Purification of ADAM 10 from bovine spleen as a TNFα convertase

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Abstract We have purified a protease with characteristics of TNFα convertase from bovine spleen membranes. Peptide sequencing of the purified protein identified it as ADAM 10 (Genbank accession no. Z21961). This metalloprotease cleaves a recombinant proTNFα substrate to mature TNFα, and can cleave a synthetic peptide substrate to yield the mature TNFα amino terminus in vitro. The enzyme is sensitive to a hydroxamate inhibitor of MMPs, but insensitive to phosphoramidon. In addition, cloned ADAM 10 mediates proTNFα processing in a processing-incompetent cell line.

Key words: Tissue necrosis factor α; TNFα convertase; ADAM 10; Metalloprotease

1. Introduction

The pro-inflammatory cytokine TNFα [1–3] is synthesized as a 233 amino acid precursor. Conversion of this membrane-bound precursor to a secreted 17 kDa mature protein [4,5] is carried out by an unidentified protease, termed the TNFα convertase. Serine proteases are capable of releasing proTNFα from the membrane-bound form, although the resulting amino-terminus is not identical with that observed in vivo [6]. Many investigators believe TNFα convertase is a membrane-bound metalloprotease [7–9]. This activity is inhibited by EDTA and many prototypic MMP inhibitors but not by phosphoramidon, thiorphan, captopril and TIMP-1. This activity cleaves the proTNFα at the correct position, yielding an amino-terminal valine. We have purified one activity from bovine spleen with enzymatic characteristics consistent with TNFα convertase. In addition, we show that this protein, identified as ADAM 10, can reconstitute TNFα processing in a processing-incompetent cell line.

2. Materials and methods

2.1. In vitro assay for TNFα convertase activity

A protein-based assay for TNFα convertase activity was based on described procedures [7], using a ^{125}I-labelled peptide-tagged recombinant proTNFα polypeptide substrate (Flag-proTNFα). A membrane protein sample was mixed with 2 ng of ^{125}I-polypeptide substrate (approx. 50 000 cpm) in the presence of inhibitors (0.2 mM PMSF, 2 μM pepstatin, 200 μM leupeptin, 1 mM *N*-methoxysuccinyl-AAPV-chloromethyl ketone [AAPVCMK]). Following incubation at 37°C overnight, samples were fractionated by SDS-PAGE on a 16% gel. Polypeptides were visualized by autoradiography.

A peptide-based assay for TNFα convertase activity was carried out similarly to the procedure described in [7] using an amino-terminal dinitrophenylated peptide substrate (DNP-SPLAQAVRSSSR-NH_2). A membrane protein sample was mixed in 50 μl with 1 μg peptide substrate in the presence of inhibitors (see above). Following incubation at 37°C, the protein was precipitated by cold 5% trichloroacetic acid, 20% acetonitrile and the soluble peptide fraction was applied to a YMC 120 Å C-18 ODS-AQ column (4.6×100 mm). The column was eluted isocratically at 1 ml/min using 40% acetonitrile plus 0.06% trifluoroacetic acid. The elution of DNP-peptide was monitored at 360 nm using a Waters 625 LC system.

2.2. Purification from bovine spleen

Bovine spleens were purchased from Pel-Freez. The washed tissue was homogenized in a Waring blender, resuspended in lysis buffer (20 mM Tricine, pH 7.8, 8% sucrose) containing 0.1% PMSF, then homogenized using a Brinkmann Polytron tissue homogenizer. The cell debris was removed by two low-speed centrifugations and the membranes isolated by ultracentrifugation. The isolated membranes were washed in 10 mM HEPES, pH 7.5.

The membrane fraction was resuspended to 8 mg/ml in buffer A (20 mM Tris, pH 7.5, 1 mM $MgSO_4$, 10 mM NaCl, 10 μM $ZnSO_4$) plus 2% Brij 35, and incubated at 4°C for 30 min. The membranes were collected by ultracentrifugation, then resuspended to 4 mg/ml in buffer A plus 2% Thesit (Boehringer Mannheim). The insoluble protein was removed by ultracentrifugation.

