The transferrin receptor-1 membrane stub undergoes intramembrane proteolysis by signal peptide peptidase-like 2b

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Keywords
ectodomain shedding; iron metabolism; regulated intramembrane proteolysis; signal peptide peptidase-like proteases; transferrin receptor-1

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The successive events of shedding and regulated intramembrane proteolysis are known to comprise a fundamental biological process of type I and II membrane proteins (e.g. amyloid precursor protein, Notch receptor and pro-tumor necrosis factor-α). Some of the resulting fragments were shown to be involved in important intra- and extracellular signalling events. Although shedding of the human transferrin receptor-1 (TfR1) has been known for > 30 years and soluble TfR1 is an accepted diagnostic marker, the fate of the remaining N-terminal fragment (NTF) remains unknown. In the present study, we demonstrate for the first time that TfR1-NTF is subject to regulated intramembrane proteolysis and, using MALDI-TOF-TOF-MS, we have identified the cleavage site as being located C-terminal from Gly-84. We showed that the resulting C-terminal peptide is extracellularly released after regulated intramembrane proteolysis and it was detected as a monomer with an internal disulfide bridge. We further identified signal peptide peptidase-like 2a and mainly signal peptide peptidase-like 2b as being responsible for the intramembrane proteolysis of TfR1-NTF.

Introduction

Transferrin receptor-1 (TfR1) is a homodimeric type II transmembrane protein that is expressed on every dividing cell [1]. Because of its important function in cellular iron uptake, TfR1 is essential for survival. It consists of a large ectodomain (residues 89–760), which is kept by a stalk at a distance of 2.9 nm from the plasma membrane [2], a small transmembrane region (residues 68–88) and a cytoplasmic domain (residues 1–67). The soluble form of the TfR1 (sTfR1) is generated by proteolytic cleavage (shedding) of TfR1 C-terminal of Arg100 within the juxtamembrane region of the ectodomain [3] by a disintegrin and metalloprotease [4]; the remaining N-terminal fragment (TfR1-NTF) thus consists of 100 amino acids and therefore includes the cytoplasmic domain, the transmembrane domain and a part of the extracellular stalk. The fate of TfR1-NTF was unknown so far. The sTfR1 is a diagnostic marker for erythropoietic activity, in particular to discriminate between iron deficiency anaemia and anaemia of inflammation [5];

Abbreviations
Cp, C-terminal peptide; DAPT, *N*-[*N*-(3,5-difluorophenylacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; FasL, Fas ligand; HAS, human serum albumin; HEK, human embryonal kidney; HRP, horseradish peroxidase; ICD, intracellular domain; I-CLiP, intramembrane cleaving protease; NTF, N-terminal fragment; RIP, regulated intramembrane proteolysis; SPPL, signal peptide peptidase-like; SPP, signal peptide peptidase; TfR1, transferrin receptor-1; TNF, tumour necrosis factor.

however, the biological function of sTfR1 is unclear. The cytoplasmic domain of TfR1 interacts with a number of different proteins, including adapter complex-2, transferrin receptor trafficking protein, γ-aminobutyric acid type A receptor-associated protein, ADP-ribosylation factor GTPase-activating protein with coiled coil, ankyrin repeat and Pleckstrin homology domains, heat shock protein HSC70 and programmed cell death 6 interacting protein [6–10]. However, to date, remains unclear how TfR1 ectodomain shedding affects these interactions.

The cleavage of membrane proteins in the interior of lipid bilayers constitutes a new mechanism for promoting the controlled release of signal molecules and transcription factors. This regulated intramembrane proteolysis (RIP) is mediated by a two-step proteolytic process [11–15]: first, ectodomain shedding and, second, cleavage in or close to the transmembrane region mediated by intramembrane cleaving proteases (I-CLiPs). Three classes of I-CLiPs are known so far: intramembrane metalloproteases (site-2 proteases) [11], intramembrane serine proteases (rhomboids) [16,17] and GxGD-type intramembrane aspartyl proteases, as represented by presenilins (γ-secretase) [18,19] and the more recently identified signal peptide peptidase (SPP)/signal peptide peptidase-like (SPPL)-family [20]. These proteases differ in their location, function and substrate requirements. Presenilins and rhomboid-like proteases are specialized on type I topology substrates, whereas SPP/SPPLs selectively cleave type II membrane proteins. For site-2 proteases cleavage of membrane proteins with both orientations has been described [21]. Recent studies identified three type II transmembrane proteins that were substrates for the SPPL-family. Tumour necrosis factor (TNF)α [22,23] and the British dementia protein Bri2 [24] are substrates for SPPL2a and SPPL2b, and Fas ligand (FasL) is a substrate for SPPL2a [25]. With the exception of rhomboid-like proteases, all I-CLiPs characterized so far require an initial shedding event prior to RIP [11,26–28].

Analogous to TNFα that is cleaved by SPPL2b, TfR1 also fulfils the general requirements for SPP-substrates, as defined by Lemberg and Martoglio [29]: (a) The ectodomain of TfR1 is subject to shedding by cleavage at position Arg100 by a TNFα protease inhibitor-2-sensitive shedding protease [4]; (b) there are no positively-charged amino acids C-terminal of the transmembrane domain; and (c) the transmembrane region contains helix destabilizing residues (Gly69, Gly80, Gly84 and Gly87). Therefore, in the present study, we investigated the role of TfR1 as a potential substrate of I-CLiPs.

