

# Shedding of the Transferrin Receptor Is Mediated Constitutively by an Integral Membrane Metalloprotease Sensitive to Tumor Necrosis Factor $\alpha$ Protease Inhibitor-2\*

Received for publication, April 10, 2002, and in revised form, August 1, 2002  
Published, JBC Papers in Press, August 5, 2002, DOI 10.1074/jbc.M203461200

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**The transferrin receptor (TfR) is a transmembrane protein that mediates cellular uptake of iron. Although the serum concentration of the soluble TfR (sTfR) is altered in several diseases and used for diagnostic purposes, the identity and regulation of the shedding protease is unknown. In this study we quantified sTfR release from microsomal membranes and leukocytic cell lines in the presence of numerous protease inhibitors and cell activating compounds. We show that sTfR release is mediated by an integral membrane metalloprotease and can be inhibited by matrix metalloproteinase inhibitor 2 and tumor necrosis factor  $\alpha$  protease inhibitor-2 (TAPI-2). Cleavage is also inhibited by a specific furin inhibitor, indicating that the protease is activated by a furin-like proprotein convertase. Whereas stimulation of the cells by the ectodomain shedding activator phorbol 12-N-myristate 13-acetate did not alter sTfR release significantly, the phosphatase inhibitor pervanadate led to an increase of TfR shedding in several leukocytic cell lines. Our results suggest that TfR shedding is constitutively mediated by a member of the metalloprotease family known as ADAM (for a disintegrin and metalloprotease).**

The transferrin receptor (TfR)<sup>1</sup> is a type II transmembrane protein that mediates uptake of iron by binding the iron carrier protein transferrin (Tf). Following internalization of the com-

plex, iron is released in the acidic endosomes and the TfR-Tf complex recycles back to the cell surface where apotransferrin is released at neutral pH. The TfR is composed of two homologous peptide chains of 760 amino acids linked by two disulfide bonds (Cys-89 and Cys-98) close to the transmembrane domain. Each polypeptide is divided into a large C-terminal extracellular domain of 672 amino acids, a transmembrane domain of 21 amino acids, and an N-terminal cytoplasmic domain of 67 amino acids. The extracellular domain that binds Tf is kept by a juxtamembrane stalk at 2.9 nm from the plasma membrane (1). Within the stalk Thr-104 is O-glycosylated (2, 3), but detailed structure and function of the stalk remain unknown.

Although the appearance of the soluble form of the transferrin receptor (sTfR) in human serum has been known for quite a long time (4) and its concentration is accepted as diagnostic marker for erythropoietic activity (5, 6), little is known about the molecular basis of the shedding process, in particular the nature of the shedding protease involved. In diseases accompanied by enhanced erythropoiesis or TfR expression, like iron deficiency anemia, increased serum sTfR levels are observed (7–10), whereas lower levels of serum sTfR were determined in patients with aplastic anemia (8). The sTfR level remains unchanged in anemia arising from chronic disease, so that it is a reliable marker to distinguish iron deficiency anemia from the anemia of chronic inflammation and liver disease (11). It was shown that a human red blood cell fraction does not have an own TfR-specific proteolytic activity, whereas a white blood cell fraction does have (12). The latter was also observed in human primary granulocytes, activated mononuclear blood cells, HL-60 cells, and K562 cells (13–16).

A mixture of protease inhibitors consisting of leupeptin, pepstatin, EDTA, and PMSF was used to block the release of a TfR fragment from HL-60 membranes (15). Cleavage of purified TfR by HL-60 plasma membrane fractions could be inhibited by the serine protease inhibitors PMSF and diisopropyl fluorophosphate at concentrations of 1 mM but not with inhibitors of other classes of proteases. Furthermore, four matrix metalloproteinases (MMP1, MMP2, MMP3, and MMP9) did not cleave TfR (17). Using sheep exosome-bound TfR as substrate for a protease present on human granulocytes, release of a TfR fragment was inhibited by 1 mM PMSF (13). However, the actual cleavage site was not determined.

