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Journal

Nature Cell Biology, 8(8)

ISSN

1465-7392

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Publication Date

2006-08-01

DOI

10.1038/ncb1450

Peer reviewed

A γ-secretase-like intramembrane cleavage of TNFα by the GxGD aspartyl protease SPPL2b

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γ-secretase and signal peptide peptidase (SPP) are unusual GxGD aspartyl proteases, which mediate intramembrane proteolysis. In addition to SPP, a family of SPP-like proteins (SPPLs) of unknown function has been identified. We demonstrate that SPPL2b utilizes multiple intramembrane cleavages to liberate the intracellular domain of tumor necrosis factor α (TNFα) into the cytosol and the carboxy-terminal counterpart into the extracellular space. These findings suggest common principles for regulated intramembrane proteolysis by GxGD aspartyl proteases.

Extensive studies on sterol-regulated proteolysis, rhomboids, γ-secretase and SPP[1] led to the concept of regulated intramembrane proteolysis. In addition to SPP, several SPP homologues (SPPLs) of unknown function were identified by database searches[2,3]. We previously mutated the aspartate residue within the GxGD motif of SPPL2 and SPPL3 in zebrafish and found that overexpression of these dominant–negative constructs phenocopies loss of function[4]. This strongly indicates that all SPPLs are members of the GxGD protease family. Although SPP is known to be involved in the clearance of signal peptides, as well as in the processing of hepatitis C viral core protein[3], substrates for SPPLs have not been found. To identify putative substrates for SPPLs, we focused on SPPL2a and SPPL2b, as they are of specific interest due to their unexpected localization to late endosomes and at the plasma membrane[4] (and see note added in proof), which suggests a function independent of signal peptide cleavage. Knowledge of the subcellular localization[4] and previous work on the substrate requirements for SPP[5] led to the identification of TNFα as a candidate substrate (see note added in proof). To verify selective TNFα processing by SPPL2a and SPLL2b, cell lysates from human embryonic kidney 293 cells co-expressing TNFα and SPP, or members of the SPPL family, were investigated for the presence of the TNFα intracellular domain (ICD; for a schematic representation of TNFα processing see Fig. 1f). TNFα ICD generation was only observed when SPPL2a or SPPL2b were coexpressed, whereas SPP and SPPL3 did not support TNFα proteolysis (Fig. 1a). As SPPL2a and SPPL2b seem to be redundant (Fig. 1a), and the zebrafish genome encodes only one *SPPL2* gene[4], we focused exclusively on SPPL2b. Liberation of the TNFα ICD (sometimes appearing as a closely spaced doublet on western blots; Fig. 1b) was dependent on the aspartate residue (D421) within the GxGD of SPPL2b, as the TNFα amino-terminal fragment (NTF) accumulates on overexpression of SPPL2b^{D421A}, a proteolytically inactive mutant of SPPL2b (Fig. 1b). Moreover, as it is the case for γ-secretase substrates, the TNFα NTF coimmunoprecipitates with SPPL2b when it is inactivated either by mutagenesis (Fig. 1c) of the critical aspartate within the GxGD motif, or by use of selective inhibitors (Fig. 1c). Surprisingly, and in contrast with γ-secretase[6], significant amounts of full-length TNFα also specifically copurified with SPPL2b (Fig. 1c). As full-length TNFα also copurified with SPPL2b, it may be cleaved, to some extent, directly by SPPL2b. This is consistent with the observation that SPPL2b acts independently of a docking protein (for example, Nicastrin, which is responsible for the size selection of γ-secretase substrates[7]).

TNFα ICD generation predicts that its C-terminal counterpart (the TNFα C-domain) is secreted into the media, analogous with processing of γ-secretase substrates[1]. To facilitate isolation of the TNFα C-domain, we generated TNFα$^{\Delta E}$, where the ectodomain is deleted. Similar constructs greatly facilitated the investigation of Notch, APP and CD44 endoproteolysis by γ-secretase[1]. As shown in Fig. 1d cells expressing proteolytically active SPPL2b secreted the TNFα C-domain, whereas large amounts of uncleaved TNFα$^{\Delta E}$ were observed on expression of SPPL2b^{D421A} (Fig. 1d).

