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Identification and Characterization of a Pro-tumor Necrosis Factor-α-processing Enzyme from the ADAM Family of Zinc Metalloproteases*

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Tumor necrosis factor-α (TNF) is initially expressed as a 26-kDa membrane-bound precusor protein (pro-TNF) that is shed proteolytically from the cell surface, releasing soluble 17-kDa TNF. We have identified human ADAM 10 (HuAD10) from THP-1 membrane extracts as a metalloprotease that specifically clips a peptide substrate spanning the authentic cleavage site between Ala^{76} and Val^{77} in pro-TNF. To confirm that HuAD10 has TNF processing activity, we cloned, expressed, and purified an active, truncated form of HuAD10. Characterization of recombinant HuAD10 (rHuAD10) suggests that this enzyme has many of the properties (*i.e.* substrate specificity, metalloprotease activity, cellular location) expected for a physiologically relevant TNF-processing enzyme.

Tumor necrosis factor-α (TNF)[1] is a cytokine that is produced primarily by activated monocytes and macrophages in response to a variety of physiological stresses such as infection or injury (1). Clinical and experimental evidence has also identified TNF as a mediator of chronic autoimmune diseases such as rheumatoid arthritis (2) and Crohn's disease (3), as well as being involved in the pathology associated with sepsis (1). Accordingly, TNF has become a primary target for therapeutic intervention of several inflammatory diseases.

TNF is initially synthesized as a 26-kDa membrane-bound protein (pro-TNF) that is subsequently cleaved to release soluble 17-kDa TNF with an NH_2 terminus of Val^{77} (4). The identity of the protease(s) responsible for TNF processing remains controversial. Robache-Gallea *et al.* (5) detected a serine protease activity (PR3) in monocyte membrane preparations which was able to generate a 17-kDa active TNF with an NH_2 terminus of Arg^{78}. In 1994, the partial isolation and characterization of a membrane-bound activity capable of generating the 17-kDa form of TNF were reported (6). The TNF-processing enzyme was thought to be a non-matrix metalloprotease since it was not inhibited by TIMP-1,2 or phosphoramidon, and no calcium requirement was detected. More recently, two members of the ADAM family (*T*NF-α *c*onverting *e*nzyme (TACE) and bovine ADAM 10 (BoAD10)) have been shown to possess pro-TNF processing activity (7–9).

In this report we describe the isolation, cloning, and characterization of a TNF-processing enzyme from the human monocytic cell line THP-1. The purified recombinant enzyme, rHuAD10, specifically recognizes the authentic cleavage site in pro-TNF and is sensitive to metalloprotease inhibitors that block soluble TNF production (6).

MATERIALS AND METHODS

Reagents—Dinitrophenol-labeled polypeptides were synthesized by the Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)/*t*-butyl-based solid phase peptide chemistry method using an Applied Biosystems, Inc. 431A peptide synthesizer (10). All peptides were purified by reversed phase HPLC, and molecular weights were verified by mass spectrometry.

HPLC Peptide Assay—TNF processing activity was measured as the ability to cleave a 12-residue peptide spanning the Ala^{76}-Val^{77} site in pro-TNF. The chromatophoric peptide substrate DNP-SPLAQA-VRSSSR-$CONH_2$ was dissolved to a final concentration of 0.5 mg/ml in 40 mM Tris-HCl, 100 μM $ZnCl_2$, 10% glycerol, 0.5% octylglucoside. Protein samples were diluted 1:1 with the peptide substrate and allowed to incubate for 1 h or longer at 37 °C, depending on the relative activity of the sample. The unhydrolyzed substrate and cleaved peptides were separated using a Beckman System Gold HPLC with a Vydac C_{18} reversed phase column (4 mm × 30 cm), and the eluate was monitored at 350 nm. The peptides were eluted using linear gradients of CH_3CN/water with 0.1% trifluoroacetic acid. Mass spectrometry confirmed that the peptide fragments produced by the partially purified protein or rHuAD10 had the correct molecular weight. One milliunit of enzyme activity is defined as 1 mmol of peptide substrate cleaved/min/mg of protein at 37 °C.

Cells and Culture Conditions—THP-1 cells (American Type Culture Collection, Rockville, MD) were grown in RPMI (Life Technologies, Inc.), 1% glutamine penicillin-streptomycin (Life Technologies, Inc.), and 10% heat-inactivated fetal bovine serum.

Membrane Extraction—THP-1 membranes were prepared essentially by the method of Maeda *et al.* (11) except that dithiothreitol was omitted, and 200 μM phosphoramidon was added to all of the buffers. Membranes were homogenized in buffer containing 10 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM Pefabloc™, 200 μM phosphoramidon, 1.3 μM aprotinin, 5 μM leupeptin, 30 mM NaCl. Octylthioglucoside and NaCl were next added to final concentrations of 2% and 0.5 M, respectively. The mixture was rocked at 4 °C for 3 h and then centrifuged at 180,000 × *g* for 1 h. The supernatant was recovered and assayed for TNF processing activity.

