containing 1% Nonidet P-40, and Complete® protease inhibitors (Roche Applied Science). Immunoprecipitations were performed on both media and cell lysates, using rabbit polyclonal anti-ADAM8 antiserum, or mouse monoclonal anti-V5 antibody, and protein A-Sepharose. The media, cell lysates, or immunoprecipitates were subjected to SDS-PAGE (5–15% acrylamide) and transferred to nitrocellulose, followed by immunoblotting with the anti-ADAM8 polyclonal antiserum or anti-V5 antibody (for proteins expressed from Genestorms clones), secondary antibodies conjugated to horseradish peroxidase, and fluorography using ECL substrate (Amersham Biosciences).

CD23 Release in Macrophage, B Cell, and Transfected HEK293 Lines—U937 cells were harvested, washed, and incubated in HBS or HBS containing soluble recombinant ADAM8 or ADAM17 for 1 h at 37 °C. The cells were then chilled, washed, and stained for surface expression of intact, endogenous CD23 with FITC anti-human CD23.

RPMI8866 cells, JY cells, or HEK293 cells transfected with ADAM8 and CD23 were incubated overnight at 37 °C, in the absence or presence of 0.4–12.5 μM MMP Inhibitor II (Calbiochem), after which the supernatant was analyzed for the presence of soluble CD23 by enzyme-linked immunosorbent assay (Pharmingen).

RESULTS

Peptide Substrate Screening of Soluble, Recombinant ADAM8, ADAM15, MDC-L, and ADAM17—Soluble pro-domains and protease domains of ADAM8, ADAM15, MDC-L, and ADAM17, tagged with C-terminal FLAG epitopes, were produced in Sf9 insect cells and purified from the media by FLAG affinity chromatography. Soluble ADAM17 appeared as a doublet at an apparent molecular mass of 40 kDa on SDS-PAGE (results not shown), corresponding to the predicted size for the metalloprotease domain after furin cleavage of the pro-domain. Activity of the recombinant protein was confirmed by cleavage of a peptide containing the TNFα cleavage site (results not shown). Purified soluble ADAM8 had an apparent molecular mass of 44 kDa (results not shown), corresponding to the predicted size for the uncleaved precursor form, containing the pro-domain and metalloprotease domain. This form of the protein showed no cleavage activity on any of 49 different peptides tested. Upon storage for a few weeks at 4 °C, a lower band of ~25 kDa appeared, and cleavage activity could be demonstrated (as described below) for a number of synthetic peptide substrates. Similar molecular weights (~50 and ~34 kDa) were observed by Schlomann *et al.* (20) for recombinant murine ADAM8, expressed from a construct coding for the pro-domain and protease domain. The lower 25-kDa band we obtained was subjected to N-terminal protein sequencing. The resulting sequence was RPRPGD, which corresponds to the

