Mutational suppression of transferrin receptor shedding can be compensated by distinct metalloproteases acting on alternative sites

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Abstract The human transferrin receptor (TfR) is proteolytically cleaved at R100 within the juxtamembrane stalk and to a lesser extent at an alternative site. We examined the effect of stalk mutations on human TfR shedding in transfected CHO cells. Point mutations at R100 led to an increase in alternative shedding while the R100 cleavage product was undetectable. Replacing the TfR-stalk by the corresponding sequences from tumor necrosis factor-α or interleukin-6 receptor also led to TfR ectodomain shedding. These results show that cleavage at alternative sites can compensate for suppressed cleavage at the major site and inhibitor studies reveal that at least three metalloproteases are involved in the shedding process.

Key words: Ectodomain shedding; Metalloprotease; Serum transferrin receptor; Interleukin-6 receptor; Tumor necrosis factor-α; ADAM

1. Introduction

Many cell-surface proteins, including membrane-anchored growth factors and precursors of cytokines, receptors, ectoenzymes, cell adhesion molecules and the Alzheimer precursor protein, are proteolytically cleaved and release their ectodomains into the extracellular space [1,2]. The first shedding enzyme identified was the metalloprotease tumor necrosis factor-α (TNF-α) converting enzyme (TACE, ADAM-17) and originally found to release TNF-α [3,4], but was later shown to be involved in the shedding of a variety of other membrane proteins (e.g. L-selectin, TNF receptors, transforming growth factor-α (TGF-α)) [5]. Other important members of the same protein family play an important role in ectodomain shedding, including ADAM-9, ADAM-10 and ADAM-19 [6,7]. Besides the ADAMs, other proteases are involved in shedding processes, including matrix metalloproteinases (MMPs, e.g. stromelysin-1, matrilysin and MT4-MMP) [8–11] and serine proteases (e.g. proteinase-3, neutrophil elastase and cathepsin G) [12–14]. Ectodomain shedding is a widely branched network of competitive processes, i.e. one type of protein can be cleaved by different proteases or, vice versa, one protease can cleave several different proteins.

Although to date many shedding enzymes, substrates and cleavage sites have been identified, the mechanism of substrate recognition and cleavage regulation remains an enigma. The targets of shedding are type I and type II transmembrane proteins as well as glycosylphosphatidylinositol-anchored proteins [2]. Systematic mutational analyses of the cleavage sites by deletion mutants and complete exchange of cleavage sites between different proteins reveal no perceptible general rules for consensus sequences or target motifs, suggesting that tertiary structural features rather than sequence data are responsible for recognition [15–17].

Besides proTNF-α one of the most important type II transmembrane proteins that is subject to shedding is the human transferrin receptor (TfR). The TfR is a disulfide-linked homodimer (Cys-89 and Cys-98) [18] possessing a juxtamembrane stalk of 2.9 nm (amino acids 89–126) [19] that contains an *O*-glycan at Thr-104 [20,21]. Shedding of the TfR occurs C-terminally of Arg-100 [22] and is mediated by an integral membrane metalloprotease sensitive to TAPI-2 (TNF-α protease inhibitor 2) [23]. Recently we have identified alternative cleavage sites at Val-108 and Lys-95 within the TfR-stalk, which are processed by neutrophil elastase and cathepsin G, respectively [14]. A further cleavage site at Gly-91 was reported for two truncated mutants of TfR [24].

In the present study we mutated amino acids in the TfR around the major and alternative cleavage sites and replaced the stalk by the corresponding segment from either the type II membrane protein TNF-α or the type I membrane protein IL-6R (interleukin-6 receptor). Using transfected CHO cells or membrane preparations thereof we showed that all mutants are substrates for one or several shedding proteases. The amount and the molecular mass of the soluble products, however, as well as the inhibitor pattern differ from mutant to mutant, indicating that different proteases are involved and can compensate for each other.