After the demonstration of selective TNFα ICD and TNFα C-domain generation, we investigated whether these TNFα ICDs can be generated endogenously. As the TNFα ICD is very unstable (data not shown), making *in vivo* detection difficult, *in vitro* assays similar to those introduced to isolate the highly unstable β-amyloid precursor protein (APP) ICD (AICD)[8], were used. After demonstration of specific *in vitro* generation of TNFα ICD when SPPL2b was overexpressed (Fig. 1e), the same type of experiments were repeated with membrane extracts obtained from cells not transfected with SPPL2b. This revealed that, under endogenous

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Received 23 March 2006; accepted 5 May 2006; published online 9 July 2006; DOI: 10.1038/ncb1450

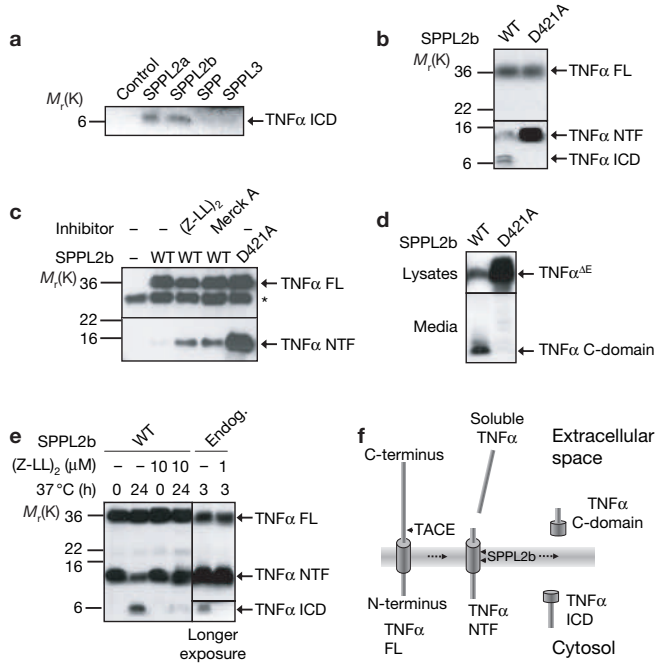

Figure 1 Endoproteolytic processing of TNFα. (**a**) The TNFα ICD is selectively generated by SPPL2a and SPPL2b. (**b**) TNFα ICD production is abolished when an inactive SPPL2b mutant is coexpressed. An uncropped image of the blot is shown in the Supplementary Information, Fig. S1a. (**c**) TNFα NTF and TNFα FL (full length) coisolated with inactivated SPPL2b. To inactivate SPPL2b either $(Z\text{-}LL)_2$-ketone (a known SPP inhibitor) or Merck A (a known γ-secretase inhibitor) was used[9]. The asterisk indicates IgG light chain. An uncropped image of the blot including input control is shown in the Supplementary Information, Fig. S2. (**d**) Generation of the TNFα C-domain by cells expressing wild-type (WT) SPPL2b, but not by cells expressing SPPL2b^{D421A}. Uncropped images of the blots are shown in the Supplementary Information, Fig. S1b. (**e**) *In vitro* generation of TNFα ICD. Endogenous TNFα ICD generation is shown in the longer exposure. (**f**) Schematic representation of the processing of TNFα. TNFα is processed by a sheddase, such as TNFα converting enzyme (TACE). The remaining NTF is futher processed by intramembrane proteolysis which liberates TNFα ICD and TNFα C-domain.

conditions, TNFα ICD was generated and its production was inhibited by the SPP–SPPL specific inhibitor $(Z\text{-}LL)_2$-ketone, which does not block γ-secretase[9] (Fig. 1e). Taken together these data strongly suggests that endogenous SPPL2a and SPPL2b generate TNFα ICD.