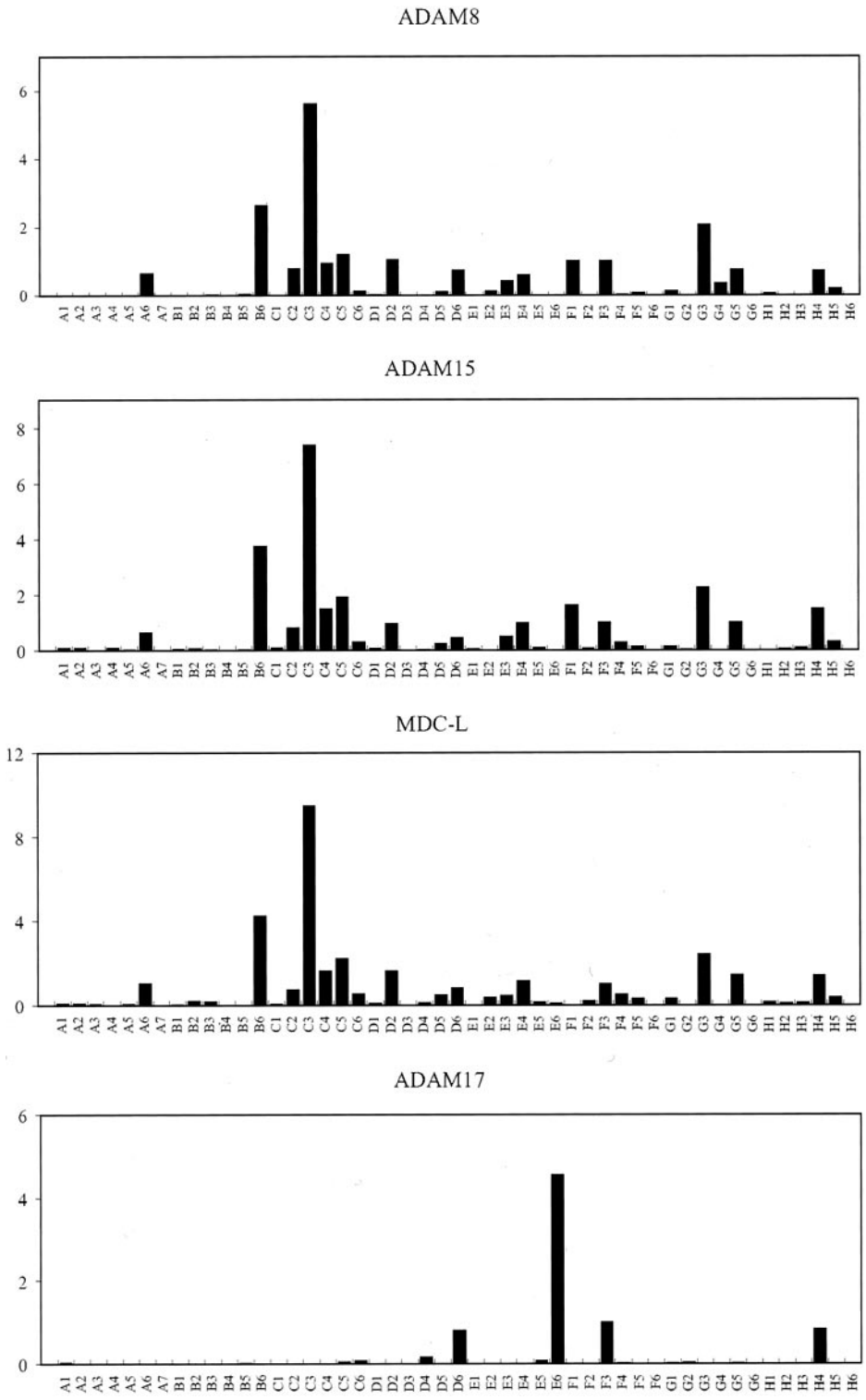

FIG. 1. **Peptide substrate screening of ADAM8, ADAM15, MDC-L, and ADAM17.** Soluble recombinant ADAM8, ADAM15, MDC-L, or ADAM17 (50–100 ng of protein, 1–2 pmol) were diluted in assay buffer, and the reaction was initiated by addition of each of 49 different peptide substrates (*numbered A1* to *H6*, every other peptide is labeled in Fig. 1) to a final concentration of 20 μM. The assays were run for 60 min at room temperature, and the rates of the reactions were determined from the slope of the kinetic increase in fluorescence for each peptide. The rates of cleavage of the peptides are plotted as relative fluorescence units per min, normalized by setting the activity for peptide F3 (cleaved by all 4 proteases) to 1 unit.

region between the pro- and metalloprotease domains, where other ADAM proteases, such as ADAM17, have a furin cleavage site. The cleavage of soluble ADAM8 upon storage suggested an autocatalytic activation mechanism, which was confirmed later by lack of processing of proteolytically inactive human ADAM8 (Fig. 5*A*) and demonstrated recently by Schlomann *et al.* (20) for murine ADAM8. Recombinant ADAM15 and MDC-L were also expressed as soluble uncleaved forms which auto-activated after storage in solution at 4 °C (not shown).

Forty nine different peptides were tested as substrates for the active soluble forms of ADAM8, ADAM15, MDC-L, and ADAM17. The peptides included a collection of substrates for other proteases, as well as a number of sequences corresponding to membrane proximal cleavage sites of various proteins postulated to be released by metalloproteases (including those published by Roghani *et al.* (21) for ADAM9/MDC9). In order to use the principle of fluorescence resonance energy transfer (FRET) for the assay, the peptides were labeled at the C terminus with Dabcyl and at the N terminus with Aedans. Cleavage of the peptides was monitored by the increase in Aedans fluorescence at 460 nm (excitation 360 nm) as a result of the