2. Materials and methods

2.1. Materials

Monoclonal mouse antibody OKT9 was prepared as previously described [25]. Other reagents: polyclonal anti-biotin antibody from goat (Sigma, St. Louis, MO, USA); secondary antibodies (Dako A/S, Glostrup, Denmark); blasticidin S, MMP inhibitor 1 and 2, TAPI-2, recombinant human tissue inhibitor of metalloproteinases TIMP-3 (Calbiochem, Schwalbach, Germany); FCI (decanoyl-Arg-Val-Lys-

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Abbreviations: ADAM, a disintegrin and metalloproteinase; FCI, furin convertase inhibitor; IL-6R, interleukin-6 receptor; MMP, matrix metalloproteinase; sTfR, soluble TfR; TAPI, TNF-α protease inhibitor; TNF-α, tumor necrosis factor-α; TfR, transferrin receptor; TIMP, tissue inhibitor of metalloproteinases; wt-TfR, wild-type transferrin receptor

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Arg-chloromethylketone), human TIMP-1 and -2 (Alexis Biochemicals, Grünberg, Germany); pefablocSC (Boehringer Mannheim, Mannheim, Germany); FuGENE 6 (Roche, Basel, Switzerland); Dulbecco's phosphate-buffered saline (PBS; Invitrogen, Karlsruhe, Germany); EZ-Link sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA). Other materials were purchased from Sigma.

2.2. Construction of TfR-stalk mutants

The TfR-cDNA was cloned from pGEM1-TR (kindly provided by Marino Zerial, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany) into *Age*I-deficient pcDNA6/V5-His B (Invitrogen). Unique *Bsu*36I and *Age*I restriction sites (corresponding to amino acids K95 and P107, respectively) were introduced into the stalk. The R100 and L101 mutations, as well as replacement of the TfR-stalk (segment T96 to S106) by the corresponding TNF-α (71-SPLAQAVRSSSR-82) and IL-6R (352-TSLPVQDSSSVP-363) stalk (mutants referred to as TfR-Tα and TfR-IR) were introduced by ligation of synthetic dimeric oligonucleotides. The mutations V108G and R109G were introduced into a TfR-segment (up to base pair 431) by ligation of synthetic oligonucleotides using the *Age*I and natural *Pst*I restriction sites. The segment was cloned into pcDNA6 containing full length TfR via *Xho*I/*Btr*I.

2.3. Transfection of CHO-TRVb cells and selection

CHO-TRVb cells (designated hereafter as TRVb; cells were kindly provided by Timothy E. McGraw, Cornell University, New York, USA), a cell line that does not express functional transferrin receptor at their cell surface, were cultured at a 5% CO_2 atmosphere and 37°C in Dulbecco's modified Eagle's medium with Glutamax supplemented with penicillin/streptomycin and 5% fetal calf serum. Transfection was performed with FuGENE 6 according to the manufacturer's instructions and cells selected in the presence of blasticidin S.

2.4. Detection of soluble TfR (sTfR) from cell culture medium

For sTfR collection, 1×10^6 transfected TRVb cells were seeded on uncoated 150 mm dishes and cultured for 4 days (final cell number 3×10^7) in 30 ml medium. The cells were used for membrane preparation (see Section 2.5), the medium was centrifuged ($500\times g$, 4°C, 10 min) to remove cellular debris and incubated overnight with 20 μl ferri-transferrin–Sepharose (prepared as described in [26]). The Sepharose was washed three times with PBS (150 mM NaCl, 10 mM phosphate, pH 7.5) containing 1% Triton X-100, once with PBS and boiled for 5 min with two-fold concentrated sodium dodecyl sulfate (SDS) sample buffer. The samples were separated on a 7.5% polyacrylamide gel and TfR was detected by Western blotting using OKT9 directed against the extracellular domain.

2.5. Detection of sTfR release from membranes

After removal of the medium the TRVb cells were placed on ice, washed twice with 30 ml Dulbecco's PBS and then incubated for 15 min with 1 ml 1:10 diluted Dulbecco's PBS. After detaching the cells from the dish, they were homogenized by douncing 30 times and differentially centrifuged at $500\times g$ for 15 min, followed by $2600\times g$ for 15 min and finally at $100\,000\times g$ for 45 min. The membrane pellet was washed once in 1 ml PBS and centrifuged at $20\,000\times g$ for 20 min. The membrane pellet was resuspended in PBS to a final concentration of 6 mg/ml and 30-μl aliquots were incubated at 4°C or 37°C for 18 h or stored at −20°C. The samples were then centrifuged at $20\,000\times g$ for 20 min at 4°C and the supernatants analyzed by SDS gel electrophoresis and Western blotting as described above.