S-Sepharose (HP) Column Chromatography—Membrane extracts were diluted 25-fold with buffer A (20 mM NaOAc, 20 mM monobasic sodium phosphate, pH 5.0, 10% glycerol, 0.5% octylglucoside, 50 μM phosphoramidon) and brought to a NaCl concentration of 20 mM. The mixture was clarified by filtration before being loaded onto an S-Sepha-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF009615.

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[1] The abbreviations used are: TNF, tumor necrosis factor α; ADAM, *a d*isintegrin *a*nd *m*etalloprotease; BoAD10, bovine ADAM 10; bp, base pair(s); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DNP, dinitrophenol; HA, hydroxyapatite; HPLC, high performance liquid chromatography; HuAD10, human ADAM 10; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; rHuAD10, recombinant human ADAM 10; TACE, *T*NF-α *c*onverting *e*nzyme; TIMP, tissue inhibitor of metalloprotease; TNFR, tumor necrosis factor receptor.

rose column previously equilibrated with buffer A. The proteins were eluted in 10 column volumes with a linear gradient to 100% of buffer B (20 mM NaOAc, 20 mM dibasic sodium phosphate, pH 10.0, 10% glycerol, 0.5% octylglucoside, 50 μM phosphoramidon). The pH of the collected fractions was adjusted immediately to 8.0 with 1 M Tris base.

Wheat Germ Lectin Affinity Chromatography—The active S-Sepharose fractions were pooled and diluted 1:1 with buffer C (30 mM Tris-HCl, pH 7.0, 10% glycerol, 150 mM NaCl). Additional protease inhibitors were added to final concentrations of 1 μg/ml pepstatin, 1 mM Pefabloc™, 2 μg/ml aprotinin, and 50 μM phosphoramidon. The protein pool was next applied to a wheat germ lectin-Sepharose column equilibrated in buffer C. The column was washed with additional buffer C and eluted with 500 mM *N*-acetylglucosamine. A majority of the original activity was present in the flow-through (69%) which was taken forward onto the next column.

Hydroxyapatite (HA) Chromatography—The wheat germ flow-through pool was concentrated, diluted 1:4 with buffer D (10 mM sodium phosphate, pH 7.6, 10% glycerol, 0.5% octylglucoside, 50 μM phosphoramidon), loaded onto an HA-Ultragel column, and eluted with a linear gradient from 10 to 400 mM sodium phosphate in buffer D. A majority of the activity was detected in the flow-through, whereas many of the contaminating proteins bound to the HA resin.

Protein Sequencing—The HA flow-through was concentrated and the components separated by SDS-PAGE using a 10% Tris-glycine gel (NOVEX™, San Diego) followed by transfer to polyvinyldifluoride membrane and Coomassie R-250 Brilliant Blue staining. NH_2-terminal sequencing of the proteins was performed by automated Edman degradation on a Procise™ 494 microsequencer (Applied Biosystems, Inc.).

cDNA Cloning—HuAD10 cDNA was amplified by PCR using an oligonucleotide primer (5′-ACT TAT TAA GAT CTC GGA AGA TGG TGT TGC (CT)GA (CG)AG TG) derived from the 5′-ends of bovine (12) and murine sequences[2] and a primer (5′-AAG CTA ATT GCG GCC GCC AAA AGC TGC AGT TAG CGT CTC ATG) derived from the 3′-end of the human sequence (12). The PCR was carried out using Advantage KlenTaq™ DNA polymerase as described by the manufacturer (Clontech, Palo Alto, CA), with 2×10^7 plaque-forming units of a human macrophage cDNA library cloned in λZAPII™ (Stratagene, La Jolla) as template. A single band of the expected size, 2.2 kilobases, was generated using these conditions. The PCR was then repeated in the presence of radiolabeled nucleotide, and the ^{32}P-labeled product was used to probe the human macrophage cDNA library used in the PCR. Positive hybridizing clones were plaque-purified, and phagemid DNA (pHuAD10) was excised as described by the manufacturer (Stratagene).

Northern Blot Analysis—A human immune system multiple tissue blot (Clontech) was probed with an [α-^{32}P]dCTP-labeled TNF cDNA probe using conditions described by the manufacturer. The blot was then stripped and probed with an [α-^{32}P]dCTP-labeled HuAD10 cDNA probe.

Expression of rHuAD10—HuAD10 cDNA lacking the transmembrane domain and cytoplasmic tail coding sequences was fused in-frame to a human IgGγ1 heavy chain hinge and CH2 and CH3 domains. The HuAD10 portion was prepared by PCR amplification of a 2,036-bp DNA fragment spanning the initiation codon to the codon corresponding to Glu^{672}. The 5′-DNA oligonucleotide primer (5′-GTG GCA CCA AGC TTG CCA CCA TGG TGT TGC TGA GAG TGT TAA TTC) contained a *Hin*dIII restriction site and a Kozak sequence, whereas the 3′-oligonucleotide primer (5′-GAG CTG GAC CGC GGC CGC TTC AGC AAT GTT TTC ATA GAG CTC) contained a *Not*I restriction enzyme site. PCR was performed on pHuAD10 using *Pfu* polymerase as described by the manufacturer (Stratagene). The resulting PCR product was then digested with *Hin*dIII and *Not*I and then ligated into the human IgG vector, pFC-A3.[3] The *Hin*dIII-*Xho*I fragment containing HuAD10-Ig was then cloned into the mammalian expression vector, pCEP4 (Invitrogen, San Diego) to create pHuAD10-Ig.