The N terminus of sTfR isolated from human serum starts with Leu-101 revealing a cleavage site C-terminal of Arg-100 in the stalk region 11 amino acids distal to the plasma membrane (18). 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 (19). A further cleavage site at Gly-91 was reported for two N-terminal

\* This work was supported by Deutsche Forschungsgemeinschaft Grants FU 408/1-1 and FU 408/1-3 and by a Fonds der Chemischen Industrie grant (to K. D.). The work at the Institute of Biochemistry (C. W., Neurochemistry Department, Prof. F. Hucho) was supported by the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: TfR, transferrin receptor; ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; E64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; EGF, epidermal growth factor; FCI, furin convertase inhibitor (Alexis Biochemicals); GPI, glycosylphosphatidylinositol; mAb, monoclonal antibody; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; PBSB, PBS + Brij 58; PBST, PBS + Tween 20; 8-POE, octylpolyoxyethylene; PMA, phorbol 12-N-myristate 13-acetate; sTfR, soluble transferrin receptor; TAPI-2, tumor necrosis factor  $\alpha$  protease inhibitor-2; Tf, transferrin; TIMP, tissue inhibitor of metalloproteinases; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PKC, protein kinase C; TACE, tumor necrosis factor  $\alpha$ -converting enzyme; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; mTfR, membrane fragment of transferrin receptor; PMSF, phenylmethylsulfonyl fluoride.

truncated TfRs lacking either the entire cytoplasmic domain or the proximal 31 amino acids of the transmembrane domain. Pulse-chase analysis revealed that this cleavage occurs during the biosynthetic pathway (20). In contrast, the wild-type TfR that was cleaved at Arg-100, when transfected in Chinese hamster ovary cells, was shown to be processed during cycling through an endosomal compartment, although cleavage was not dependent on acidification of the endosomes (21).

Elimination of the *O*-linked carbohydrate at Thr-104 may enhance the susceptibility of TfR to cleavage (22). Nevertheless, purified sTfR from human serum was sensitive to *O*-glycanase and could bind to jacalin lectin, indicating that the sTfR contains an *O*-linked oligosaccharide (3). This suggests that the glycan at Thr-104 does not modulate the shedding process of TfR *in vivo*.

Several members of the metalloprotease family known as ADAM (for a disintegrin and metalloprotease), including tumor necrosis factor  $\alpha$  (TNF $\alpha$ )-converting enzyme (TACE or ADAM-17), ADAM-9, ADAM-10 (KUZ), and ADAM-19, play an important role in ectodomain shedding (reviewed in Ref. 23). The ADAM proteins fall within the metzincin superfamily that is characterized by an extended zinc binding consensus sequence (HEXXHXXGXXH) and a common tight Met-containing turn and includes the matrix metalloproteinases (MMPs). Besides these structural similarities, the proteolytic activities of most MMPs and several ADAMs could be inhibited by hydroxamate-based inhibitors and four endogenous tissue inhibitors of metalloproteinases (TIMPs). However, the TIMP inhibitor profile of ADAMs is different from that of MMPs (25–27). TACE was originally found to release TNF $\alpha$  (28, 29), 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  $\alpha$  (Ref. 30)). Like TACE, ADAM-10 releases TNF $\alpha$  (31), participates in the nonamyloidogenic pathway of APP processing to its soluble form (32), and is required for Notch signaling (33). ADAM-9 is responsible for the shedding of heparin-binding epidermal growth factor (EGF)-like growth factor (34) and ADAM-19 for the release of neuregulin- $\beta$ 1, a member of the EGF family (35). Besides the ADAMs, other types of proteases are involved in shedding processes. Thus, the matrix metalloproteinase stromelysin-1 (MMP-3) releases active heparin-binding EGF-like growth factor (36) and matrilysin (MMP-7) generates active soluble Fas ligand (37) and is required for TNF $\alpha$  release from macrophages (38). In addition, the membrane type 4 matrix metalloproteinase (MMP-17) and the serine protease proteinase-3 may participate in TNF $\alpha$  processing (39, 40).