2.6. Inhibition experiments

For inhibition experiments the membranes were incubated in the presence of various inhibitors at the following final concentrations: pefablocSC 1 mM, elastatinal 0.2 mM, 1,10-phenanthroline 3 mM, MMP inhibitors 0.5 mM, TAPI-2 0.5 mM, TIMPs 0.5 μM, FCI 1 mM and phosphoramidon 0.82 mM.

2.7. Detection of biotinylated cell surface TfR

Cell surface proteins of CHO cells were labeled with sulfo-NHS-LC-biotin as described by Volz et al. [27]. After biotinylation, 3×10^7 cells were cultured for 24 h. sTfR was precipitated by ferri-transferrin–Sepharose as described in Section 2.4, separated by SDS–PAGE and detected by Western blotting using an anti-biotin antibody. Subsequently the blot membrane was stripped of bound antibody by incubating at 50°C for 30 min in stripping buffer (62 mM Tris–HCl, pH 6.7, 2% SDS (w/v) and 100 mM β-mercaptoethanol), and probed with OKT9.

3. Results

3.1. Shedding of TfR mutants from cells and cell membranes

In HL-60 cells TfR is cleaved at the major cleavage site Arg-100, but a second cleavage product of the same apparent molecular mass as the alternative Val-108 product released by neutrophil elastase in U937 cells could also be detected [14,23]. To better characterize the shedding process we transfected TRVb cells with human wild-type TfR (wt-TfR), TfR point mutants (R100E, R100G, L101D, L101G, V108G, R109G) and TfR where the stalk was replaced by either the TNF-α-stalk or the IL-6R-stalk.

Untransfected TRVb cells did not express any detectable TfR (data not shown). The TRVb cells transfected with wt-TfR released mainly the major sTfR and a minor product of lower molecular mass (Fig. 1A, lane 2), as shown for HL-60 cells [23]. Since the *O*-glycosylation at Thr-104 contributes to a gel shift the different cleavage sites can be clearly distinguished. In contrast to wt-TfR, transfected cells expressing the R100E or R100G mutant released mainly the alternative product, indicating that suppression of cleavage at the major site can be compensated by cleavage at the alternative site (Fig. 1A, lanes 3 and 4). Unlike the R100 mutants, expression of L101D and L101G led to release of the major sTfR (Fig. 1A, lanes 5 and 6). Furthermore, the amount of released sTfR was dramatically increased (not recognizable in Fig. 1 since a sample of the same dilution as used for R100 mutants would strongly overload the gel) in comparison to wt-TfR whereas expression of the R100 mutants did not significantly alter the total amount of released sTfR. The V108G and R109G mu-

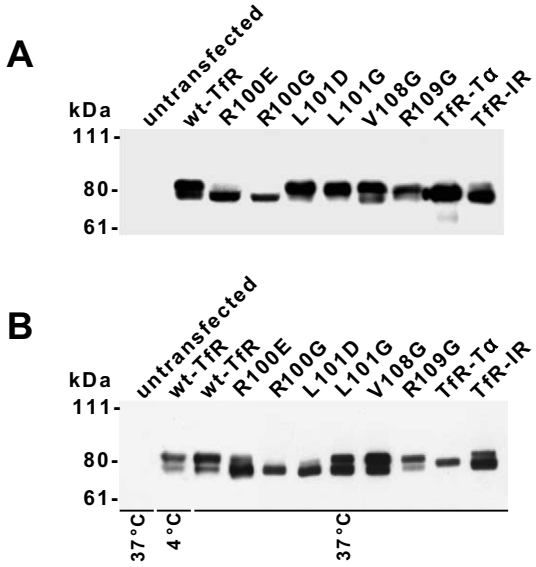

Fig. 1. Anti-TfR Western blots showing the shedding of different TfR mutants from transfected cells and cell membranes. A: sTfR was precipitated from the supernatant of transfected TRVb cells by ferri-transferrin–Sepharose. Lane 1 shows sTfR from untransfected cells, lane 2 from wt-TfR, all other lanes from the mutants indicated. B: sTfR from the supernatant of incubated TRVb membranes. Lane 1 shows sTfR from untransfected cells, lanes 2 and 3 from wt-TfR incubated either at 4 or 37°C, all other lanes from the mutants indicated (incubation at 37°C).