For mammalian expression, 2×10^6 293-EBNA cells (Invitrogen) were transfected with 10 μg of pHuAD10-Ig using LipofectAMINE™ as described by the manufacturer (Life Technologies, Inc.). After 24 h, the cells were split 1:5 into medium containing 100 μg/ml hygromycin. The drug-resistant cells were expanded and used to seed Nunc cell factories at a density of 1.4×10^5 cells/cm^2. Factories were incubated at 37 °C with 10% CO_2 in serum and hygromycin-supplemented medium for 6 days. After reaching confluence (approximately 10^6 cells/cm^2), the cells were washed with phosphate-buffered saline and then incubated in serum-free Dulbecco's modified Eagle's medium containing 1 × L-glutamine and 100 μg/ml hygromycin. The medium was conditioned for 7 d at 37 °C before harvesting.

[2] Amgen cDNA data base, unpublished results.

TABLE I
Purification recoveries for a TNF-processing enzyme

THP-1 cells were used as starting material for the isolation and identification of a protease responsible for the TNF processing activity present in the cell membranes. Activity as determined by the HPLC peptide assay is described under "Materials and Methods."

Sample	Total protein	Total activity	Specific activity	Purification
	mg	*units × 10^{-3} (milliunits)*	*milliunits/mg total protein*	*−fold*
Membrane extract	175	273	1.56	1
S-Sepharose pool	0.83	101.5	127	81
Wheat germ pool	0.14	72.4	517	331
HA pool	0.06	29.4	507	325

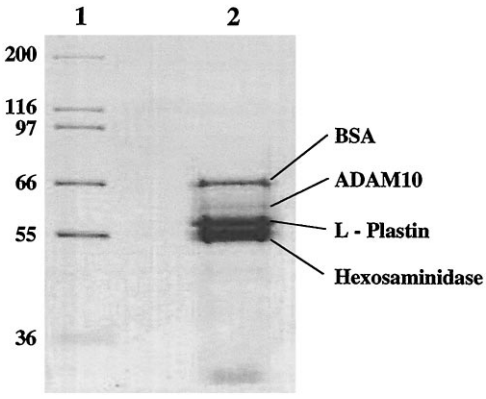

FIG. 1. **Protein blot used for the identification of HuAD10.** The HA pool representing a highly enriched protein preparation was analyzed by SDS-PAGE before electroblotting as described under "Materials and Methods" (*lane 2*). Proteins were identified by NH_2-terminal sequencing. Molecular mass standards (in kDa) are shown in *lane 1*. *BSA*, bovine serum albumin.

Purification of rHuAD10—6 liters of conditioned medium from THP-1 cells was concentrated 10 × using a Filtron 50-kDa filter and dialyzed extensively against 10 mM sodium phosphate, pH 7.0, 10% glycerol (buffer E). The protein mixture was applied to an HA-Ultragel column previously equilibrated in buffer E. rHuAD10 was eluted from the resin using a phosphate gradient from 10 to 400 mM phosphate in buffer E. The active HA fractions were pooled and dialyzed against 50 mM sodium phosphate, 1.5 M ammonium sulfate, pH 7.0 (buffer F) before being loaded onto a butyl-Sepharose HP column equilibrated in buffer F. rHuAD10 was recovered from the resin using an inverse salt gradient from 1.5 M to 0.5 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0. Active fractions from the butyl-Sepharose column were concentrated, buffer exchanged in 150 mM ammonium bicarbonate, 0.3% CHAPS, pH 8.1, then applied to a Superdex 200 (HR 10/30) gel filtration column.

HuAD10 Immunoblotting—A peptide (DANQPEGRKCKLKPGKQ) derived from amino acids 486–502 in rHuAD10 was synthesized and injected into rabbits to generate polyclonal antiserum AMG1961. For immunoblotting, samples were subjected to SDS-PAGE, and proteins were transferred electrophoretically to nitrocelluose. Filters were incubated in blocking buffer (5% skim milk in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature and then treated with AMG1961 (1:1,000) in blocking buffer for 2 h at room temperature. After washing, filters were incubated with anti-rabbit IgG (Fc) AP conjugate (Promega, Madison, WI) in blocking buffer for 1 h at room temperature. Immune complexes were visualized by incubating with Western Blue stabilized substrate for alkaline phosphatase (Promega) according to the manufacturer's specifications.