The cleavage sites of the ICD and the TNFα C-domain were then determined by mass spectrometry and radiosequencing. Mass spectrometry of purified ICDs revealed major intramembrane cleavage sites after amino acids 34 and 39 (Fig. 2a, c). Additional peptides ranging from 25–29 amino acids (Fig. 2a, c) suggested degradation by a cytoplasmic carboxypeptidase (Fig. 2c, blue arrows). This is supported by the observation that TNFα ICD can be detected as a doublet in some cases (Fig. 1b). Furthermore, over time, the higher molecular mass peptide is converted to the smaller peptide (data not shown). Radiosequencing of the TNFα C-domain demonstrated a major cleavage after Cys 49 (as was evident from large peaks of ^{3}H-Phe at cycle 4, ^{3}H-Val at cycle 6, ^{3}H-Leu at cycle 1 and 2 and ^{3}H-Ile at cycle 7), in addition to a less abundant cleavage after Leu 51 (evident from a ^{3}H-Phe peak at cycle 2,a ^{3}H-Val peak at cycle 4 and a ^{3}H-Ile peak at cycle 5; Fig. 2b, c). Both cleavage sites were also confirmed by mass spectrometry (data not shown). Similar data were obtained from sequencing of a ^{3}H-Phe-labelled TNFα C-domain generated from cells expressing endogenous SPPL2 (see Supplementary

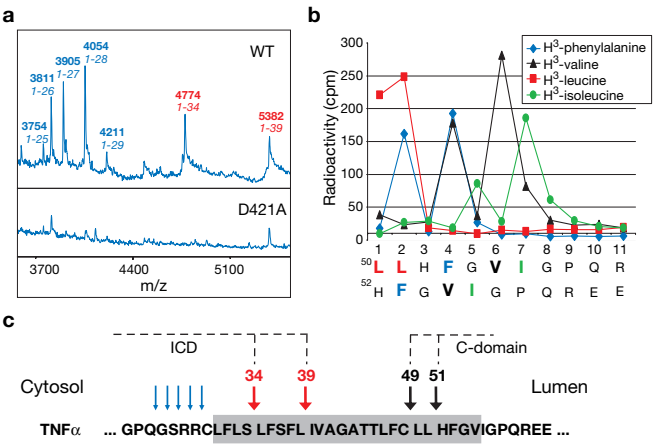

Figure 2 Intramembrane cleavage of TNFα. (**a**) MALDI–TOF–MS analysis of TNFα ICDs. Calculation of the respective masses is shown in the Supplementary Information, Fig. S3a. (**b**) Cells cotransfected with TNFα and wild-type SPPL2b were labelled with either ^{3}H-phenylalanine, ^{3}H-valine, ^{3}H-leucine or ^{3}H-isoleucine. Radiosequencing of the isolated TNFα C-domain revealed a major cleavage after Cys 49 and a lesser cleavage after Leu 51. (**c**) Schematic representation of the multiple cleavages of TNFα by SPPL2b.

Information. Fig. S3b). ICDs with C-termini of approximately 49 or 51 amino acids were not obtained. Likewise, no evidence for longer TNFα C-domains was obtained.

Our data suggest a similarity in the cleavage pattern of γ-secretase and SPPL2b, although both proteases have fundamental differences with respect to complex formation (γ-secretase requires four different subunits for its activity[10], whereas SPPL2 activity is massively increased when a single cDNA is overexpressed) and substrate orientation, and have limited sequence homology. SPPL2b, similarly to γ-secretase, performs multiple intramembrane cleavages separated by a number of amino acids (Fig. 2c). It is tempting to speculate that this may include an ε- (after amino acid 34)[11] and ζ-like cut (after amino acid 39)[12], in addition to a γ-like cleavage (after amino acids 49 and 51) at the luminal side of the membrane. Interestingly, the latter cleavage occurs as two cuts very similar to the γ-secretase cleavage, which liberates the 40 and 42 amino acid amyloid β-peptide. Moreover, similarly to γ-secretase, regulated intramembrane proteolysis of TNFα by SPPL2 generates a cytoplasmic cleavage product that is required for cellular signalling (see note added in proof). Strikingly, the active sites for SPPL2b are reversed within the membrane to accommodate type-2-oriented substrates. As for γ-secretase, the question arises of how one protease activity can cleave its substrate at several sites. Similarly to presenilin[13], the catalytically active component of the γ-secretase complex[1], SPP and all SPPLs seem to occur as homodimers[4,14]. Moreover, at least for SPP, it has been shown that dimerization facilitates the binding of an active-site-directed photoaffinity-labelled γ-secretase inhibitor, suggesting that dimerization is required to form the fully active catalytic site of SPP[14]. In summary, we demonstrate a common multiple intramembrane cleavage by GxGD-type aspartic proteases, which is required for the liberation of a signalling factor into the cytoplasm and the secretion of amyloid β-peptide like domains into the extracellular space. □