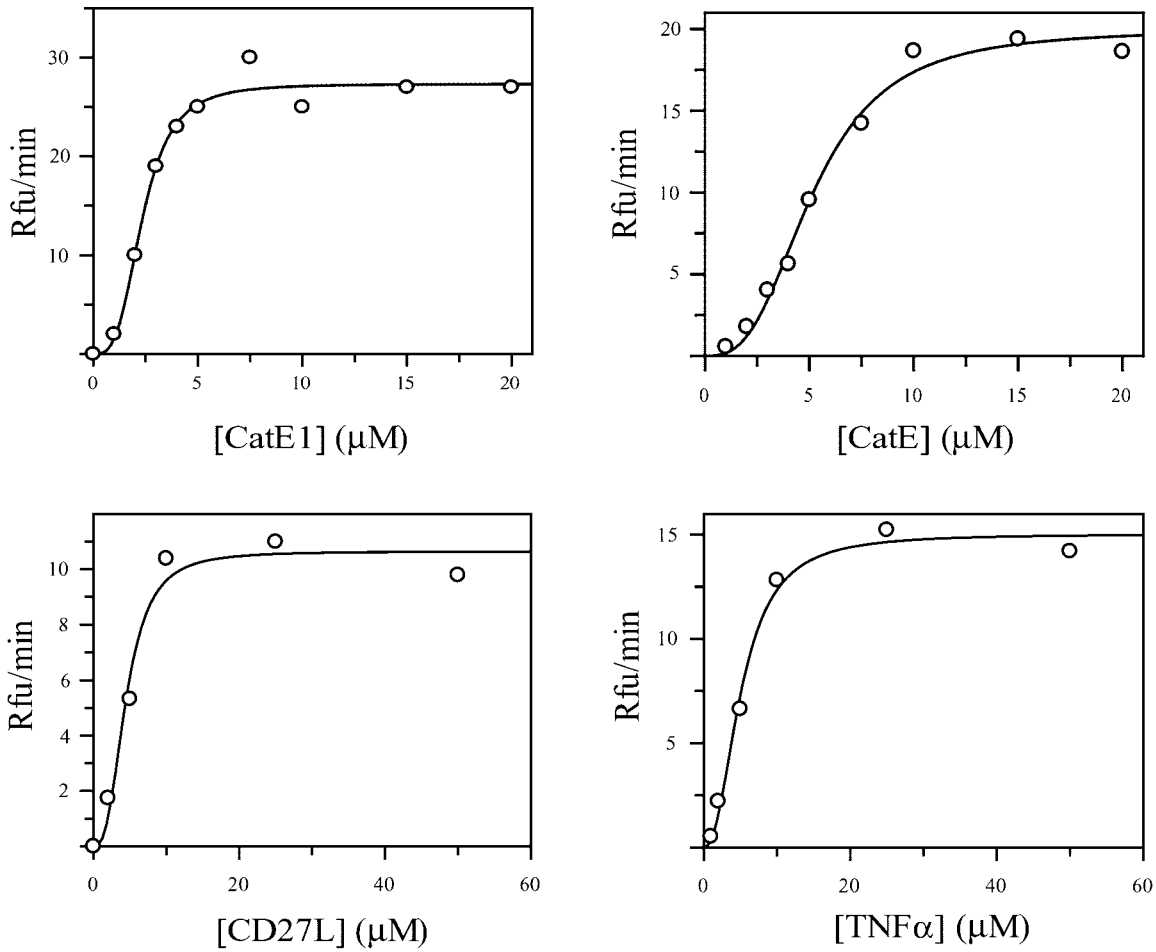

FIG. 2. **Kinetic analysis of ADAM8 cleavage for four different peptide substrates.** Soluble recombinant ADAM8 was diluted in assay buffer, and the reaction was initiated by addition of the substrate to different final concentrations. The assay was run for 30 min at room temperature. The proteolytic activity for each of the peptides is shown in relative fluorescence units (*Rfu*) per min as a function of substrate concentration. The curves were fitted to the data using the program Grafit (Erithacus Software). ADAM8 reached 50% of maximum activity at 2.5 μM for CatE1 peptide, 5 μM for CatE, 6.8 μM for CD27L, and 6.5 μM for TNFα peptide.

decrease in proximity of the Dabcyl quencher at the opposite end of each peptide. Soluble recombinant ADAM8, ADAM15, MDC-L, or ADAM17 (50–100 ng of protein, 1–2 pmol) were diluted in assay buffer, and the reactions were initiated by addition of each of 49 different peptide substrates, respectively, to a final concentration of 20 μM.

Fig. 1 shows the relative activities for the different peptides (numbered A1 to H6) expressed in arbitrary units, normalized by setting the activity for peptide F3 to one unit. This peptide was cleaved by all enzymes and thus was chosen to represent a standard by which to compare the relative activity of the various enzymes for their potential to cleave the different peptide substrates. Whereas ADAM8, ADAM15, and MDC-L showed a very similar activity profile for the various peptides, ADAM17 appeared to have a significantly different specificity and was able to cleave fewer of the peptides than the other three proteases. ADAM8, ADAM15, and MDC-L showed the highest activity for peptide C3, followed by B6 and G3. In contrast, the specificity of peptide cleavage for ADAM17 was quite distinct, with maximal cleavage being observed for peptide E6, followed by F3 and D6, both containing the authentic TNFα cleavage site, and peptide H4.

In order to compare directly the concentration dependence of cleavage of different peptides by ADAM8, initial rates of cleavage were measured for peptides C3 (named CatE1), a closely related peptide D2 (CatE), peptide B6 (corresponding to the cleavage site for CD27 ligand), and peptide F3 (corresponding to the TNFα release cleavage site). Fig. 2 shows the proteolytic activity (in relative fluorescence units per min) as a function of peptide concentration for peptides CatE1, CatE, CD27L, and TNFα, respectively. The curves were fitted to the data using the program Grafit (Erithacus Software). The best fits for the enzyme kinetic data were obtained using the Hill equation for allosteric behavior. Hill coefficients of 3, for peptides CatE1 and CatE, and 2, for CD27L and TNFα, respectively, were derived, suggesting the existence of cooperative active sites for ADAM8. Substrate inhibition was observed at peptide concentrations higher than those shown on the graphs (data not shown). The peptide for which ADAM8 had the highest apparent affinity was CatE1, and half-maximal cleavage activity was observed at 2.5 μM. Half-maximal cleavage for the other 3 peptides was observed between 5 and 6 μM. Table I shows the major cleavage site, determined by liquid chromatography-mass spectrometry, for ADAM8 within each peptide, as indicated by a caret within the peptide sequence.

Screening for Ectodomain Cleavage of Transfected Proteins by Soluble ADAM8, ADAM15, MDC-L, and ADAM17—Peptide substrate screening demonstrated catalytic activity for ADAM8, ADAM15, and MDC-L, with specificity distinct from that for ADAM17. In order to investigate substrate specificity for protein substrates, the ability of ADAM8 to cleave a number of membrane-bound proteins was tested. HEK293 cells were transfected with vector alone or cDNA for expression of V5 epitope-tagged CD27 ligand, CD40 ligand, CD30 ligand, or

TABLE I
Cleavage sites for ADAM8 within synthetic peptides
(X = Aedans-E, Z = Dabcyl-K)

Peptide name	Cleavage site	Ref.
CatE1	*X*KP**AKF^FRL**Z	This study (C3)
CatE	*X*KP**AAF^FRL**Z	This study (D2)
CD27L	*X*RF**AQA^QQQ**LPZ	This study (B6)
TNFα	*X*PLAQA**VRS^SSZ**	This study (F3)
TNFα	S**PLA^QAV**RSSSRK	3
TNFα	SPL**AQA^VRS**SSRK	3
IL-1Rc	TV**KEAS^STF**SWG	3
KL	LPP**VAA^SSL**R	3
APP	YE**VHH^QKL**VFF	3