In Vitro Translation and Cleavage of Pro-TNF—The human pro-TNF coding sequence was inserted into the *Eco*RI site of pCRII (Invitrogen). Plasmid DNA was transcribed and translated using a rabbit reticulocyte lysate translation kit (TNT™ SP6-coupled Reticulocyte Lysate System, Promega) with [^{35}S]cysteine (Amersham Corp.), according to the manufacturer's recommendations. The lysate was dialyzed against a 250-fold excess volume of buffer consisting of 50 mM Tris-HCl, pH 8.5, 10% glycerol, and 10 mM NaCl for 3 h at 4 °C. After dialysis, pro-TNF

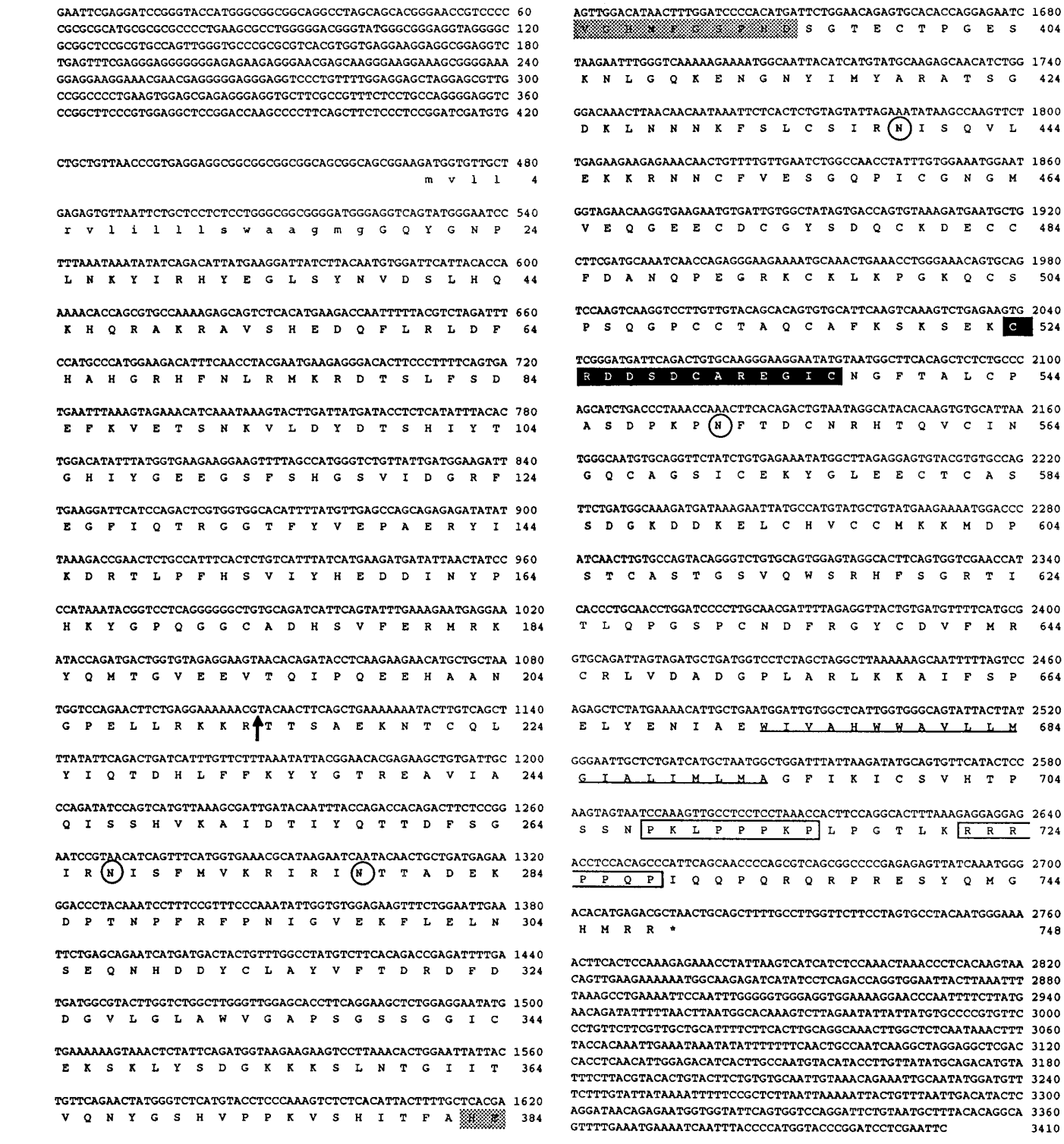

FIG. 2. **Nucleotide and deduced amino acid sequence for HuAD10.** The putative signal sequence, transmembrane sequence, and propeptide cleavage site are indicated by *lowercase letters*, *underlining*, and a *solid arrow*, respectively. *Light shading* is indicative of the metalloprotease active site, and *dark shading* is indicative of the disintegrin loop. Four putative *N*-linked glycosylation sites are *circled*, and two potential SH3 (Src homology 3) domains are *boxed*.

was incubated with a partially purified protein preparation with TNF processing activity (S-Sepharose pool) or rHuAD10 + 50 μM $ZnCl_2$ for 10 h at 37 °C. The reaction mixtures were immunoprecipitated with rabbit anti-human TNF polyclonal antibody (Amgen Inc.) before SDS-PAGE analysis.