The detergent-solubilized fraction was applied to a chelating Sepharose (Pharmacia) column charged with nickel sulfate in buffer A with 0.3 M NaCl/0.1% octyl glucoside (all subsequent steps included 0.1% octyl glucoside). The 50 mM imidazole eluate was concentrated, then applied to a Sephacryl 300 (Pharmacia) sieving column equilibrated in buffer A. A retained fraction (approx. 60 kDa) was pooled, then applied to a wheat germ agglutinin column in 0.3 M NaCl and eluted with buffer A containing 0.5 M *N*-acetylglucosamine and 0.5 M NaCl. The eluate was dialyzed vs. 1 mM $NaPO_4$ pH 7, then applied to an hydroxyapatite (BioRad) column. An eluted fraction (40–50 mM $NaPO_4$) was then dialyzed vs. buffer A and passed successively over HS-20 and HQ-20 (Perseptive Biosystems) ion exchange columns. The unbound fraction from both columns contained the TNFα convertase activity.

The final protein fraction (approx. 100 μg) was fractionated by SDS-PAGE on an 8% gel (Novex), then electrophoretically transferred to an Immobilon filter membrane in 10 mM CAPS buffer, pH 10, 10% methanol. After staining the membrane with 0.1% Amido Black in 40% methanol, 10% acetic acid, the major protein band was excised and used for microsequencing.

2.3. Cloning and in vivo activity of ADAM 10

Bovine ADAM 10 (Genbank accession no. Z21961) was cloned from 5 μg poly(A)$^+$ RNA (Clontech) using a GibcoBRL SuperScript Preamplification System and an oligo(dT) primer. The resulting cDNA was used directly for PCR using the listed primers:

CTTCCGGGTACCCGGAAGATGGTGTTGCTGAGAGTG
(5′ forward)
CGTTAAAAGCTTTTAACGTCTCATGTGTCCCATCTG
(3′ reverse).

PCR conditions were: 94°C, 30 s/60°C, 30 s/72°C, 2 min for 30 cycles. The PCR product was digested with *Kpn*I and *Hin*dIII restriction enzymes, and was ligated into pCEP4 vector (Invitrogen)

Human 293EBNA cells (Invitrogen) were seeded into 96-well plates at 10^4 cells/100 μl per well in DMEM containing 10% fetal bovine serum, and incubated at 37°C, 5% CO_2 for 20 h. Cells were transfected with 50 ng pSRαSPORT/proTNFα vector with or without 100 ng pCEP4/ADAM 10 using Lipofectin (Gibco-BRL). After 20 h, human TNFα was detected by ELISA using a human TNFα capture antibody (Pharmingen no. 18631D) and a biotinylated anti-TNFα (Pharmingen no. 18642D). The bound biotinylated antibody was detected using streptavidin-HRP (BioSource) in concert with ABTS sub-

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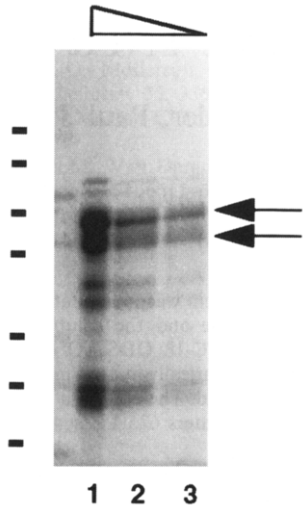

Fig. 1. Characterizing the TNFα convertase pool by SDS-polyacrylamide gel electrophoresis. TNFα convertase activity was purified from bovine spleen as described. Serial dilutions of final preparation were fractionated on an 8–16% polyacrylamide gel in SDS. Polypeptides were visualized by Coomassie Blue staining. Black arrows denote major protein species.

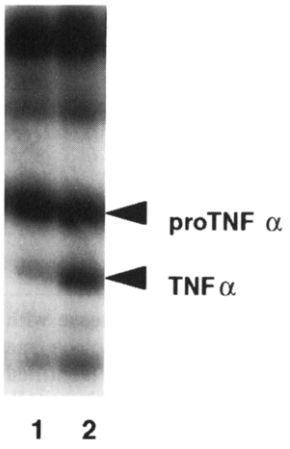

Fig. 3. ADAM 10 cleaves a proTNFα substrate. Aliquots of purified ADAM 10 were mixed with ^{125}I-Flag-proTNFα in a reaction containing 0.2 mM PMSF, 2 μM pepstatin, 0.2 mM leupeptin and 1 mM AAPVCMK. Following incubation at 37°C overnight, samples were fractionated by SDS-PAGE on a 16% gel and radioactivity detected by autoradiography. Lanes: 1, no enzyme control; 2, ADAM 10. The upper arrow indicates the uncut proTNFα, about 21 kDa; the lower arrow denotes the 17 kDa processed TNFα band.

strate (KP Labs). Visible color development was quantitated at 405 nm vs. standards.