CD23. After 48 h, the cells were harvested, washed, and incubated in HBS or soluble recombinant ADAM8 protease domain diluted in HBS for 1 h at 37 °C. The cells were then chilled, washed, and stained for surface expression of the intact membrane protein with FITC anti-V5. As shown in Fig. 3*A*, CD27 ligand and CD40 ligand were resistant to cleavage by ADAM8. However, CD30 ligand and, in particular, CD23, the low affinity IgE receptor, were susceptible to cleavage by ADAM8, as shown by the downward shift in cell-surface FITC anti-V5 staining. CD30 ligand staining intensity decreased by 26% after treatment with ADAM8. CD23 appeared to be the most susceptible of the proteins tested to ADAM8 ectodomain cleavage, resulting in a 64% decrease in mean fluorescence intensity for CD23 staining. Therefore, cleavage of CD23 by ADAM15, MDC-L, and ADAM17 was compared with that by ADAM8. The data in Fig. 3*B* show that CD23 was similarly susceptible to cleavage by soluble recombinant ADAM15 and MDC-L but not by an amount of soluble ADAM17 with equivalent activity on a peptide substrate, once again distinguishing their specificity, similarly to that observed with synthetic peptide substrates.

In order to show cleavage of endogenous CD23 as well as in the transfected cells, cells from the U937 macrophage line were harvested, washed, and incubated in HBS, or HBS containing soluble recombinant ADAM8 or ADAM17, for 1 h at 37 °C. The cells were then chilled, washed, and stained for surface expression of intact, endogenous CD23 with FITC anti-human CD23. As shown in Fig. 4*A*, exogenous ADAM8 was able to cleave endogenously expressed CD23 on U937 cells. However, cleavage was not observed (Fig. 4*B*) for exogenous addition of an amount of soluble ADAM17 with equivalent activity to ADAM8 on a synthetic peptide.

In order to demonstrate proteolytic ectodomain cleavage of CD23 by ADAM8 in *cis*, *i.e.* when expressed in the same cells, HEK293 cells were co-transfected with CD23, and either vector (pCDNA3), full-length ADAM8, or catalytically inactive ADAM8 (H604A, H608A), respectively. After 48 h, the medium from each transfection was removed, and the cells washed and lysed. Samples from the cell lysate and medium from each transfection were run on SDS-PAGE gels, transferred to nitrocellulose, and analyzed by Western blotting for the presence of cellular, intact CD23, active or inactive ADAM8, and soluble CD23 in the medium, respectively.

The *1st panel* in Fig. 5*A* shows staining of all the cell lysates with anti-V5 for the presence of V5-tagged CD23, whereas the *3rd panel* shows anti-V5 staining of the medium from each transfection, in order to detect any soluble forms of V5-tagged CD23. The *middle panel* shows staining with a polyclonal anti-ADAM8 antibody, directed against the C-terminal of full-length ADAM8. In contrast to the truncated soluble precursor and active forms of ADAM8, described earlier in this study with molecular masses of 44 and 25 kDa, respectively, the full-length, membrane-bound ADAM8 comprised a number of species with major bands at apparent molecular masses of 100,