Results

Characterization of Flag-TfR1-NTF-V5

To investigate the fate of TfR1-NTF after shedding, we decided to establish a double-tagged TfR1-NTF possessing an N-terminal Flag tag and a C-terminal V5 tag (Fig. 1A), which allowed us to trace fragments of a potential intramembrane cleavage. Flag-TfR1-NTF-V5 expressed in human embryonal kidney (HEK) 293 cells appeared as a disulfide-linked dimer of approximately 28 kDa, as demonstrated by the application of reducing sample buffer, resulting in a 14-kDa monomer (Fig. 1B). We next determined the subcellular localization and membrane orientation of Flag-TfR1-NTF-V5 by immunofluorescence using either affinity purified monoclonal anti-Flag or anti-V5 IgG. In unpermeabilized cells, no signal in cells stained with monoclonal anti-Flag IgG was observed, whereas the anti-V5 IgG resulted in plasma membrane staining. This indicates that only the C-terminus of Flag-TfR1-NTF-V5 is exposed to the extracellular space. By contrast, signals in the plasma membrane and in vesicles were observed after treatment with either anti-Flag or anti-V5 IgG in permeabilized cells. We therefore conclude that Flag-TfR1-NTF-V5 is expressed and distributed in the cells in the same manner as has been shown for the endogenous membrane stub of TfR1 after shedding [3] (i.e. it exists as a dimer, is located in the plasma membrane and probably in endocytic vesicles and is presented in the correct orientation exposing the N-terminal Flag tag to the cytoplasm and the C-terminal V5 tag to the extracellular space).

Identification of the TfR1-C-terminal peptide (Cp)

Intramembrane proteolysis of TfR1-NTF should result in an intracellular domain (TfR1-ICD) and an extracellular Cp (TfR1-Cp) (Fig. 2). To identify the TfR1-Cp, the conditioned media of HEK293 cells expressing Flag-TfR1-NTF-V5 were used for immunoprecipitation with monoclonal anti-V5 IgG. TfR1-Cp was clearly detected by western blotting under nonreducing conditions with polyclonal anti-V5 IgG (Fig. 3C); the apparent molecular mass of 4.6 kDa corresponds to the monomer as shown under reducing conditions (see below and Fig. S1). The use of nonreducing conditions allowed us to judge which of three possible TfR1-Cp isoforms is generated: a monomeric reduced TfR1-Cp, a monomeric oxidized TfR1-Cp or a dimeric TfR1-Cp. MALDI-TOF-MS analysis resulted in a peak at 3276 Da (Fig. 3B). Two possible monomeric peptides of Flag-TfR1-NTF-V5 are represented by this mass. One is generated by cleavage between Phe81 and Met82

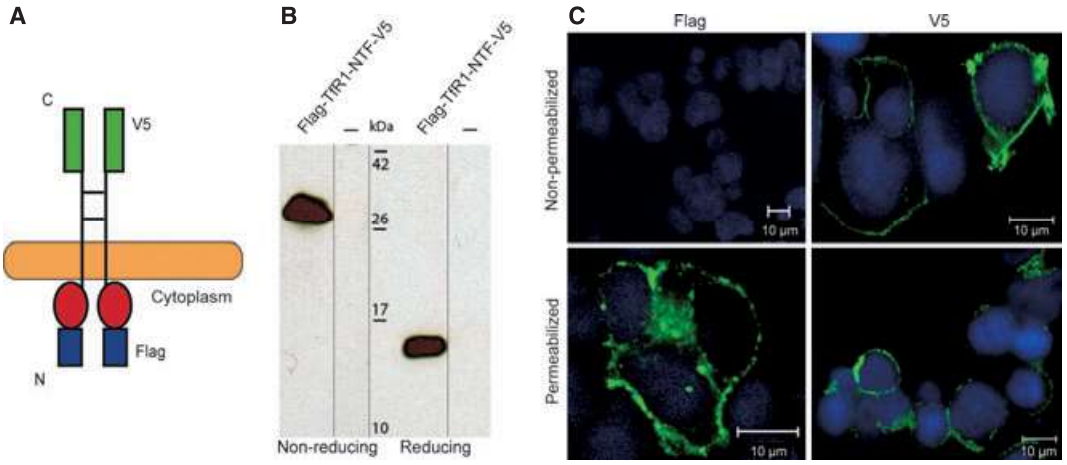

Fig. 1. Characterization of Flag-TfR1-NTF-V5. (A) Schematic illustration of the Flag-TfR1-NTF-V5 construct; an N-terminal Flag tag and a C-terminal V5 tag were added to amino acids 1–100 of TfR1, representing the N-terminal TfR1 stub, which remains in the membrane after ectodomain shedding of TfR1. The green box depicts the V5 tag, the blue box depicts the Flag tag and the red circle indicates the intracellular domain of TfR1. (B) Flag-TfR1-NTF-V5 expressed in transfected HEK293 cells appears as a dimer at 28 kDa under nonreducing conditions and as monomer at 14 kDa in reduced cell lysates (right lane). (C) Localization and orientation of Flag-TfR1-NTF-V5 in HEK293 cells transiently expressing this construct. The indicated cells were permeabilized with 0.1% Triton X-100; the samples were either stained with monoclonal Flag- or V5-specific IgG and visualized by confocal laser scanning microscopy.

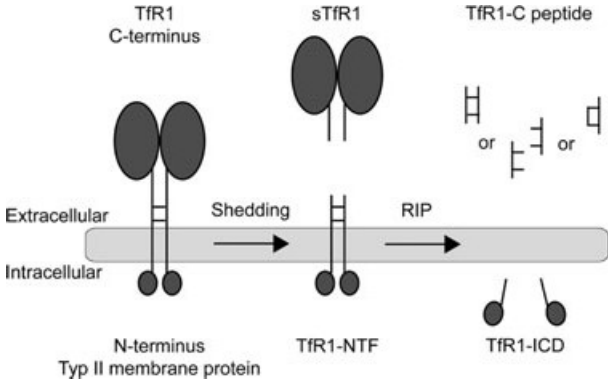

Fig. 2. Schematic presentation of TfR1 shedding and fragments after potential intramembrane proteolysis. Because two disulfide bridges preserve the dimeric form of the TfR1-NTF, three different redox forms of the TfR1-C peptide (TfR1-Cp) can be assumed.