RESULTS

Purification of TNF Processing Activity—A purification scheme for the isolation of a TNF-processing enzyme from THP-1 membrane extracts was optimized using an assay based on the specific proteolysis of a peptide spanning the pro-TNF cleavage site. Purification recoveries after cation exchange, lectin affinity, and hydroxyapatite chromatographies are shown in Table I. During the final column step, we saw a decrease in specific activity which was indicative of protein instability, even in the presence of detergents and glycerol. A protein blot from a final preparation with TNF processing

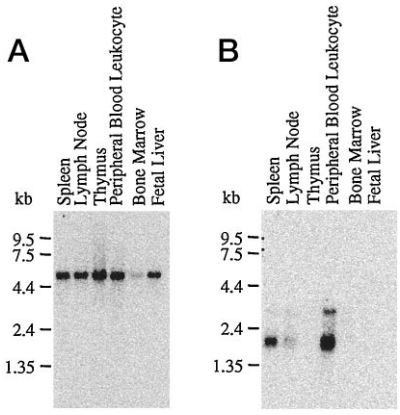

FIG. 3. **Northern blot analysis of HuAD10 and TNF in human tissues.** A Northern blot of RNA extracted from different human tissues was probed with a HuAD10- (*panel A*) or TNF- (*panel B*) specific probe as described under "Materials and Methods." The source of the mRNA is indicated for each *lane*. *kb*, kilobases.

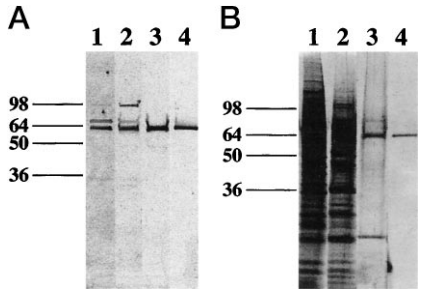

FIG. 4. **Purification of rHuAD10.** Samples from each purification step were analyzed by SDS-PAGE and visualized by immunoblotting (*panel A*) or silver staining (*panel B*) as described under "Materials and Methods." *Lane 1*, conditioned medium; *lane 2*, HA pool; *lane 3*, butyl-Sepharose pool; and *lane 4*, S-200 pool.

activity is shown in Fig. 1. Although we were unable to purify the active component to homogeneity, the correct proteolytic activity consistently tracked with the presence of a 62-kDa protein having an NH_2 terminus of SAEKNTXQLYIQTDH-LFFKYYG. A search of this sequence against GenBank revealed a high homology with the NH_2 terminus of BoAD10. This protease is the 10th member of a family of proteins known to contain both "*a d*isintegrin *a*nd *m*etalloprotease *d*omain." ADAM 10 was previously purified from bovine brain, but its physiological function was not known (12). Copurifying with HuAD10 were nonproteolytically active proteins including bovine serum albumin (from the growth medium), L-plastin (an actin-binding protein), and β-*N*-acetylhexosaminidase α-chain.

Cloning of HuAD10 cDNA—The initial identification of HuAD10 in an active, highly purified THP-1 cell extract prompted us to clone and express rHuAD10 for further characterization studies. A search of GenBank revealed that a partial HuAD10 cDNA lacking the 5′-end had been deposited by P. Glynn (12). PCR and hybridization techniques were used to identify a full-length cDNA from a human macrophage library.

The HuAD10 cDNA is 3,410 bp (Fig. 2) consisting of a 469-bp 5′-untranslated region, a 2,244-bp sequence encoding a protein of 748 amino acids, and a 697-bp 3′-untranslated region. Northern blot analysis (Fig. 3*A*) demonstrated that a single transcript of approximately 5.0 kilobases (also detected by Howard *et al.* (12) in five human cell lines) was present in all tissues examined. When the same blot was hybridized with a TNF-specific probe, TNF mRNA was detected in three of the six tissues tested (Fig. 3*B*). The deduced amino acid sequence of our clone is identical to the published partial sequence of HuAD10 except that we observed a glycine at amino acid 296 instead of a serine. Overall, HuAD10 is 96.7% identical to BoAD10 and 97.1% identical to a partial cDNA encoding rat ADAM 10 (12).

Comparisons with other family members indicate that HuAD10 is most closely related to TACE (49.8% overall similarity (7, 8)) followed by murine ADAM 8, a macrophage surface antigen (49.4% overall similarity (13)). Analogous to other ADAM family members, HuAD10 possesses propeptide, metalloprotease, disintegrin, and transmembrane domains (Fig. 2) (14). The HuAD10 propeptide is predicted to end at amino acid 213 with a tetrabasic motif (RKKR) that might serve as a cleavage site for furin-like proteases in the Golgi (15). The propeptide of rHuAD10 contains a single cysteine at amino acid 173 which may be regulating the activity of the enzyme through a cysteine switch mechanism as with other metalloproteases (16). Mature HuAD10 is predicted to have 535 amino acids with a calculated M_r of 59,286. The difference between the calculated M_r of HuAD10 and its apparent M_r of 62,000 on SDS-PAGE is likely because of glycosylation, as HuAD10 has four potential sites for *N*-glycosylation (Fig. 2).