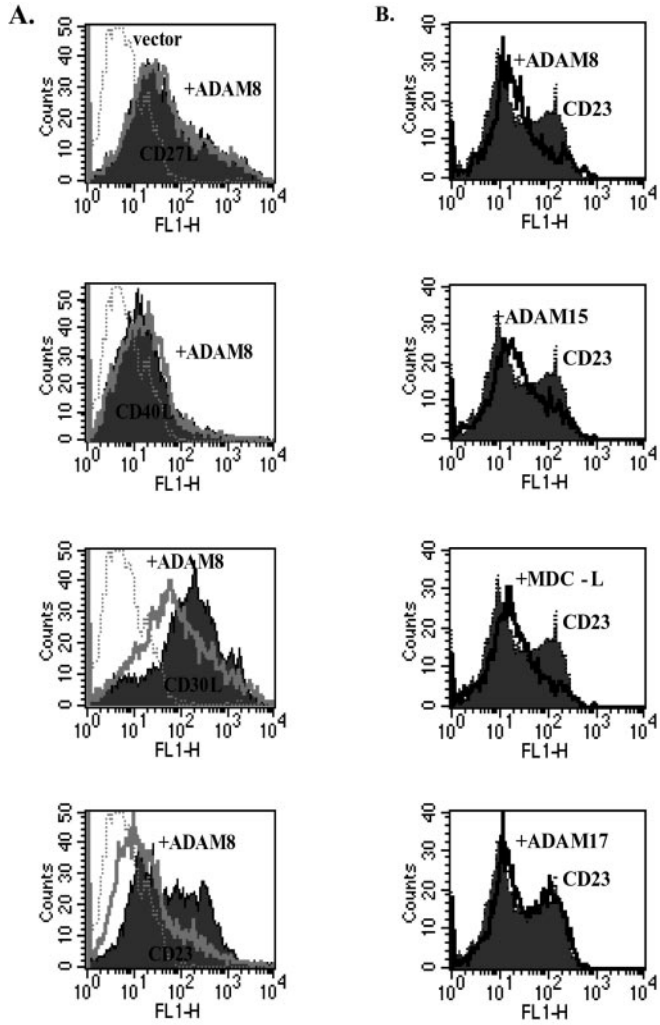

FIG. 3. **Screening for ectodomain cleavage of transfected CD23 and TNF family proteins by soluble ADAM8, ADAM15, MDC-L, and ADAM17.** HEK293 cells were transfected with vector alone or cDNA for expression of CD27L, CD40L, CD30L, or CD23 (Genestorms expression clones). After 48 h, the cells were harvested, washed, and incubated in HBS, or soluble recombinant ADAM8 diluted in HBS, for 1 h at 37 °C. The cells were then chilled, washed, and stained for surface expression of the intact TNF family protein with FITC anti-V5. *A* shows the flow cytometric analysis of the samples (*dotted line,* vector alone; *filled histogram*, TNF family protein or CD23 staining; *solid line,* staining after ADAM8 treatment). *B* shows the flow cytometric analysis of cells expressing CD23 incubated in the absence or presence of ADAM8, ADAM15, MDC-L, or ADAM17. Each panel is a histogram representing cell numbers (*y* axis, counts) *versus* log FITC fluorescence intensity (*x* axis, FL1-H), which is proportional to the amount of surface CD23 or TNF family member.

70, and 60 kDa. The theoretical molecular mass for full-length ADAM8 is ~92 kDa. The higher apparent molecular mass (100 kDa) for the largest species observed is likely to be due to post-translational modifications, as shown by Schlomann *et al.* (20) for murine ADAM8. Removal of the pro-domain (theoretically 19.4 kDa) was shown by Schlomann *et al.* (20) to result in a species of 72 kDa, presumably the same species as the 70-kDa band we observed. The 60-kDa band was shown by Schlomann *et al.* (20) to correspond to a "remnant" lacking the metalloprotease domain.

In cells transfected with vector plus CD23, no soluble forms of CD23 were observed in the medium (Fig. 5*A, 1st 2 lanes, lower panel*). However, when co-transfected with full-length, membrane-bound ADAM8, soluble CD23 could be detected in the medium (*lower panel, middle 2 lanes*). When a catalytically

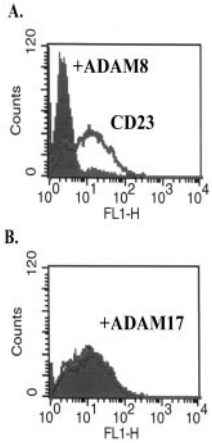

FIG. 4. **Ectodomain cleavage of endogenous CD23 in U937 cells by exogenous soluble ADAM8 *versus* ADAM17.** U937 cells were harvested, washed, and incubated in HBS or HBS containing soluble recombinant ADAM8, or ADAM17, for 1 h at 37 °C. The cells were then chilled, washed, and stained for surface expression of intact, endogenous CD23 with FITC anti-human CD23 (*solid line,* CD23 staining; *filled histogram,* staining after ADAM8 (*A*) or ADAM17 (*B*) treatment). Each panel is a histogram representing cell numbers (*y* axis, counts) *versus* log FITC fluorescence intensity (*x* axis, FL1-H), which is proportional to the amount of surface CD23.

inactive form of ADAM8 was co-transfected, no soluble CD23 was formed, and interestingly, the mutant ADAM8 was present mainly as the high molecular weight precursor, suggesting that, similar to MDC-L (ADAM28) (17), ADAM8 undergoes autocatalytic cleavage. Similar observations have recently been made by Schlomann *et al.* (20) for murine ADAM8. Thus, we observed the ectodomain cleavage of CD23, which was dependent on the presence of catalytically active ADAM8 protein. Preliminary results suggest that the soluble forms of CD23 released by ADAM8 were biologically active, as shown by their ability to stimulate TNFα release from THP-1 cells (not shown).

To determine the specificity of this ectodomain cleavage, HEK293 cells stably transfected with ADAM8 were transiently transfected with a number of TNF family proteins, namely CD27 ligand, Fas ligand, CD40 ligand, CD30 ligand, TRAIL and TNFα. The proteolytic susceptibility of these proteins to ADAM8 was then compared with that of CD23. Expression of all the transfected proteins could be detected in the cell lysates, (Fig. 5*B*, *upper panel*), although the levels varied, with FasL expression being particularly low. Whereas traces of soluble FasL and CD30L could be detected in the medium, the most significant appearance of soluble ectodomains was for CD23 (Fig. 5*B*, *lower panel*), showing that the cleavage is specific and not general, in line with the cytometry results in Fig. 3*A*.

Physical Association of ADAM8 with CD23—We looked for evidence of physical association of ADAM8 and its substrate, CD23, by immunoprecipitation of either CD23 or ADAM8 from transfected cells, and then Western blotting for the other protein in each case (Fig. 5*C*). In cells transfected only with vector or CD23, immunoprecipitation of ADAM8 was associated with a very faint band of immunoreactive CD23, which probably represented nonspecific background co-precipitation (*upper panel*, *lanes 5* and *6*). However, in cells co-transfected with two different clones of ADAM8, a much more significant band of co-precipitating CD23 was observed, indicating a physical association of the two proteins in these cells (*lanes 7* and *8*). Similarly, immunoprecipitation of CD23 was associated with a significant band of immunoreactive ADAM8 in co-transfected cells, confirming their co-precipitation (Fig. 5*C*, *lower panel*). Interestingly, only the highest molecular weight form of ADAM8 was associated with CD23. This is probably the precursor, inactive form of ADAM8, suggesting that the autocatalytic activation of ADAM8 may happen in association with substrate, and then ectodomain cleavage of CD23 may take place, disrupting the complex and releasing soluble CD23.