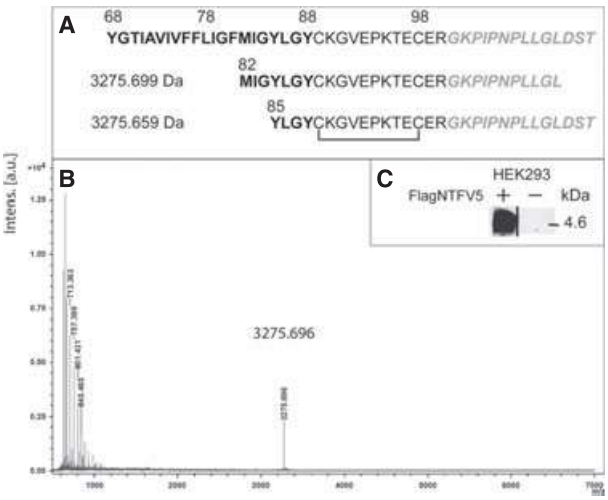

Fig. 3. Detection of the TfR1-Cp. (A) The amino acid sequence of the transmembrane region (bold), the stalk (regular font) and the V5 tag (grey, bold italic), as well as sequence numbering, are indicated. Using FindPept tool of EXPASY, two possible peptides representing the measured mass shown in (B) were identified and are depicted above. (B) Under nonreducing conditions, immunoprecipitated TfR1-Cp-V5 was subjected to MALDI-TOF-MS and detected as a peak at 3275.696 Da. (C) Immunoprecipitated TfR1-V5-Cp was detected by western blotting under nonreducing conditions.

with a V5 tag shortened by three amino acids, the other is generated by cleavage between Gly84 and Tyr85 with a complete V5 tag but a loss of two protons, which indicates a disulfide bridge between Cys89 and Cys98 (Fig. 3A). The existence of dimeric peptides was excluded because no other signals were obtained by MALDI-TOF-TOF-MS analysis. To confirm the sequence of the discovered TfR1-Cp, we analyzed the peptide by MALDI-TOF-TOF-MS. Breakage of the amino acid backbone by MALDI-TOF-TOF-MS analysis results in six possible types of fragment ions. The most common cleavage sites are at the CO-NH bonds, which give rise to b and y ions with the charge retained on the NTF and C-terminal fragment, respectively. The mass difference between two adjacent b or y ions represents the amino acid at this site. Thus, the b26 to b29

ions in a fragment analysis of TfR1-Cp (Fig. 4) show the fragmentation of a full-length V5 tag, whereas the y13 to y19 ions show a complete V5 tag and an N-terminal fragmentation. These fragments therefore represent a peptide with a full-length V5 tag and cleavage between Gly84 and Tyr85. The data thus revealed a 16 amino acid long TfR1 peptide, which appeared as an oxidized monomer. These investigations demonstrate the existence and the sequence of a TfR1-Cp released from the N-terminal membrane stub of TfR1 by intramembrane proteolysis.

Identification of human serum albumin (HAS) as interaction partner of TfR1-Cp

The cleavage of TfR1-NTF in cell culture results in the release of TfR1-Cp into the media, which lead us to expect that TfR1-Cp is circulating in human serum. However, we were unable to detect it there during a first attempt. The main serum protein albumin binds a number of hydrophobic substances, including hydrophobic peptides [30]. We therefore investigated whether the TfR1-Cp is masked by binding to albumin. We conducted a pull-down assay with streptavidin agarose and biotinylated serum albumin in the presence or absence of TfR1-Cp and detected TfR1-Cp by western blotting (Fig. 5). To confirm the success of the pull-down procedure and detection system, we used biotinylated TfR1-Cp (75 ng; Fig. 5, lane 1) in NaCl/P_i. Unbiotinylated serum albumin (Fig. 5, lane 2) and TfR1-Cp in NaCl/P_i (without albumin; Fig. 5, lane 6) served as a control to show that these molecules do not bind to streptavidin agarose. Notably, TfR1-Cp coprecipitated with biotinylated serum albumin and was detected between 75 and 55 kDa under nonreducing conditions (Fig. 5, lanes 4 and 5), demonstrating that TfR1-Cp binds to albumin. The precipitation of albumin and identity of the albumin band with the 55/75-kDa band was verified by detecting the same blot with a purified anti-albumin IgG after stripping and stripping control (Fig. 5, bottom). When chemically synthesized TfR1-Cp is directly applied to the gel, it appears