Note added in proof: an accompanying manuscript by Friedmann, E. et al. *(*Nature Cell Biol. ***8,*** *doi: 10.1038/ncb1440; 2006) is also published in this issue.*

Note: Supplementary Information (including Methods) is available on the Nature Cell Biology website.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 596), the Gottfried Wilhelm Leibniz Award to C.H., the APOPIS Program (Abnormal proteins in the pathogenesis of neurodegenerative disorders - An integrated project funded by the EU under the SIXTH FRAMEWORK PROGRAMME; to C.H. and B.M.), National Genome Research Network 2 (NGFN-2) to C.H. and National Institute of Health (NIH, Bethesda, MD) grants NS38328, NS4414, and AG18921 to D.B.T. We thank S. Lichtenthaler and P.-H. Kuhn for providing TNFα cDNA and for helpful discussion.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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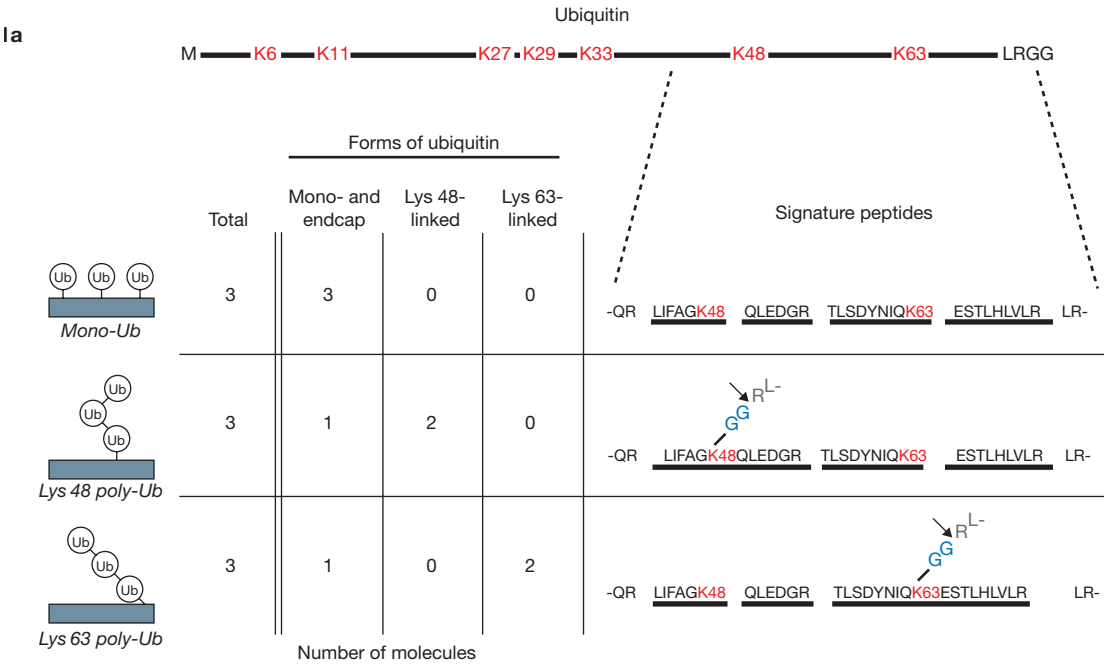
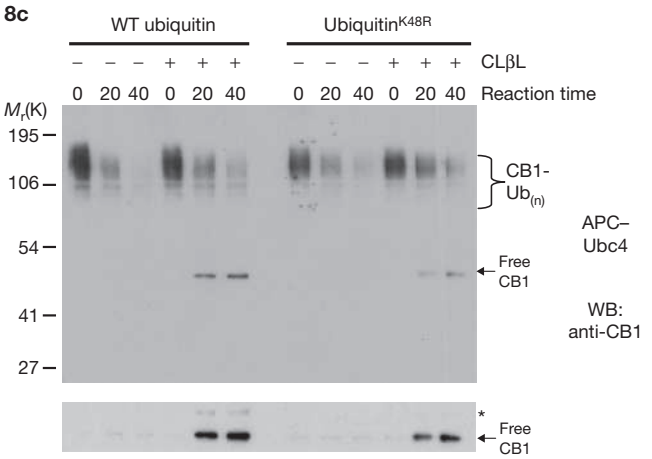
Erratum