Expression and Purification of rHuAD10—To obtain rHuAD10, a soluble ADAM 10-Ig construct was generated and transfected into 293-EBNA cells. Immunoblot analysis with an antibody specific to HuAD10 demonstrated the expression of 98-, 72-, and 62-kDa proteins in the conditioned medium of transfected cells (Fig. 4*A*). Isolation and characterization of these proteins revealed that TNF processing activity tracked only with the 62-kDa protein. Since the 98-kDa band cross-reacts with an antibody against human Fc, but the 62- and 72-kDa proteins do not (data not shown), we suspect that the proteolytically sensitive Fc hinge region was cleaved from the 62- and 72-kDa proteins by proteases present in 293-EBNA cells or conditioned medium.

A silver-stained gel demonstrating purification of the 62-kDa rHuAD10 to homogeneity is shown in Fig. 4*B*. rHuAD10 has a specific activity of 1,000 milliunits/mg of protein and an NH_2 terminus that is identical to that of HuAD10 purified from THP-1 cells.

Characterization of rHuAD10—To characterize the proteolytic activity of rHuAD10 *versus* a partially purified protein preparation (S-Sepharose pool), we assayed for TNF processing activity in the presence of a variety of protease inhibitors. Relative activities, shown in Table II, reveal that the S-Sepharose pool and rHuAD10 have comparable profiles. Results indicate that the serine protease inhibitors had little effect on proteolytic activity, and no significant inhibition was observed with the matrix metalloprotease inhibitors phosphoramidon and TIMP-1. In contrast, known metal chelators (EDTA and 1,10-phenanthroline) inhibited the proteolytic activity in both protein preparations.

The pH optima were examined for both rHuAD10 and partially purified HuAD10 (S-Sepharose pool). Maximum TNF processing activity for both protein preparations was between pH 9 and 10 (data not shown).

Substrate Specificity of rHuAD10—The substrate specificity of rHuAD10 was examined by testing its ability to cleave a variety of peptides with substitutions surrounding the Ala-Val cleavage site. The influence of these substitutions on peptide cleavage is shown in Table III. Alterations at Ala^{76} except for A76Y and A76K resulted in a significant decrease in cleavage efficiency. rHuAD10 was better able to tolerate changes at Val^{77}. Substitutions with Leu, Ile, Arg, Tyr, and Ser had no effect on the ability of rHuAD10 to catalyze peptide cleavage.

We examined peptide sequences spanning the cleavage sites for other proteins known to be shed proteolytically from the cell surface (Table IV). Proteins were chosen based on sequence

TABLE II
Characterization of rHuAD10

Protease inhibitors were added to each protein preparation at a concentration recommended by the manufacturer. Activity was assayed by the HPLC peptide assay as described under "Materials and Methods."

Inhibitors	Protease class	rHuAD10	S-Sepharose pool
		% inhibition	*% inhibition*
Phenylmethylsulfonyl fluoride (10 μM)	Serine	0	14.5
Leupeptin (1 μM)	Serine, thiol	0.5	5.4
Aprotinin (0.3 μM)	Serine	3	8.6
Pepstatin (1 μM)	Aspartic	0	0
EDTA (1 mM)	Metallo	88	53
1,10-Phenanthroline (1 mM)	Metallo	100	86
Phosphoramidon (10 mM)	Metallo	0	0
TIMP-1 (20 nM)	Metallo	0	12

TABLE III
Influence of substitutions at the pro-TNF cleavage site

DNP-peptides were synthesized and assayed as described under "Materials and Methods." rHuAD10 was used as the enzyme source. Samples were incubated for 17 h at 37 °C and quenched with 1 mM EDTA before HPLC analysis.

Wild type sequence										
72	73	74	75	76	77	78	79	80	81	82
Pro	Leu	Ala	Gln	Ala	Val	Arg	Ser	Ser	Ser	Arg

Substitutions			
Substitution	% cleavage	Substitution	% cleavage
Wild type	100	V77G	0
A76D	0	V77E	0
A76L	0	V77P	0
A76G	11	V77W	49
A76T	27	V77L	100
A76S	39	V77I	100
A76P	63	V77R	100
A76Y	95	V77Y	100
A76K	100	V77S	100
R78K	33	S79E	10
R78T	94	S79T	77
R78Q	100	S79R	100

homology to the pro-TNF cleavage site (c-*kit* ligand-1, pro-transforming growth factor-α, TNFR-75) or previous reports of metalloprotease-dependent shedding as determined by inhibitor studies (interleukin-6 receptor, TNFR-55, β-amyloid precursor protein, angiotensin converting enzyme, L-selectin). In each case rHuAD10 was unable to affect the proposed proteolytic clip with the single exception of TNFR-75 where a trace of activity was detectable.

rHuAD10 Will Cleave Pro-TNF to a 17-kDa Species—The purification of HuAD10 from THP-1 cells was based on pooling active fractions as determined by the HPLC peptide assay. To verify that HuAD10 is capable of processing full-length pro-TNF, *in vitro* translated ^{35}S-labeled pro-TNF was treated with a partially purified HuAD10 (S-Sepharose pool) or rHuAD10. After immunoprecipitation, the reaction mixtures were subjected to SDS-PAGE followed by autoradiography. Fig. 5 shows that both protein preparations cleaved a portion of the 26-kDa pro-TNF to a 17-kDa species corresponding to the molecular weight of soluble mature TNF. It is not known if the processing efficiency of HuAD10 is enhanced when colocalized with pro-TNF on cell membranes.