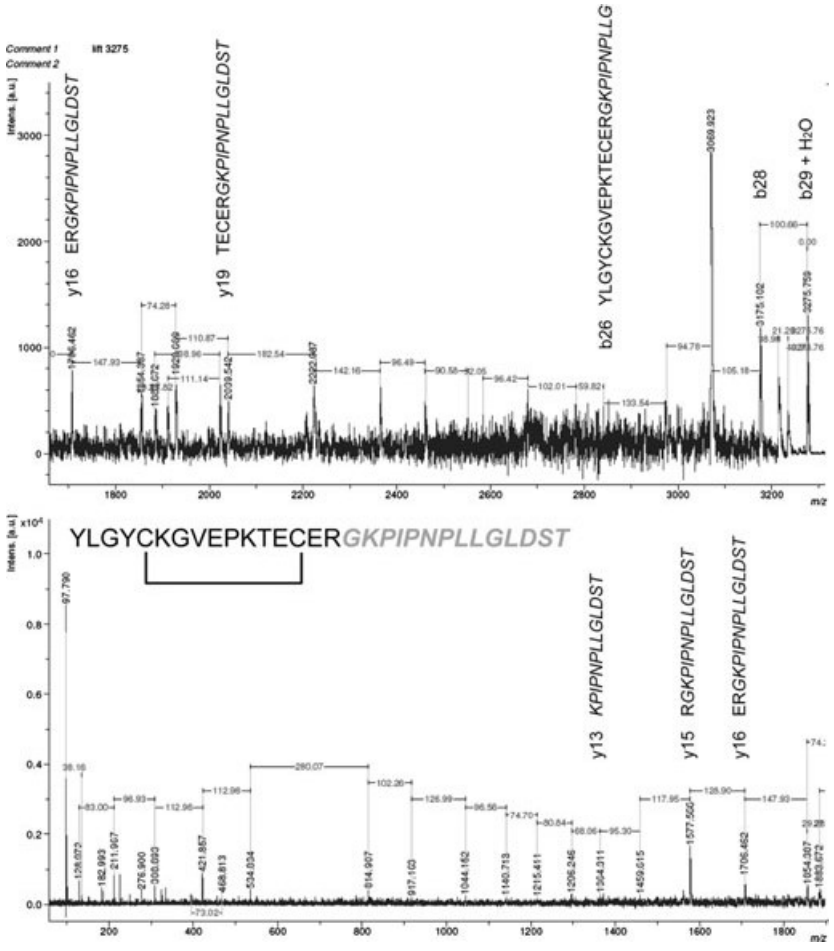

Fig. 4. MALDI-TOF-TOF-MS analysis of the TfR1-Cp. The b and y ions and some internal fragments designated by their sequence have been clearly identified. The masses indicate that the peptide appears as an oxidized monomer (i.e. it contains a disulfide bridge between Cys89 and Cys98 of TfR1). The identified sequence is shown at the top of the second panel. Black, TfR1-Cp; grey italic, V5 tag. For clarity, the mass range is split into two panels.

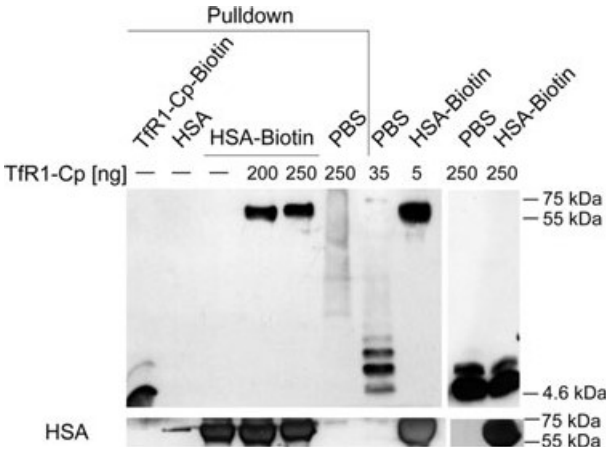

Fig. 5. HSA as interaction partner for TfR1-Cp. Pull-down with streptavidin agarose and biotinylated HSA (HSA-biotin), which was incubated in presence or absence of TfR1-Cp. The TfR1-Cp was detected by western blotting under nonreducing conditions using polyclonal anti-TfR1-Cp IgG. After stripping and stripping control, the same membrane was used to detect the HSA with a monoclonal anti-human albumin IgG (bottom panel). To show where TfR1-Cp should appear in the presence or absence of serum albumin, it was directly applied to the gel under nonreducing conditions (lanes 7 and 8) and under reducing conditions (the furthest two lanes to the right). NaCl/P_i is indicated as PBS in the figure.

as a monomer, dimer and multimer under nonreducing conditions (Fig. 5, lane 7) and mainly as a monomer under reducing conditions (Fig. 5, second lane to the right). In the presence of albumin, it appears again as a 55/75-kDa band under nonreducing conditions (Fig. 5, third lane to the right) but at 4.6 kDa under reducing conditions (Fig. 5, furthest lane to the right), clearly indicating binding via a disulfide bond. Furthermore, we showed that 5 ng of TfR1-Cp is measurable as conjugate with serum albumin, whereas TfR1-Cp alone is not detectable < 20 ng (Fig. S1), reflecting an increasing diffusion of the unconjugated peptide during gel electrophoresis and immunoblotting. Nevertheless, it remains critical to detect TfR1-Cp directly from blood serum because only a minute ratio of the albumin molecules is expected to carry a TfR1-Cp molecule.

TfR1-NTF is a substrate for intramembrane proteolysis by SPPL2b

To determine the class of intramembrane proteases that is responsible for the release of the TfR1-Cp, we tested different inhibitors targeting I-CLiPs. After treatment of HEK293 cells with the indicated inhibitors, the TfR1-Cp generated from endogenous I-CLiPs was isolated from conditioned media using anti-V5 IgG. The metalloprotease inhibitor 1,10-phenanthroline, which inhibits site-2 proteases [31], did not change the amount of TfR1-Cp released to the supernatant compared to control cells (Fig. 6A). Different studies have shown that $(Z\text{-}LL)_2$-ketone inhibits SPP and SPPL2a/b but not γ-secretase [20,22–24], whereas L-685,458 targets γ-secretase, SPP and SPPL2a/b [21,23,24,32]. *N*-[*N*-(3,5-difluorophenylacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) only inhibits γ-secretase activity but fails to block SPP and SPPL2b activity [21,23,32]. In our experiments, $(Z\text{-}LL)_2$-ketone and L685,458 strongly reduced TfR1-Cp secretion from HEK293 cells, whereas treatment with DAPT had no detectable effect (Fig. 6A). Moreover, the inhibition of TfR1-Cp secretion by $(Z\text{-}LL)_2$-ketone was concentration-dependent (Fig. 6B). These results suggest that GxGD intramembrane aspartyl proteases of the SPP/SPPL-family are involved in the intramem-