All these data show that one type of protein can be processed by different proteases or, *vice versa*, one protease can process several different proteins. Recently the idea emerged that regulating steps may determine which protease processes the target protein. In general it can be differentiated into (i) regulated shedding mediated by activating protein kinase C (PKC) by phorbol esters like phorbol 12-*N*-myristate 13-acetate (PMA) and (ii) constitutive shedding, which is present at a basal level. The stimulation of PKC by treating cells with PMA leads to a reduction of cell surface expression of L-selectin and p75 TNF receptor only from wild-type cells but not TACE inactive cells (30). The PMA-induced shedding of interleukin-6 receptor is also strongly reduced in TACE-deficient fibroblasts, whereas a basal release could still be detected (41). In addition disruption of the TACE gene also abolishes regulated  $\alpha$ -cleavage of APP in cultured cells, whereas the basal secretion of soluble APP is unaffected in cells derived from TACE knockout mice (42). Thus, TACE appears to play a central role in regulating shedding processes in most cases, although it was reported that

constitutive  $\alpha$ -secretase cleavage could also be increased in cells transfected with TACE (43). In contrast to TACE, ADAM-10 is reported to be involved in the constitutive shedding of APP (32, 44), whereas overexpression of ADAM-10 enhanced PKC-stimulated  $\alpha$ -secretase cleavage (32).

In the present study we developed a cell-free assay to detect the release of sTfR from membrane-bound TfR. Membranes of the shedding active cell line HL-60 were isolated, and following incubation at 37 °C a TfR fragment of ~80 kDa could be purified that lacked the cytoplasmic and transmembrane domain. N-terminal sequencing of the fragment revealed that the TfR is cleaved C-terminal of Arg-100; thus, this fragment is identical to the major sTfR found in human serum. We could show that the TfR-shedding protease is an integral membrane metalloprotease that can be inhibited by specific metalloprotease inhibitors. In addition to inhibiting release of sTfR from HL-60 membrane preparations, the inhibitors also effectively diminish constitutive TfR shedding from HL-60 cells.

#### EXPERIMENTAL PROCEDURES

**Reagents and Antibodies**—Mouse mAb OKT9 directed against the extracellular domain of TfR was prepared from a hybridoma cell line as previously described (45), and mouse mAb H68.4 directed against the intracellular domain of TfR was purchased from Zymed Laboratories (San Francisco, CA). Polyclonal rabbit antibody pAB063 was generated by immunization with purified human placental TfR (in cooperation with R. Geßner, Charité, Berlin, Germany). Horseradish peroxidase-labeled anti-mouse and anti-rabbit antibodies were obtained from Dako A/S (Glostrup, Denmark). MMP inhibitor 1, MMP inhibitor 2, TAPI-2, and recombinant human TIMP-3 were purchased from Calbiochem (Schwalbach, Germany). Furin convertase inhibitor (FCI; decanoyl-Arg-Val-Lys-Arg-chloromethylketone), human TIMP-1 (isolated from stimulated neutrophils), and human TIMP-2 (isolated from rheumatoid synovial fibroblasts) were obtained from Alexis Biochemicals (Grünberg, Germany). PefablocSC and *n*-octylglucoside were from Roche Molecular Biochemicals (Mannheim, Germany), and octylpolyoxyethylene (8-POE) was from Bachem Biochemica GmbH (Heidelberg, Germany). Other inhibitors and reagents were purchased from Sigma. Pervanadate was generated immediately before use by mixing equivalent volumes of 2 M sodium vanadate (Sigma) and 1 M hydrogen peroxide (Sigma) to obtain 0.5 M pervanadate. Sodium vanadate was used in excess to completely eliminate hydrogen peroxide, because remaining hydrogen peroxide in the pervanadate solution turned out to be toxic to the leukocytic cells.

**Cell Culture**—HL-60, Jurkat, and U937 cells were cultured in RPMI 1640 medium supplemented with 5% (v/v) fetal calf serum in the presence of 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 1 $\times$  Glutamax. The cell cultures were maintained at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere.