DISCUSSION

Characterization of rHuAD10 suggests that it has many of the physical and biochemical properties required of a TNF-processing enzyme. Our data indicate that HuAD10 specifically recognizes pro-TNF and is not a general protease involved in the shedding of other cell surface proteins. rHuAD10 processed

TABLE IV
Specificity of rHuAD10 for peptides spanning the cleavage site of shed proteins

DNP-peptides spanning the proposed cleavage sites of a variety of membrane-bound proteins were assayed in the HPLC assay in place of the DNP-pro-TNF cleavage peptide as described under "Materials and Methods." rHuAD10 was used as the enzyme source. Samples were incubated for 17 h at 37 °C and quenched with 2.5 mM EDTA before analysis. * represents a proposed proteolytic cleavage site.

Protein	Sequence	% cleavage by rHuAD10
Pro-TNF	LAQA*VRSS	100
IL-6 receptor (17, 18)	LPVQ*DSSS	0
TNFR-55 (17)	QIEN*VKGT	0
TNFR-75 (19)	APGA*VHLP	5
Pro-TGF-α (18)	DLLA*VVAA	0
c-*kit* ligand-1 (20)	PVAA*SSLR	0
β-Amyloid precursor protein (18)	HHQK*LVFF	0
Angiotensin-converting enzyme (21)	AGQR*LATA	0
L-Selectin (17)	KLDK*SFSM	0

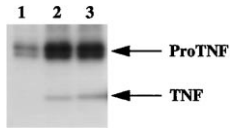

FIG. 5. **In vitro cleavage of human pro-TNF by rHuAD10.** The *in vitro* translated pro-TNF (*lane 1*) was incubated for 10 h at 37 °C with 1 mg of protein from an S-Sepharose pool (*lane 2*), 1 μg of rHUAD10 (*lane 3*). Human TNF (2 μg) was added to an aliquot of the *in vitro* translated pro-TNF and immunoprecipitated as control for its migration on a 16% SDS-PAGE gel. The gel was visualized using autoradiography as described under "Materials and Methods."

full-length pro-TNF to a 17-kDa form and cleaved the pro-TNF peptide sequence at a site that corresponds to the authentic NH_2 terminus (Val^{77}) of soluble TNF. We also show that substitutions surrounding the cleavage site adversely affect the ability of rHuAD10 to cleave the pro-TNF peptide. Furthermore, rHuAD10 failed to recognize and process peptides spanning the cleavage sites found in other membrane-associated proteins known to be shed proteolytically. These data are consistent with the expectation that a TNF-processing enzyme would have a preferred specificity for its substrate, pro-TNF.

Our inhibitor studies demonstrate that HuAD10 is a functional metalloprotease since it is effectively inhibited by EDTA and 1,10-phenanthroline. Interestingly, two metalloprotease inhibitors, phosphoramidon and TIMP-1, had no effect on TNF processing activity. These results agree with previous experiments that showed an essentially identical inhibition profile for a partially purified TNF-processing enzyme (6).

The protease responsible for TNF processing is likely to be membrane-bound and present on cells that secrete high levels of TNF. Accordingly, THP-1 cells were chosen for purification of the protease. HuAD10 appears to be an integral membrane protein since it fractionated with cell membranes rather than the cytosol. Additionally, it has a classical transmembrane domain. Attempts to verify its presence on the cell surface using fluorescence-activated cell sorting with AMG1961 have not been successful since this antibody does not recognize native HuAD10. We are generating monoclonal antibodies against rHuAD10 to address this question in the future. Northern analysis indicates that HuAD10 mRNA is found in tissues that have detectable TNF mRNA (*e.g.* spleen, lymph node, peripheral blood leukocytes). Thus, the expression and presumed localization of HuAD10 in cells are consistent with a potential role for this protein in TNF processing. It is not known if HuAD10 has additional functions in tissues that do not express TNF.

Recently, Lunn *et al.* (9) have shown that a protein preparation enriched in BoAD10 cleaves pro-TNF or a peptide spanning the pro-TNF cleavage site. We have purified rHuAD10 to homogeneity and have shown that this protein possesses high intrinsic activity for cleaving similar substrates. Lunn *et al.* (9) also reported that 293-EBNA cells processed pro-TNF only when cotransfected with BoAD10 cDNA. We observed that 293-EBNA cells produced substantial amounts of soluble TNF when transfected with pro-TNF. Cotransfection with HuAD10 did not significantly increase soluble TNF above the already high background. We did observe small increases in soluble TNF when insect cells were cotransfected with HuAD10 and pro-TNF, compared with insect cells singly transfected with pro-TNF (data not shown).

Interestingly, other ADAM family members have been recently shown to process pro-TNF (7, 8, 22). Of this group, TACE is the only member that is membrane-bound and found in mammals. Significantly, mutant T-cells lacking TACE were found to be greatly impaired in secreting TNF when stimulated by anti-CD3e (7). However, it has not been shown if TACE is also an important regulator of soluble TNF production in human T-cells or monocytes that are activated with physiological stimuli. Also yet to be determined is what role, if any, TACE may play in the release of other membrane-bound proteins such as L-selectin, interleukin-6 receptor, or the TNF receptors.