Effects of a Metalloprotease Inhibitor on CD23 Release in ADAM8-CD23-transfected Cells and JY and RPMI8866 B Cell Lines—A metalloprotease activity responsible for soluble CD23 release has been identified in the RPMI8866 B cell line (22). We have shown that MMP Inhibitor II (Calbiochem) potently inhibits this activity in RPMI8866 cells. In order to explore the possibility that this protease activity was ADAM8, we compared the inhibition by this compound of CD23 release from HEK293 cells co-transfected with ADAM8 and CD23 with inhibition of the endogenous release from both JY and RPMI8866 B cell lines.

As shown in Fig. 6, ADAM8-dependent CD23 release in transfected cells was potently inhibited by MMP inhibitor II, for which greater than half-maximal inhibition was observed at 1 μM compound. The inhibitor showed similar inhibition of endogenous CD23 release in both JY and RPMI8866 cells.

To verify the expression of ADAM8 in RPMI8866 and JY cell lines, immunoblotting with an ADAM8-specific rabbit antiserum was performed on lysates from equal cell numbers of the two lines, using actin as a control for loading of total protein. The results in Fig. 7 show a number of immunoreactive bands in both cell lines that correspond to molecular weights similar to the specific bands in ADAM8-transfected cells, as shown in Fig. 5, *A* and *C*. Thus, ADAM8 is expressed in both B cell lines, consistent with a potential role for this protease in the endogenous release of soluble CD23 from these lines.

DISCUSSION

ADAM8, ADAM15, and MDC-L (ADAM28) are members of the ADAM family of disintegrin metalloproteases, believed to play important roles in "ectodomain shedding." This study represents the first demonstration of their substrate specificity, as well as a comparison to that for ADAM17, the TNFα-concerting enzyme. By screening a library of synthetic peptides for cleavage by their metalloprotease domains, we have shown that the first three enzymes have very similar proteolytic substrate specificity, which is clearly distinct from that of ADAM17. In line with this observation, alignment of the amino acid sequences of their respective protease domains shows that the sequences of ADAM8, ADAM15, and MDC-L are much more closely related to one another than they are to that of ADAM17, shown by the phylogenetic tree in Fig. 8.

The cleavage sites found for ADAM8 within four synthetic peptides in our study are shown in Table I, compared with those found by Amour *et al.* (3), who also recently examined cleavage sites for recombinant soluble ADAM8 within synthetic peptides. The only peptide examined in both studies was TNFα. Whereas Amour *et al.* (3) found that ADAM8 cleaved between the Ala and Gln and between Ala and Val residues, we found cleavage between Ser and Ser residues, similar to that found for ADAM9 by Roghani *et al.* (21). Interestingly, the recombinant ADAM8 in the study by Amour *et al.* (3) cleaved between Ser and Ser in another peptide from IL-1Rc, and we found cleavage by ADAM8 between Ala and Gln in a peptide from CD27L. A peptide from the proposed region for ectodomain cleavage of CD40 ligand was not cleaved by ADAM8, consistent with our data suggesting that membrane-bound CD40 ligand was resistant to cleavage by ADAM8. From the limited set of cleavage sites determined for ADAM8 in Table I, it appears that this enzyme cleaves mainly between hydrophobic or polar amino acids. Apart from the P3 site in peptide IL-1Rc, the P3 through P3′ sites in peptides cleaved by ADAM8