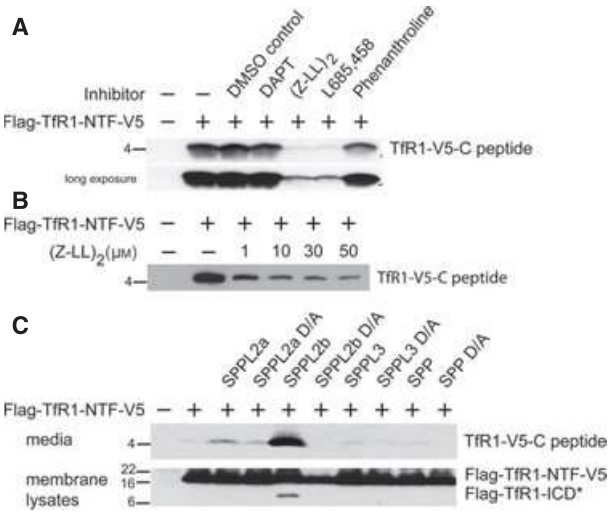

Fig. 6. Intramembrane proteolysis of TfR1-NTF. (A, B) The conditioned media of HEK293 cells expressing Flag-TfR1-NTF-V5 were subjected to immunoprecipitation with anti-V5 IgG and TfR1-V5-Cp was detected by western blotting. (A) The cells were incubated overnight with the indicated inhibitors. The solvent dimethysulfoxide served as a control. (B) Concentration-dependent inhibition of the endogenous protease by the inhibitor $(Z\text{-}LL)_2$-ketone. (C) HEK293 cells, stably expressing the indicated SPPL variants, were transfected with Flag-TfR1-NTF-V5; D/A indicates dominant negative mutants (D421A for SPPL2a and SPPL2b, D272A for SPPL3, D265A for SPP). The conditioned media were used for immunoprecipitation with anti-V5 IgG to detect TfR1-Cp, whereas a membrane preparation of the cells was applied to identify the intracellular domain of the TfR1 (TfR1-ICD). The band designated as Flag-TfR1-ICD was identified as a result of the N-terminal Flag tag and a molecular mass of 10 kDa. An unspecific signal can be excluded because the control lanes do not show this signal; however, we cannot exclude the possibility that this Flag-TfR1-ICD is C-terminally shortened for some amino acids.

brane cleavage of the TfR1-NTF. To further support this hypothesis, HEK293 cells, stably expressing the indicated members of the SPP/SPPL-family or their catalytically inactive mutants (D/A) were transiently transfected with Flag-TfR1-NTF-V5 and conditioned media were analyzed for TfR1-Cp secretion (Fig. 6C). The exposure time of the western blotting was optimized to the TfR1-Cp signal obtained after the overexpression of SPPLs. Therefore, the bands of TfR1-Cp released by endogenous proteases are only very faint (Fig. 6C, lane 2) and identical in intensity to cells expressing SPPL3 and SPP, demonstrating that these proteases have no impact on TfR1-NTF cleavage. Only the expression of catalytically active SPPL2a and SPPL2b resulted in increased release of TfR1-Cp, strongly indicating that these two I-CLiPs are involved in processing of the TfR1-NTF, with SPPL2b being the most prominent enzyme (Fig. 6C). Expression of the inactive mutant SPPL2a D/A resulted in TfR1-Cp release comparable to the endogenous level, whereas SPPL2b D/A led to the complete abolishment of this fragment, suggesting that this mutant is able to suppress endogenous SPPL2a by a feedback mechanism. To detect the corresponding intracellular cleavage product of TfR1-NTF (TfR1-ICD), we analyzed membrane lysates, cytosol and nuclei of the cells but were unable to find them during a first attempt. A sophisticated methanol/chloroform precipitation of the membrane lysates was finally required to detect the TfR1-ICD as a low-molecular weight fragment in cells co-expressing SPPL2b and TfR1-NTF (Fig. 6C, lane 5), which is in line with the existence of two palmitoylation sites within TfR1-ICD. By contrast to SPPL2b, the expression of SPPL2a did not result in a detectable generation of TfR1-ICD, indicating that TfR1-NTF is mainly processed by SPPL2b in HEK293 cells. This is further corroborated by confocal immunofluorescence microscopy experiments, demonstrating a clear colocalization between SPPL2b and TfR1-NTF, whereas colocalization with SPPL2a can only be detected sporadically (Fig. S2). Taken together, these results demonstrate that TfR1-NTF is mainly cleaved by SPPL2b and, to a lesser extent, by SPPL2a to release a 16 amino acid long Cp and a corresponding ICD.

Discussion

Intramembrane proteases are important for a variety of processes, such as transcriptional control, cellular signalling, control of mitochondrial membrane remodelling, parasite invasion and bacterial protein translocation. They are also involved in a broad range of diseases, such as Alzheimer's disease, hepatitis C virus infection, tuberculosis and malaria [33,34]. The GxGD-aspartyl proteases SPPL2a and SPPL2b are important for cleaving the membrane stubs of type II membrane proteins after shedding, as observed for TNFα, FasL and Bri2. The length of the remaining ectodomain negatively correlates with the efficiency of intramembrane proteolysis, and the primary sequence determinants within the intracellular domain and the transmembrane domain, together with short luminal juxtamembrane sequences, are also required for efficient cleavage [27]. The liberated ICD of TNFα and FasL are involved in reverse signalling [25,35].