TACE and HuAD10 have significant sequence and structural homology and may represent two members of a family of TNF-processing enzymes. We have screened cDNA libraries for novel ADAM family members based on conserved sequences from known members including HuAD10 and have isolated unique ADAM family cDNAs including TACE (data not shown). Recombinant proteins expressed from these cDNAs are now being compared with respect to their relative abilities to process pro-TNF.

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REFERENCES

1. Beutler, B. (ed) (1992) *Tumor Necrosis Factors*, Raven Press, New York
2. Elliott, M. J., Maini, R. N., Feldmann, M., Kalden, J. R., Antoni, C., Smolen, J. S., Leeb, B., Breedveld, F. C., MacFarlane, J. D., Bijl, H., and Woody, J. N. (1994) *Lancet* **344,** 1105–1110
3. Van Dullemen, H. M., Van Deventer, S. J. H., Hommes, D. W., Bijl, H. A., Jansen, J., Tytgat, G. N. J., and Woody, J. (1995) *Gastroenterology* **109,** 129–135
4. Pennica, D., Nedwin, G. E., Hayflick, J. S., Seeburg, P. H., Derynck, R., Palladino, M. A., Kohr, W. J., Aggarwal, B. B., and Goeddel, D. V. (1984) *Nature* **312,** 724–729
5. Robache-Gallea, S., Morand, V., Bruneau, J. M., Schoot, B., Tagat, E., Realo, E., Chouaib, S., and Roman-Roman, S. (1995) *J. Biol. Chem.* **270,** 23688–23692
6. Mohler, K. M., Sleath, P. R., Fitzner, J. N., Cerretti, D. P., Anderson, M., Kerwar, S. S., Torrance, D. S., Otten-Evans, C., Greenstreet, T., Weerawarna, K., Kronheim, S. R., Petersen, M., Gerhart, M., Kozlosky, C. J., March, C. J., and Black, R. A. (1994) *Nature* **370,** 218–220
7. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Cerretti, D. P. (1997) *Nature* **385,** 729–733
8. Moss, M. L., Jin, S., Milla, M. E., Burkhart, W., Carter, H. L., Chen, W., Clay, W. C., Didsbury, J. R., Hassler, D., Hoffman, C. R., Kost, T. A., Lambert, M. H., Leesnitzer, M. A., McCauley, P., McGeehan, G., Mitchell, J., Moyer, M., Pahel, G., Rocque, W., Overton, L. K., Schoenen, F., Seaton, T., Su, J., Warner, J., Willard, D., and Becherer, J. D. (1997) *Nature* **370,** 733–736
9. Lunn, C. A., Fan, X., Dalie, B., Miller, K., Zavodny, P. J., Narula, S. K., and Lundell, D. (1997) *FEBS Lett.* **400,** 333–335
10. Merrifield, R. B. (1963) *J. Am. Chem. Soc.* **85,** 2149–2154
11. Maeda, T., Balakrishnan, K., and Mehdi, S. G. (1983) *Biochim. Biophys. Acta* **731,** 115–120
12. Howard, L., Lu, X., Mitchell, S., Griffiths, S., and Glynn, P. (1996) *Biochem. J.* **317,** 45–50
13. Yoshida, S., Setoguchi, M., Higuchi, Y., Akizuki, S., and Yamamoto, S. (1990) *Int. Immunol.* **2,** 585–591
14. Wolfberg, T. G., Primakoff, P., Myles, D. G., and White, J. M. (1995) *J. Cell Biol.* **131,** 275–278
15. Watanabe, T., Nakagawa, T., Ikemizu, J., Nagahama, M., Murakami, K., and Nakayama, K. (1992) *J. Biol. Chem.* **267,** 8270–8274
16. Woessner, J. F. (1991) *FASEB J.* **5,** 2145–2154
17. Mullberg, J., Durie, F. H., Otten-Evans, C., Alderson, M. R., Rose-John, S., Cosman, D., Black, R. A., and Mohler, K. M. (1995) *J. Immunol.* **155,** 5198–5205
18. Arribas, J., Coodly, L., Vollmer, P., Kishimoto, T. K., Rose-John, S., and Massague, J. (1996) *J. Biol. Chem.* **271,** 11376–11382
19. Crowe, P. D., Walter, B. N., Mohler, K. M., Otten-Evans, C., Black, R. A., and Ware, C. F. (1995) *J. Exp. Med.* **181,** 1205–1210
20. Pandiella, A., Bosenberg, M. W., Huang, E. J., Besmer, P., and Massague, J. (1992) *J. Biol. Chem.* **267,** 24028–24033
21. Oppong, S. Y., and Hooper, N. M. (1993) *Biochem. J.* **292,** 597–603
22. Moura-da-Silva, A. M., Laing, G. D., Paine, M. J. I., Dennison, J. M. T. J., Politi, V., Crampton, J. M., and Theakston, R. D. G. (1996) *Eur. J. Immunol.* **26,** 2000–2005

ENZYMOLOGY:

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