In the article by Kirkpatrick *et al.* (*Nature Cell Biol.* **8,** 700–710; 2006), the labelling in Fig. 1a and the size of the lower panel in Fig. 8c were incorrect. These figures have been corrected online and are shown below.





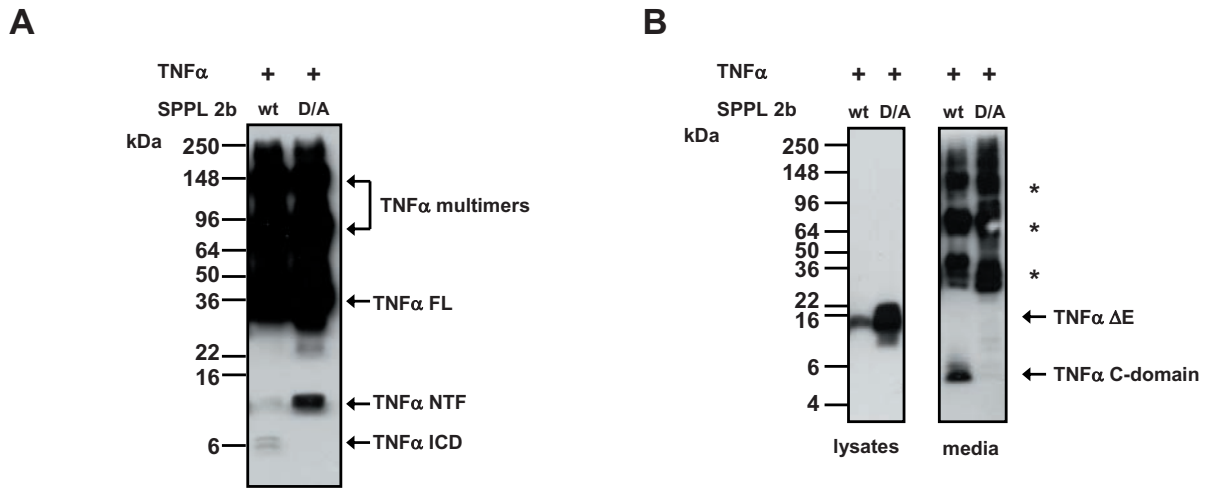


Figure S1 Endoproteolytic processing of TNFα **(A)** The TNF(ICD is selectively generated by SPPL2a/b. **(B)** TNF(ICD production is abolished upon co-expression of an inactive SPPL2b mutant. Full length blot is presented as Suppl. Fig. S1 A. **(C)** TNF(NTF (lower panel) and TNFαFL (upper panel) co-isolate with inactivated SPPL2b. Asterisk: IgG light chain. Full length blot including input control is presented as Suppl. Fig. S2. **(D)** Generation of the TNF(C-domain by cells expressing wt SPPL2b but not by cells expressing SPPL2b D421A. Full length blots are presented as Suppl. Fig. S1 B. **(E)** In vitro generation of TNF(ICD. Note endogenous TNFα ICD generation (longer exposure in the right bottom panel). **(F)** Model illustrating the processing of TNFα.