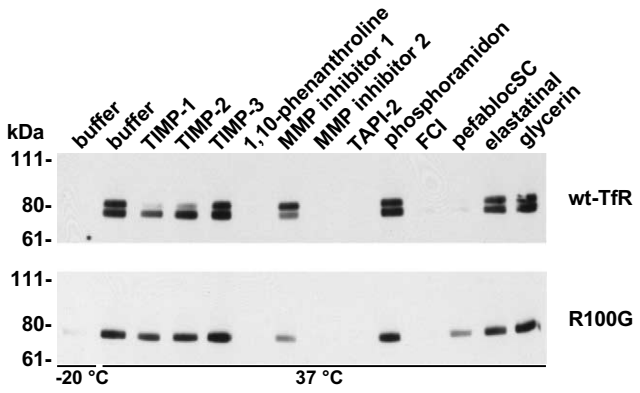

Fig. 2. Anti-TfR Western blots showing the inhibition of sTfR release from cell membranes containing either wt-TfR (upper panel) or the R100G mutant (lower panel). sTfR was detected in the supernatant of TRVb membranes. Lanes 1 and 2 show the sTfR after incubation at −20°C and 37°C without inhibitor, all other lanes after incubation at 37°C with the inhibitors indicated (concentrations, see Section 2.6). Lane 14 served as a control since TIMP-3 is delivered in 50% glycerin.

tations at the alternative TfR-cleavage site processed by neutrophil elastase neither altered the ratio between major and alternative sTfR nor the total amount of cleavage, even though in some preparations the amount of the alternative product appeared to be slightly reduced (Fig. 1A, lanes 7 and 8). TfR-Tα was shed to a high degree from the cells (not recognizable for the same reason stated above) and a single product of a molecular mass between that of the major TfR and the alternative TfR was released (Fig. 1A, lane 9). Since it is unclear whether the TNF-α-stalk is *O*-glycosylated in the present context the apparent molecular mass did not allow a direct comparison to the cleavage site of wt-TfR. In contrast to TfR-Tα, two shedding products were released from TfR-IR exhibiting the same molecular mass as observed for wt-TfR (Fig. 1A, lane 10). The total amount of sTfR released was similar to that of wt-TfR; however, the ratio was shifted to the lower molecular mass product.

Studies performed recently with membrane preparations of HL-60 cells revealed that the different sTfR products and their amounts released are identical to those of whole cells [23]. Advantageously, for inhibitor studies (see Sections 3.2 and 3.3) the membrane assay is not affected by the cytotoxicity of inhibitors and guarantees an increased accessibility to the enzyme. Therefore, we compared also for transfected TRVb cells the results obtained for whole cells with those from the membrane assay (method, see Section 2.5). Fig. 1B shows that the shedding of wt-TfR and of R100E, R100G, V108G, R109G, TfR-Tα and TfR-IR is identical to that of living cells. In contrast, the results for the L101 mutants differ from those of living cells. L101D almost only released the alternative product in the membrane assay and L101G released both products (see Section 4).

3.2. Inhibitor pattern for the shedding of TfR point mutants

To further characterize sTfR release from membranes of transfected TRVb cells we tested several protease inhibitors using wt-TfR (cleaved predominantly at the major site) and the R100G mutant (cleaved exclusively at the alternative site). Since in U937 cells neutrophil elastase is responsible for alternative TfR-cleavage [14] we first tested elastatinal; however, it had neither an effect on the major nor the alternative sTfR product (Fig. 2, lane 13). Unlike elastatinal the general metalloprotease inhibitor 1,10-phenanthroline blocked the release of both shedding products (Fig. 2, lane 6). Testing more specific inhibitors for MMPs and several ADAMs we showed that both shedding processes are inhibited by MMP inhibitor 2, TAPI-2 and TIMP-1 (by the latter only weakly for the alternative product) but not by TIMP-3 (Fig. 2). Interestingly, TIMP-2 weakly inhibited only the release of the major sTfR whereas MMP inhibitor 1 solely reduced the release of the alternative product. The observation that the furin inhibitor FCI also blocked sTfR release supports the assumption that ADAMs are involved in TfR shedding since ADAMs are activated by furin or furin-like proteases. The observed inhibition by high concentrations of pefablocSC can be attributed to the same mechanism [28]. Low concentrations of pefablocSC had no effect (data not shown). The results were confirmed when the sTfR bands from the R100G mutant treated with various inhibitors were compared with the corresponding lower band from the wt-TfR.