In the present study, we describe for the first time the intramembrane cleavage of the TfR1 membrane stub (TfR1-NTF) and have identified the sequence of the Cp by fragmentation with MALDI-TOF-TOF-MS. The TfR1-Cp is 16 amino acids long and is released from TfR1-NTF by cleavage in the transmembrane domain between Gly84 and Tyr85. Although, in principle, other redox forms are possible, in all of our samples, the peptide appeared as an oxidized monomer under nonreducing conditions, suggesting a rearrangement of the two intermolecular disulfide bridges in the TfR1-NTF to intramolecular bridges during RIP.

In the present study, we further demonstrated that TfR1-NTF is proteolytically processed by SPPL2a and SPPL2b. SPPL2a is predominantly expressed in late endosomal and lysosomal compartments [10,27], whereas SPPL2b is located at the plasma membrane [22]. The TfR1 was very efficiently recycled from endosomes to the cell surface and did not return to the trans-Golgi network or to other biosynthetic compartments in detectable amounts [36]. Therefore, it can be expected that TfR1-NTF is in direct contact with SPPL2b rather than SPPL2a. A minor part of TfR1-NTF may colocalize with SPPL2a when sorted to lysosomes for final degradation. This is in line with our findings showing that expression of the inactive SPPL2b D/A mutant [20] completely abolished the release of TfR1-V5-Cp, which emphasizes a role for SPPL2b in TfR1-NTF processing. The observation that the expression of the inactive SPPL2a D/A mutant leads to a decrease (but not disappearance) of TfR1-V5-Cp release can result from the colocalization of TfR1-NTF with endogenous SPPL2b, as revealed by confocal fluorescence microscopy experiments (Fig. S2).

Synthetic ICDs of the type II membrane proteins proTNFα and Fas ligand were detected in the nucleus and shown to affect gene transcription [25,35]. We cannot exclude the possibility of a similar role for the TfR1-ICD, although we have no evidence for nuclear localization, and we detected the TfR1-ICD exclusively in membrane lysates after methanol/chloroform precipitation. Palmitoylation is important for lipid raft

association. Levental *et al.* [37] have demonstrated for the majority of integral raft proteins that enrichment in the raft phase is dependent on palmitoylation at two juxtamembrane cysteines and could be enhanced by oligomerization. Furthermore, a more recent study showed that palmitoylation of TNFα is involved in the regulation of TNF receptor-1 signalling [38]. Although full-length TfR1 is not found in lipid rafts [39] and is commonly used as non raft protein marker, the TfR1 can be palmitoylated at two juxtamembrane cysteines, which is associated with the inhibition of receptor-mediated endocytosis [40]. Because these palmitoylation sites are located within the sequence of the TfR1-ICD, it can be speculated that shedding and intramembrane proteolysis of palmitoylated TfR1 results in an assembly in lipid rafts rather than migration to the nucleus. Thus, the existence of TfR1-ICD in the membrane may indicate a possible role in lipid raft signalling. This is corroborated by the observation that other important proteins in iron metabolism (e.g. ferroportin) are distributed in rafts and detergent-resistant membranes in macrophages [41]. Therefore, a regulatory role for TfR1-ICD in lipid rafts is conceivable and should be taken into consideration in future investigations.

Interestingly, the cleavage of TfR1-NTF occurs between Gly84 and Tyr85 next to a G*XXX*G-dimerization motif. Such a motif was shown to modulate γ-secretase cleavage specificity [42,43] and was also found in the type II transmembrane protein Bri2 that is also subject to RIP [24]. The cleavage region within the G*XXX*G-motif of TfR1 is highly conserved among the species in the transmembrane domain (Fig. 7), indicating the importance of the intramembrane proteolytic process of TfR1-NTF. In the case of Bri2, mutations of Gly67 and Gly71 to alanines within the G*XXX*G-motif did not result in a significant reduction of RIP, whereas mutation of Gly60 to alanine, which is located in the N-terminal part of the transmembrane domain (amino acids 55–75) of Bri2, led to a decrease of RIP as a result of an α-helix-destabilizing effect [44]. In the transmembrane domain of TfR1 (amino acids 68–88), Gly69, which is also N-terminally located within the transmembrane domain, is highly conserved in vertebrates; therefore, the influence of the G*XXX*G-motif (amino acids 80–84) and Gly69 for the intramembrane proteolysis of TfR1 needs to be investigated further.

We identified HSA as a molecular interaction partner for TfR1-Cp. The two molecules are covalently linked via a disulfide bridge. Remarkably, serum albumin has a free cysteine, which has antioxidant activity [45] and is possibly responsible for the binding of TfR1-Cp. Because the concentration of sTfR1 reflects the balance between functional iron availability and iron demand

Species	Trivial name	TfR1-TMD
Homo sapiens	human	YGTIAVIVFFLIGFMIGYLGY
Pongo abelii	orangutan	YGTIAVIIFFLIGFMIGYLGY
Macaca mulatta	rhesus monkey	YGTIAVIIFFLIGFMIGYLGY
Bos taurus	neat	YGIIAVIVFFLIGFMIGYLGY
Sus scrofa	pig	YGIIAVITFFLIGFMIGYLAY
Felis catus	cat	YGTIAIILFFLIGFMIGYLGY
Mus musculus	mouse	FAAIALVIFFLIGFMSGYLGY
Rattus norvegicus	rat	FATIAVVIFFLIGFMIGYLGY
Gallus gallus	chicken	FLVIAAVLLLLIGFLIGYLSY
Danio rerio 1a	zebrafish	GFVVGILLLFLFGYLIGYMSN
Danio rerio 1b	zebrafish	VCVGAALLVFLVGLLIGYSVH

Fig. 7. Alignment of the amino acid sequence of the TfR1 transmembrane domain in different species. Grey shading shows the highly conserved region in which intramembrane proteolysis takes place including the G*XXX*G-motif; the line indicates the site of cleavage.