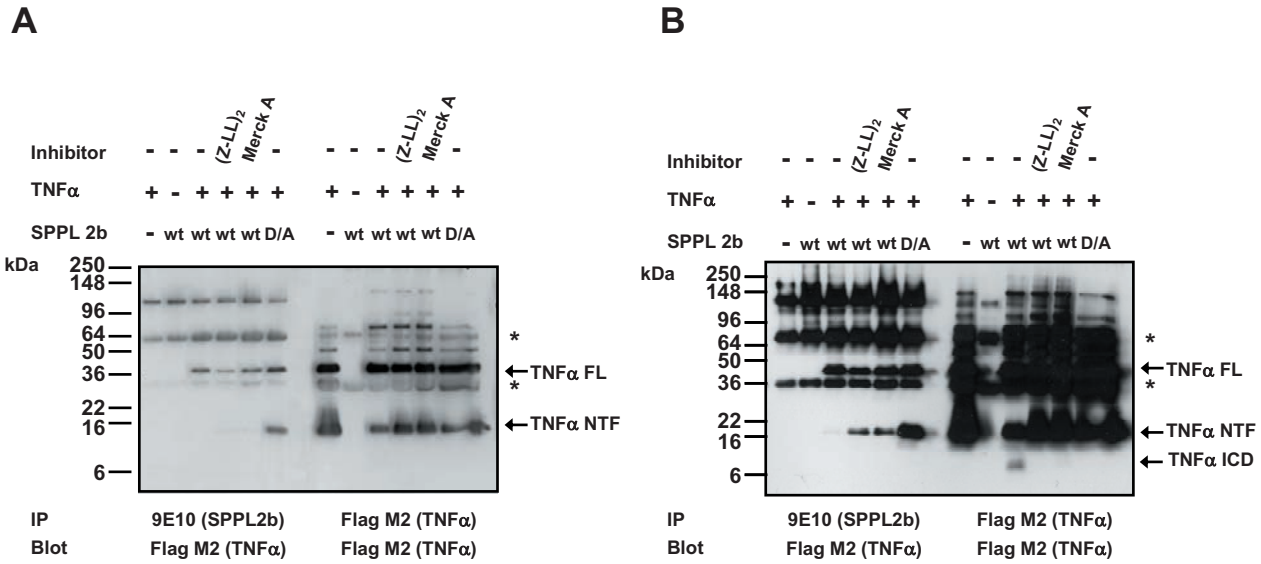


Figure S2 Intramembrane cleavage of TNFα **(A)** MALDI-TOF-MS analysis of TNF(ICDs. For calculation of the respective masses see Suppl.

A

TNFa species	*Sequence*	*mass calc. [Da]*	*mass calc. incl. acetyl group [Da]*
1-39	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQGSRRCLFLSLFSFL	5340	5382
1-34	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQGSRRCLFLS	4732	4774
1-29	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQGSRR	4169	4211
1-28	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQGSR	4013	4055
1-27	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQGS	3857	3899
1-26	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQG	3770	3812
1-25	M**DYKDDDDK**STESMIRDVELAEEALPKKTGGPQ	3713	3755

B

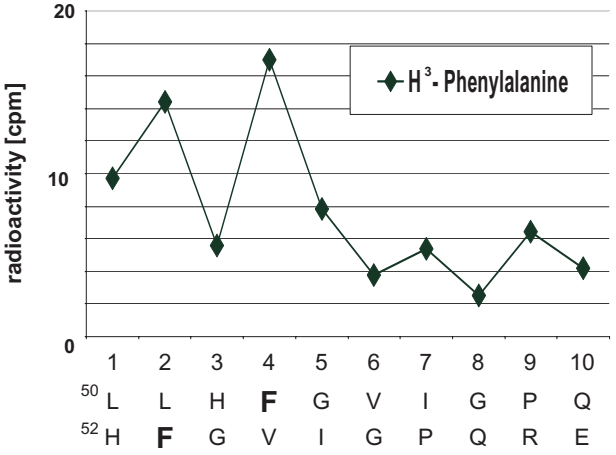


Figure S3 A. **(B)** Cells cotransfected with TNF(and SPPL2b wt were labelled with either ^{3}H-Phenylalanine (pentagon), ^{3}H-Valine (triange),^{3}H-Leucine (square) or ^{3}H-Isoleucine (star). Radiosequencing of the isolated TNF(C-domain reveals a major cleavage after Cys 49 and a lesser cleavage after Leu 51. **(C)** Model showing the multiple cleavages of TNF(by SPPL2b.

METHODS

Cell culture, cDNAs and transfection. HEK293 cells were cultured in DMEM with Glutamax (Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Invitrogen GmbH). The cell lines stably overexpressing SPP, SPPL2b and SPPL3 have been described previously[4]. The inactive SPPL2b^{D421A} mutant was generated by polymerase chain reaction mutagenesis and subcloned into the *Eco*RI and *Xho*I sites of pcDNA4–Myc–His (Invitrogen GmbH).