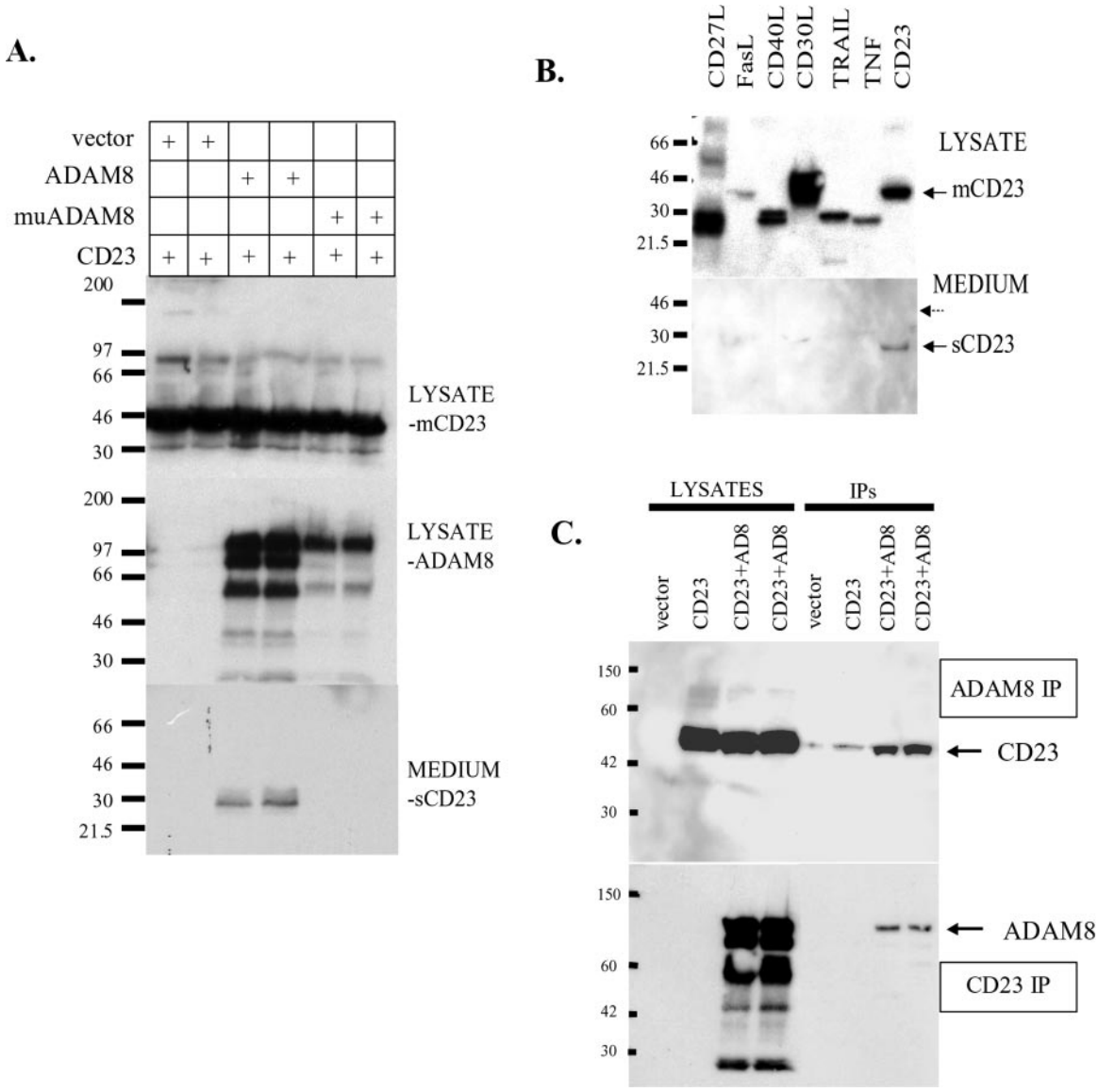

FIG. 5. **ADAM8-dependent soluble CD23 release in transfected cells.** *A*, release of sCD23 by proteolytically active ADAM8. HEK293 cells were transfected with CD23 cDNA plus either vector, ADAM8 cDNA, or the cDNA for a catalytically inactive ADAM8 (H604A, H608A = muADAM8). After 48 h, the medium from each transfection was removed, and the cells were washed and lysed. Samples from the cell lysate and medium from each transfection were run on SDS-PAGE gels, transferred to nitrocellulose, and analyzed by Western blotting for the presence of cellular, intact CD23 (*top panel*), active or inactive ADAM8 (*middle panel*), and soluble CD23 in the medium (*lower panel*). The *1st panel* shows staining of all the cell lysates with anti-V5 for the presence of V5-tagged CD23, and the *3rd* panel shows anti-V5 staining of the medium from each transfection, in order to detect any soluble forms of V5-tagged CD23. The *middle panel* shows staining with a polyclonal anti-ADAM8 antibody, directed against the C-terminal of full-length ADAM8. *B*, effect of ADAM8 on release of soluble CD23 compared with other TNF proteins. HEK293 cells stably expressing ADAM8 were super-transfected with cDNA for expression of CD27L, FasL, CD40L, CD30L, TRAIL, TNFα, or CD23. After 48 h, the medium from each transfection was removed and analyzed by Western blotting for the presence of soluble ectodomain fragments in the medium. *C*, physical association of ADAM8 with CD23. HEK293 cells were transfected with vector alone, cDNA for CD23 alone, or CD23 plus two different clones of ADAM8 cDNA. After the generation of stable lines by selection with G418 and/or Zeocin, cells from each line were lysed and subjected to immunoprecipitation (*IP*) of CD23, using anti-V5 antibody or ADAM8, using a polyclonal antiserum to the C terminus of ADAM8. The immunoprecipitates were subjected to SDS-PAGE and Western blotting with either anti-V5 to detect the presence of CD23 or the anti-ADAM8 antiserum for the presence of ADAM8. The complete lysates were also subjected to Western blotting to control for the expression of CD23 and ADAM8 in each line.

do not contain any negatively charged residues. This is in contrast to cleavages observed by Amour *et al.* (3) for MT1- and MT4-MMP, or the known preference for the aggrecanase members of the ADAMTS family for glutamic acid in the P1 site (23, 24). However, it is difficult to draw any general conclusions about sub-site specificity from such a small collection of peptide substrates.

CD23 or FcεRII, the low affinity IgE receptor, is expressed on B cells, monocytes, macrophages, and eosinophils (25) and is cleaved from the cell surface to generate a number of soluble forms. Both membrane-bound and soluble forms of CD23 have been shown to be elevated in a number of diseases such as asthma, rheumatoid arthritis, and inflammatory bowel disease (18). Soluble forms of CD23 play an important role in the up-regulation of IgE synthesis by interaction with B cells (26), as well as promoting the induction of inflammatory cytokines by macrophages (18). The identity of the metalloprotease(s) responsible for the release of soluble CD23 has not been determined. We found that ADAM8, ADAM15, and MDC-L, but not ADAM17, catalyzed ectodomain shedding of CD23. In co-transfection studies, ADAM8-dependent soluble CD23 release was observed only when proteolytically active ADAM8 was present.