[46], its soluble counterpart, TfR1-Cp, may act as a regulator in systemic iron metabolism and be important for the understanding of basic mechanisms, as well as for diagnostic and therapeutic purposes.

Materials and methods

Cell culture, transfection procedures and cDNAs

Human embryonal kidney (HEK293) cells were cultured in DMEM with Glutamax (PAA, Pasching, Austria) supplemented with 1× Anti-Anti (Invitrogen, Darmstadt, Germany) and 10% fetal calf serum at a 5% CO_2 atmosphere and 37 °C. Cells were stably transfected using Fugene HD (Roche, Basel, Switzerland) in accordance with the manufacturer's instructions and selected in the presence of 45 μg·mL^{-1} Blasticidin S (PAA). Transient transfection was conducted with 11.1 μL (0.45 mg·mL^{-1}) poly(ethylenimine) per 1 μg of DNA. The Flag-TfR1-NTF-V5 construct consists of the TfR1-NTF cDNA coding for amino acids 1–100 of TfR1, an N-terminal Flag-tag (DYKDDDDK) and a C-terminal V5-tag (GKPIPNPLLGLDST). The tags were introduced by PCR. The PCR product was ligated into pJet1.2/blunt (Fermentas; Thermo Fisher Scientific, Rockford, MD, USA), sequenced for verification and subcloned via *Xho*I and *Xba*I into pcDNA6.0-V5-HisA (Invitrogen, Darmstadt, Germany). The cDNAs coding for SPP or SPP D265A (SPP D/A) were subcloned into the *Eco*RI and *Xho*I sites of pcDNA 4/TO A (Invitrogen Life Technologies, Carlsbad, CA, USA). Using PCR, a C-terminal HA tag (AYPYDVPDYA) was added to the cDNAs of SPPL2a, SPPL2a D421A (SPPL2a D/A), SPPL2b, SPPL2b

D421A (SPPL2b D/A), SPPL3 and SPPL 3 D272A (SPPL3 D/A). PCR products were subcloned into the *Eco*RI and *Xho*I sites of pcDNA 4/TO A (Invitrogen Life Technologies, Carlsbad, CA, USA). Stable transfection of HEK293 TR cells and the induction of the respective SPP/SPPL expression was carried out as described previously [24].

Detection of Flag-TfR1-NTF-V5 in cells

For immunoprecipitation experiments 2×10^6 HEK293 cells stably transfected with Flag-TfR1-NTF-V5 were seeded and cultured for 3 days. Cells were lysed in ice-cold 1% Triton X-100 in NaCl/P_i supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany), incubated on ice for 1 h (mixed every 10 min) and centrifuged at 16 300 ***g*** for 30 min at 4 °C. The clear lysates were used for western blotting to determine the amount of β-actin (using anti-β-actin, dilution 1 : 10 000; Sigma-Aldrich, Munich, Germany) for standardization. The lysates were immunoprecipitated at 4 °C for 2 h or overnight with 2 μg of monoclonal antibody against the N-terminus of TfR1 (clone: H68.4; Zymed, Carlsbad, CA, USA) and 25 μg of protein A sepharose (GE Healthcare, Uppsala, Sweden). Subsequently, the protein A sepharose was washed one time with NaCl/P_i, three times with Triton X-100 in NaCl/P_i and twice with water and boiled for 5 min with reducing or non-reducing SDS sample buffer. The samples were separated on a 16% tricine gel, transferred to a poly(vinylidene difluoride) membrane (Immobilon-PSQ; Millipore, Billerica, MA, USA) by electroblotting, and Flag-TfR1-NTF-V5 was detected with a purified monoclonal anti-V5 IgG (dilution 1 : 3000; Invitrogen, Karlsruhe, Germany).

Detection of fragment release from cells

The C-terminal fragment (TfR1-Cp) was detected in the conditioned media from HEK293 cells expressing Flag-TfR1-NTF-V5. Samples were centrifuged (500 ***g*** for 5 min at 4 °C) to remove cellular debris and immunoprecipitated overnight at 4 °C with 1 μg of monoclonal anti-V5 and 25 μg of protein A sepharose.

For detection by MALDI-TOF-MS, the sepharose was washed three times with NaCl/P_i and three times with 50 mM ammonium acetate. TfR1-Cp was eluted with 50% acetic acid. The eluate was dried in a rotary vacuum evaporator (Centrivac; Heraeus Instruments, Frankfurt, Germany) and washed once with water. Samples were dissolved in 5 μL of water and spotted on a steel MALDI-TOF-MS target mixed with 0.5 μL of a 2-cyano-4-hydroxycinnamic acid matrix (1 mg·mL^{-1} in 70% acetonitrile/0.1% trifluoric acid) and the mixture was air-dried. MALDI-TOF-MS were recorded using an Ultraflex III mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam laser and a LIFT-MS/MS facility. Calibration was performed with a peptide standard mixture (Pepmix; Bruker Daltonics). Analyses were carried out using Findpept of EXPASY (http://web.expasy.org/findpept/).