3. Results

After screening several bovine tissues for the ability to cleave ^{125}I-Flag-proTNFα to a 17 kDa protein (membrane fractions from kidney, spleen and lung contained detectable TNFα convertase activity, while heart and liver were inactive), we selected bovine spleen for purification of TNFα convertase. 9 g of bovine spleen membranes were fractionated as listed in Section 2. The final pool (Fig. 1) contained two major protein bands centered around 60 kDa. Fig. 2 shows the protein sequences of these bands are consistent with a known protein ADAM 10 [10], a metalloprotease with an ill-defined biological activity [11,12] (the lower band is a proteolyzed product starting at residue 285). ADAM 10 could cleave ^{125}I-proTNFα to a 17 kDa protein (Fig. 3), and could cleave a peptide substrate between Ala and Val, consistent with the cleavage site in native TNFα. The peptide-cleaving activity was inhibited by EDTA (1 mM) and SCH43534, a peptide hydroxamate inhibitor of collagenase similar to BB2284 [9], but was not inhibited by phosphoramidon (300 μM) and thiorphan (100 μM).

To confirm this result, we sought to determine whether cloned bovine ADAM 10 could process proTNFα in intact cells. Screening numerous cell lines (including CHO, HeLa, IEC-6, C_2C_{12}, clone-9) showed that a human embryonic kidney cell line 293EBNA (as well as the parental cell line 293) was unable to process recombinant proTNFα, although recombinant growth hormone was effectively processed. We transfected pSRαSPORT/proTNFα with or without pCEP4/ADAM 10, then measured TNFα production 20 h later. Fig. 4 shows that 293EBNA cannot process recombinant proTNFα (sample 1) unless co-transfected with pCEP4/ADAM 10 (samples 2–5).

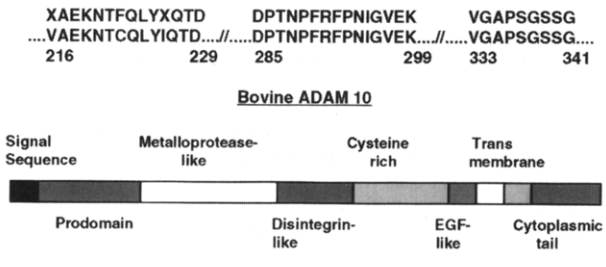

Fig. 2. Amino acid sequence analysis of the TNFα convertase polypeptide. The polypeptides (black arrows in Fig. 1) were transferred to an Immobilon filter membrane, excised and sequenced. Figure shows the alignment of the obtained sequence information with that of the known metalloproteinase ADAM 10.

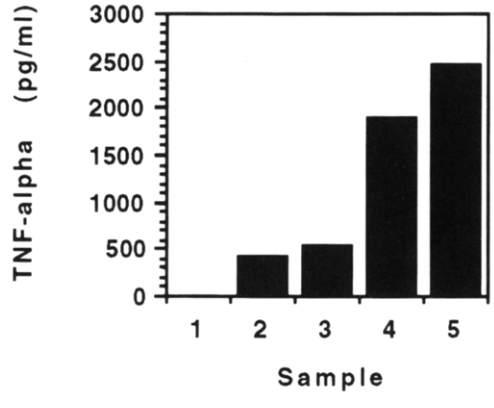

Fig. 4. ADAM 10 mediates the processing and release of proTNFα in vivo. The graph shows the amount of soluble TNFα produced with transfection of pSRαSPORT/proTNFα alone (sample 1) and after co-transfection with four independent pCEP4/ADAM 10 vector isolates (samples 2–5).

4. Discussion

We have purified an enzyme capable of proteolytically processing a recombinant proTNFα to native TNFα from bovine spleen. A homologous protein was recently purified from human Monomac 6 cells (data not shown). The protein was identified as ADAM 10 [10], a protein first purified from bovine brain based on its ability to cleave MBP [11,12]. The protein has since been found in many tissues, and is homologous to the *Drosophila kuz* protein, suggesting a role in neurogenesis [13]. We show that this protein can cleave proTNFα in vitro and in cultured human cells. This result, coupled with the numerous other proteolytic activities present in bovine tissue that conform to the inhibitor profile and cleavage site specificity established for TNFα convertase (data not shown) suggests that in vitro activity alone is not sufficient to establish a protease as TNFα convertase.

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