3.3. Inhibitor pattern for the shedding of TfR mutants with completely altered stalk

The release of the lower molecular mass sTfR is predominant in TfR-IR in the absence of inhibitor, thus the relative intensity compared to the untreated control (Fig. 3, lane 2) must be used for the comparison with wt-TfR and not the absolute. Taking this into consideration the general inhibitor pattern for the shedding of TfR-IR is very similar to wt-TfR (Fig. 3, upper panel). TIMP-1 and TIMP-2 fully blocked the cleavage of the major sTfR in TfR-IR; this may be due to the already reduced levels. In contrast, TIMP-1, TIMP-2 and MMP inhibitor 1 had no effect on TfR-Tα ectodomain release. Thus, the protease acting on TfR-Tα differs from those generating the major and alternative sTfR from wt-TfR.

3.4. Localization of the shedding process

To determine the localization of the observed shedding processes the surface proteins of the transfected CHO cells were biotinylated and after 24 h TfR was precipitated from cell lysates and cell culture supernatants. Supernatant from non-biotinylated cells served as control. Biotinylated sTfR was detected in the culture medium of all mutants examined (Fig. 4). These results supply evidence that wt-TfR and all mutants have to be directed to the cell surface before the

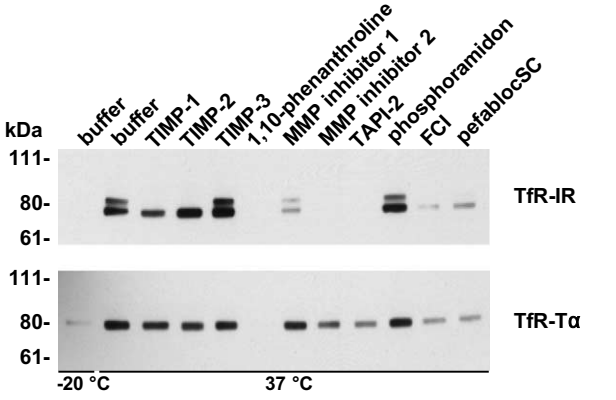

Fig. 3. Same as in Fig. 2 but cell membranes containing either TfR-IR (upper panel) or TfR-Tα (lower panel) were used.

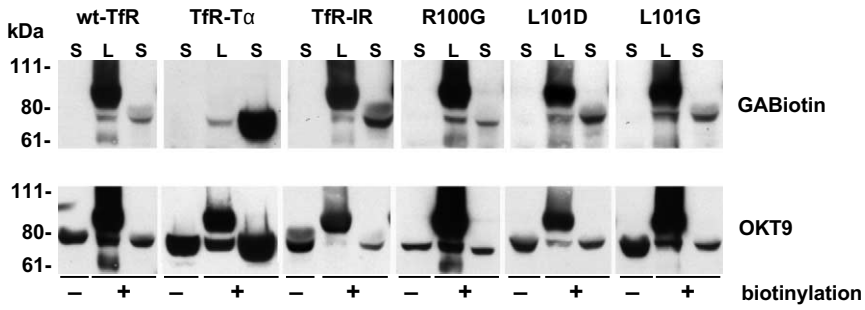

Fig. 4. Western blots of cell surface-biotinylated TfR. TfR was precipitated by ferri-transferrin–Sepharose from cell lysate (L) and cell supernatant (S) 24 h after biotinylation. Supernatant from non-biotinylated cells served as control. The blot was detected with anti-biotin antibody (GABiotin) (upper panel) and after stripping probed with OKT9 (lower panel).

release of sTfR can occur. It is conspicuous that the biotinylated TfR-Tα is completely converted to the soluble form within 24 h. The corresponding band in the OKT9 blot shows newly synthesized TfR or TfR that was in the recycling pathway during biotinylation. Thus the turnover of TfR-Tα is strongly enhanced in comparison to the wt-TfR and the other mutants.