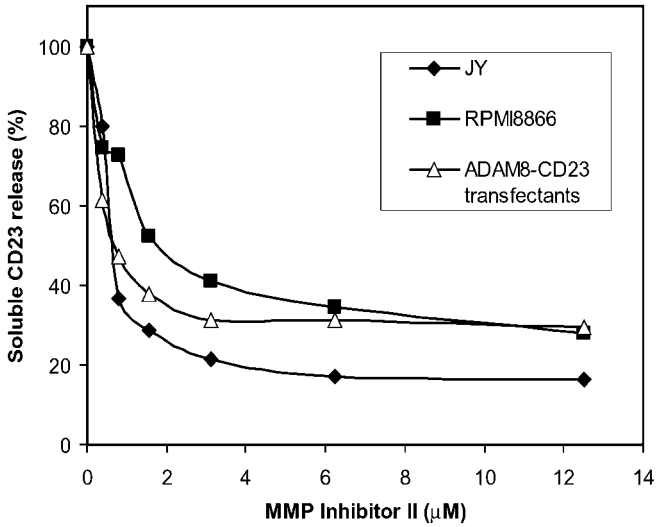

FIG. 6. **Effects of inhibitors on CD23 release in ADAM8-CD23-transfected cells and JY and RPMI8866 B cell lines.** HEK293 cells transfected with ADAM8 and CD23, JY cells, or RPMI8866 cells were incubated for 1 h at 37 °C, in the absence or presence of 0.4–12.5 μM MMP inhibitor II, after which the supernatant was analyzed for the presence of soluble CD23 by enzyme-linked immunosorbent assay. Data are expressed as a percentage of the level of soluble CD23 released in the absence of compound, and each point is the mean of duplicate wells.

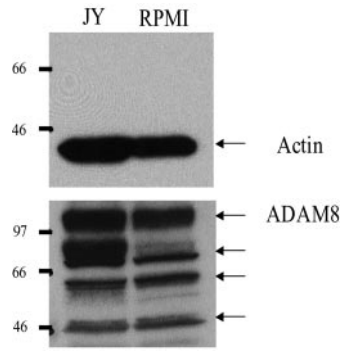

FIG. 7. **Western blot analysis of ADAM8 protein expression in JY and RPMI8866 B cell lines.** Equivalent numbers of cells from JY and RPMI8866 Epstein-Barr virus-transformed B cell lines were lysed and analyzed by SDS-PAGE and Western blotting for expression of ADAM8, as well as actin as a control for protein loading. The polyclonal antiserum used for ADAM8 protein was raised against the C-terminal peptide sequence of ADAM8, as described under "Experimental Procedures," and actin was detected with a commercially available monoclonal antibody (Roche Applied Science).

We also demonstrated a physical association of ADAM8 protease with the membrane-bound form of CD23. A similar association of Kuzbanian, the *Drosophila* homologue of ADAM10, has been observed with its substrate, ephrin-A2, a protein involved in contact-mediated axon repulsion during assembly of neural circuits (27). Upon interaction of ephrin-A2 ligand, in complex with Kuzbanian, with its receptor tyrosine kinase, the associated metalloprotease is activated and cleaves ephrin-A2, allowing for repulsion between the two cells. It is possible that activation of ADAM8 and cleavage of CD23 are similarly synchronized, either intracellularly or at the surface, because we observed only the highest molecular weight form of ADAM8, presumably the inactive precursor containing the pro-domain, in the stable complex with membrane-bound CD23. Schlomann *et al.* (20) have also shown in co-transfection experiments that ADAM8 autoactivation may require physical interaction between ADAM8 monomers. The allosteric kinetics we observed in this study for peptide cleavage also suggest activity in oligomeric forms, with co-operative active sites for ADAM8. This

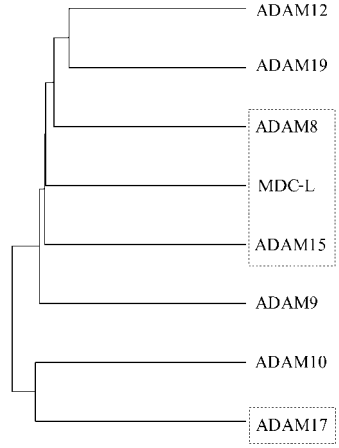

FIG. 8. **Phylogenetic tree analysis of ADAM protease domains.** The *dendrogram*, representing the degree of sequence similarity between the protease domains of different human ADAM proteins, was generated by the GCG program PILEUP, using the amino acid sequences of only their respective metalloprotease domains.

is an interesting observation in light of the fact that a number of proteins that are substrates for ectodomain cleavage, such as TNFα (28) and, in particular, CD23, exist naturally as trimers (29).

The ADAM8-dependent release of soluble CD23, and the endogenous release from two B cell lines, shown to express ADAM8, could be similarly inhibited by a metalloprotease inhibitor compound. Preliminary results suggest that the soluble forms of CD23 released by ADAM8 are biologically active, as shown by their ability to stimulate TNFα release from THP-1 cells. Although we have shown that ADAM15 and MDC-L have similar substrate specificity to ADAM8, and were also able to cleave CD23, the cell and tissue distribution of ADAM8, rather than ADAM15 or MDC-L, is most similar to that of CD23. We conclude that ADAM8 could contribute to ectodomain shedding of CD23 and may thus be a potential target for therapeutic intervention in allergy and inflammation.

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Enzyme Catalysis and Regulation:

Catalytic Activity of ADAM8, ADAM15, and MDC-L (ADAM28) on Synthetic Peptide Substrates and in Ectodomain Cleavage of CD23

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