For western blotting, the sepharose was washed three times with NaCl/P_i and twice with water, and then boiled for 5 min with nonreducing SDS sample buffer. TfR1-Cp was detected after SDS/PAGE using polyclonal anti-V5 IgG (Chemicon; Millipore) as described above.

Immunofluorescence

Stably-transfected HEK293 cells were grown on poly L-lysine coated glass cover slips to 60–80% confluence. Cells were fixed for 30 min with 4% paraformaldehyde, 0.2% glutardialdehyde in NaCl/P_i at room temperature; for permeabilization, 0.1% Triton X-100 was added to the fixation solution. Cells were washed with 200 mM glycine for 5 min and subsequently with NaCl/P_i, blocked with 5% goat serum for 30 min and stained for 1 h with either primary monoclonal anti-V5 or anti-FLAG M2 IgG (Sigma-Aldrich, Munich, Germany) at a dilution of 1 : 100 and 1 : 300, respectively. After washing the cells with NaCl/P_i, they were stained with the secondary antibody (polyclonal anti-mouse Alexa-488; Molecular Probes, Carlsbad, CA, USA; dilution 1 : 1000), washed again with NaCl/P_i and, finally, once with 100% ethanol; cover slips were then mounted in SlowFade Gold antifade reagent with DAPI (Invitrogen, Karlsruhe, Germany). Cell staining was documented by a confocal laser scanning microscope LSM510 using Plan-Neofluar ×40/1.3 Oil DIC (Zeiss, Oberkochen, Germany) and analyzed with LSM IMAGE BROWSER (Zeiss).

Pull-down with streptavidin agarose

HSA was biotinylated using sulfo-NHS-LC-biotin (Pierce; Thermo Fisher Scientific) in accordance with the manufacturer's instructions, and purified via a PD10 column (GE Healthcare). To verify successful biotinylation, an ELISA was conducted with monoclonal anti-human albumin IgG (clone HSA-11; Sigma-Aldrich, St Louis, MO, USA) as a catcher and a horseradish peroxidase (HRP) conjugated streptavidin for detection (Pierce; Thermo Fisher Scientific). For the pull-down assay, either 200 or 250 ng of TfR1-Cp was diluted in 200 μL of 20 μM biotin-HSA. Biotin-HSA (20 μM) alone, TfR1-Cp (250 ng), unbiotinylated HSA (20 μM) and TfR1-Cp-biotin (75 ng), all in NaCl/P_i, served as control. The samples had been incubated for 1 h at 4 °C before streptavidin agarose (Novagen, Merck, Darmstadt, Germany) was added for precipitation of HSA-biotin (or TfR1-Cp-biotin in the case of the corresponding control) and incubated for a further 2 h at 4 °C. For SDS/PAGE (16% tricine gel) the streptavidin agarose was washed three times with 0.1% Triton X-100 in NaCl/P_i and twice with NaCl/P_i, and then boiled for 5 min with nonreducing SDS sample buffer. TfR1-Cp was detected by western blotting with anti-TfR1-Cp IgG (dilution 1 : 1000) raised in rabbits

and purified first via protein A sepharose and then via a TfR1-Cp-NHS-sepharose column (binding of the purified antibodies to TfR1-Cp was verified via ELISA and western blotting). HRP-conjugated polyclonal goat ant-rabbit immunoglobulins (Dako, Glostrup, Denmark) were applied as secondary antibodies. HSA was also detected by western blotting on the same membrane after stripping for 40 min at 56 °C with stripping buffer (62.5 mM Tris, pH 6.7, 2% SDS and 100 mM β-mercaptoethanol) and control of successful removal of antibodies by enhanced chemiluminescence detection. Monoclonal anti-human albumin IgG HSA-11 served as a primary antibody and HRP-conjugated polyclonal rabbit anti-mouse immunoglobulins (Dako) served as secondary antibody.

Inhibitor treatment

To inhibit endogenous intramembrane proteases, 4×10^6 HEK293 cells stably transfected with Flag-TfR1-NTF-V5 were treated for 18 h with a final concentration of either 1.5 μM of L685,458, 1.5 μM DAPT, 50 μM $(Z\text{-}LL)_2$-ketone or 100 μM of the metalloprotease inhibitor 1,10-phenanthroline. Cells treated with the respective solvent, dimethylsulfoxide, served as a control. The media of the cells were then used for V5 immunoprecipitation, as described above.

Detection of TfR1-ICD

For detection of TfR1-ICD generated by intramembrane proteolysis, HEK293 cells stably expressing the indicated SPP/SPPL proteases were transiently transfected with Flag-TfR1-NTF-V5 and cellular membranes were prepared. The cell pellet was resuspended in buffer A (10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA) for 10 min on ice, passed through a fine needle (23-G¼) and centrifuged (5000 ***g*** for 5 min). The supernatant was centrifuged (13 000 ***g*** for 45 min at 4 °C). The membrane pellet was resuspended in buffer B (50 mM Tris, pH 7.8, 50 mM K-acetate, 2 mM Mg-acetate, 125 mM sucrose, 1 mM dithiothreitol) and incubated for 30 min at 37 °C at 700 r.p.m. Lipids were then extracted from the protein by chloroform/methanol (1 : 2) precipitation and incubated on ice for 30 min. Pellets were centrifuged (13 000 ***g*** for 20 min at 4°C), resuspended in 2× SDS buffer and incubated (1400 r.p.m. for 10 min at 65 °C).

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (FU 408/5-1) and by the Stiftung für Pathobiochemie und Molekulare Diagnostik.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Titration of TfR1-Cp in the presence and absence of HSA and detection of TfR1-Cp by western blotting.

Fig. S2. Colocalization of TfR1-Cp with SPPL2a and SPPL2b.