TNFα cDNA was purchased from LGC Promschem GmbH (Wesel, Germany; clone AAA61198) and after addition of a N-terminal Flag tag (DYKDDDDK) after the starting methionine and a C-terminal HA tag (YPYDVPDYA), was subcloned into the *Hind*III to Not I sites of peak 12. The TNFα$^{\Delta E}$ construct was generated by inserting a stop codon after amino acid 80. This construct also contains an N-terminal Flag tag and a C-terminal HA tag. TNFα$^{\Delta E}$ was subcloned into the *Hind*III and *Xho*I sites of pcDNA 3.1. Hygro + (Invitrogen Life Sciences). *TNFα* cDNA was cotransfected with the respective SPP or SPPLs using Lipofectamine 2000 (Invitrogen Life Sciences) according to the manufacturer's instructions. All cDNA constructs were sequenced for verification.

Antibodies, immunoprecipitation and immunoblotting. The monoclonal anti-Myc antibody 9E10 was obtained from the hybridoma bank (University of Iowa, Iowa City, IA) and the anti-HA-peroxidase coupled 3F10 antibody from Roche Diagnostics GmbH (Mannheim, Germany). The monoclonal anti-Flag M2 and the polyclonal HA 6908 antibody were obtained from Sigma (St Louis, MO). For coimmunoprecipitation assays, cells were lysed in assay buffer (25 mM HEPES–KOH at pH 7.6, 100 mM potassium acetate, 2 mM magnesium acetate, 1 mM DTT) containing 1% 3-[(3-cholamindopropyl)dimethylamino]-2-hydroxy-1-propanesulphonate (CHAPSO) on ice for 30 min. Lysates were clarified by centrifugation for 20 min at 16,000*g* and immunoprecipitated for 4 h at 4 °C.

TNFα *in vitro* assay. Membranes of HEK 293 cells stably expressing the indicated SPPL2b derivative and transiently expressing TNFα were prepared as previously described[10]. Membranes were subsequently resuspended in assay buffer and incubated for the indicated times at 37 °C. After incubation, lipids were extracted using chloroform:methanol (1:2) and proteins were subjected to SDS–PAGE and immunoblotting. Where indicated, $(Z\text{-}LL)_2$-ketone (Merck Biosciences Ltd, Nottingham, UK) a selective inhibitor of the SPP family[3] was added before incubation at 37 °C.

Mass spectrometry. TNFα *in vitro* assays were performed as described above. After incubation at 37 °C membranes were solubilized in STEN lysis buffer (50 mM Tris at pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 (NP-40), 1% Triton X-100) and TNFα ICD peptides were immunoprecipitated using anti-Flag M2 agarose affinity gel (Sigma). Immunoprecipitations were carefully washed 6 times with 1× PBS and twice with dH_2O. Proteins were subsequently eluted using formic acid. Samples were subjected to analysis by mass spectrometry using as matrix α-cyano-4-hydroxycinnamic acid (Sigma) as previously described[10]. Mass spectra were recorded on a Voyager-DE STR mass spectrometer (Applied Biosystems, Darmstadt, Germany) in the linear mode with external calibration.

Radiosequencing. HEK 293 cells stably expressing wild-type SPPL2b and transiently expressing TNFα$^{\Delta E}$ were metabolically labedled with ^{3}H-valine, ^{3}H-phenylalanine, ^{3}H-leucine or ^{3}H-isoleucine (Hartmann Analytic, Braunschweig, Germany) for 16 h. Supernatants were collected, TNFα C-domain peptides were isolated by immunoprecipitation with antibody HA6908 (Sigma), separated by SDS–PAGE and transferred to PVDF membrane. Radiolabelled proteins were detected by autoradiography, excised and subjected to automated Edman chemistry in an Applied Biosystems Model 475A sequencer. Amino-acid anilinothiazolinones were extracted with n-butyl chloride, transferred into 7 ml scintillation vials and assayed for radioactivity after the addition of 5 ml scintillation cocktail (Perking Elmer, Wellesley, MA).