4. Discussion

For the majority of proteins released by shedding, neither the protease(s) responsible nor the mechanism of regulation has been identified yet [2]. To contribute to a better comprehension of the complex network of shedding proteases we have, in the present study, systematically mutated the cleavage site of a type II transmembrane protein underlying shedding, the human TfR.

In transfected TRVb cells release of the major and alternative sTfR can be blocked by general inhibitors of metalloproteases but not by elastatinal. This suggests that neutrophil elastase that is responsible for TfR-cleavage at an alternative site in U937 cells [14] is not involved in the release of sTfR from TRVb cells. This is probably due to large quantities of neutrophil elastase in U937 cells [29] as compared to CHO cells. Although the inhibitor pattern for major and alternative cleavage was very similar, TIMP-2 inhibited cleavage only at the major site and MMP inhibitor 1 solely at the alternative site, indicating that two distinct metalloproteases are involved. The inhibition by TIMP-1, TIMP-2, TAPI-2 and FCI in the present work supports the suggestion that a member of the ADAMs is responsible for TfR cleavage at the major site in HL-60 cells [23].

Mutations in the TfR around the major cleavage site shifted the product ratio from the major to the alternative sTfR. This shows that the amino acids Arg-Leu in the P1 and P1′ position are important for correct processing and that single point mutations can markedly affect shedding. This is in accordance with the observation that a point mutation in the juxtamembrane stalk of human angiotensin I-converting enzyme invoked the action of a distinct secretase [30]. Moreover, even different stimuli can also contribute to the activation of distinct shedding activities [31]. For the TfR, the two disulfide bridges within the stalk may also play a role in receptor shedding.

The observation that L101 mutations lead to intensive shedding in cells but not in the membrane assay may be ascribed to a different accessibility of these mutants for the involved shedding protease(s). This is not due to a different subcellular localization, since all mutants show biotinylated sTfR in the cell culture supernatant. Thus, the TfR has to appear at the cell surface before it is subject to shedding, indicating that the biosynthetic pathway, which has been observed to be the location of cleavage for another TfR mutant [24], can be excluded for the main and the alternative TfR-shedding protease.

The exchange of the TfR-stalk for the corresponding sequences from proTNF-α and IL-6R showed that cleavage of the TfR does not depend on a unique structure of the stalk since both mutants are subject to shedding. This is supported by the low sequence homology in the stalk region between the TfR of different species [19]. Whereas TfR-IR revealed a very similar cleavage and inhibitor pattern as compared to wt-TfR, TfR-Tα substantially differed from both. This is in so far surprising as proTNF-α is, as TfR, a type II transmembrane protein whereas the IL-6R is type I. However, previous studies have shown that replacement of the stalk in TNF-α by the IL-6R-stalk in either orientation leads to resistance to shedding, whereas IL-6R-mutants containing the TNF-α-stalk (in either orientation) are subject to shedding [15], indicating that the orientation is not the crucial point.

A set of mutations within the stalk of L-selectin did not reveal any recognizable systematic dependence between primary structure and cleavability [32]. Insertion of the L-selectin stalk into an unrelated unshed protein (B7.2) led to constitutive non-inducible shedding and insertion of both the L-selectin stalk and EGF domain led to constitutive and phorbol ester-inducible shedding [17]. Remarkably, interchange of the juxtamembrane sequences of two proteins that are not subject to shedding led to a construct exhibiting phorbol ester-inducible cleavage [16]. In summary, all results show that it is predominantly the overall structure that appears to determine where the protein is cleaved and how cleavage is regulated.

In the present study, we show that suppression of TfR cleavage evoked by mutations exclusively pertaining to the TfR-stalk can be compensated by alternative shedding. In the case of these mutations, inhibitor studies reveal that at least three different metalloproteases are involved in the shedding process.

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