**Isolation of the Soluble Transferrin Receptor from HL-60 Culture Medium**—The cell culture (3500 ml) was centrifuged (210  $\times$  g, 4 °C, 15 min), and the supernatant was passed through a ferri-transferrin-Sepharose affinity column and eluted under nondenaturing conditions with 2 M KCl, 1% 8-POE in PBS (150 mM NaCl, 10 mM phosphate, pH 7.5), as described recently (46). TfR was detected by Western blotting using OKT9 mAb directed against the C-terminal extracellular domain of the TfR. sTfR-containing fractions were dialyzed against 1 mM phosphate, pH 7.4, 15 mM NaCl and concentrated ~10 times by lyophilization. For amino acid sequencing, proteins were transferred onto a polyvinylidene difluoride sequencing membrane (Millipore, Bedford, MA) with blotting buffer A (50 mM boric acid/NaOH, pH 9.0, 10% (v/v) methanol) for 1 h at a constant voltage of 50 V according to the method of Xu and Shively (47). The band of interest was cut out and sequenced by Edman degradation either commercially (Peptide Specialty Laboratories GmbH, Heidelberg, Germany) or by using an Applied Biosystems type 473A automated protein sequencer.

**Preparation of Membrane Fractions**—A 300-ml cell suspension of actively growing HL-60 cells (~1  $\times$  10<sup>6</sup> cells/ml) was centrifuged at 130  $\times$  g for 15 min and washed once with 30 ml of Dulbecco's PBS (Invitrogen GmbH, Karlsruhe, Germany). All following steps were carried out at 4 °C. The pelleted cells were resuspended in 10 ml of Dulbecco's PBS, homogenized by douncing 15 times in a Dounce homogenizer, and differentially centrifuged at 500  $\times$  g for 15 min, followed by 2600  $\times$  g for 15 min and finally at 20,000  $\times$  g for 30 min. The membrane pellet was washed once in 5 ml of PBS and again centrifuged at

20,000 × *g* for 30 min. The microsomal membrane pellet was resuspended in PBS to a final concentration of 6 mg/ml protein.

**Digestion of Membrane-bound Transferrin Receptor**—In order to detect membrane-associated TfR cleaving activity, 50 μl of HL-60 membrane fraction was incubated for 18 h at 4 °C or 37 °C. The samples were applied to a 12% SDS-polyacrylamide gel under either reducing or nonreducing conditions, and TfR fragments were detected by immunostaining with OKT9 or H68.4 as described below.

**sTfR Release Assay**—HL-60 membrane fractions were incubated in aliquots of 50 μl for 18 h at 4 °C as negative control or 37 °C in the absence or presence of reagents. The samples were centrifuged at 20,800 × *g* for 15 min at 4 °C, the supernatants analyzed by 12% SDS-polyacrylamide gel electrophoresis under nonreducing conditions, and sTfR detected by immunostaining with OKT9 as described below. For quantitation of the chemiluminescence signals, membranes were scanned with a FujiFilm LAS-1000 system and analyzed with the Image Gauge version 3.2 software. pH-dependent release was determined by resuspending the HL-60 membranes in 50 mM BisTris buffer, 150 mM NaCl (pH 6.0–7.5) or 50 mM Tris buffer, 150 mM NaCl (pH 8.0–9.5).

**Western Blot Analysis**—Samples were prepared according to Laemmli (48) by boiling for 5 min in sample buffer containing 2% SDS with or without 2% 2-mercaptoethanol and separated by electrophoresis. For immunostaining, electrotransfer of proteins to a nitrocellulose membrane, pore size 0.2 μm (Sartorius AG, Göttingen, Germany), was performed with blotting buffer B (25 mM Tris, 192 mM glycine, 10% (v/v) ethanol). After blotting the membrane was blocked with Dulbecco's PBS containing 5% dry skim milk. TfR was detected either with OKT9 (6 μg/ml) or H68.4 (0.5 μg/ml) in Dulbecco's PBS containing 0.2% Brij 58 (PBSB) and 1% dry skim milk and a subsequent incubation with peroxidase-conjugated polyclonal rabbit anti-mouse antibody (60 μg/ml). Each antibody incubation step was followed by intensive washes with PBSB. Secondary antibody detection was carried out by the enhanced chemiluminescence system from PerkinElmer Life Sciences (Boston, MA).

**sTfR-ELISA**—Using a 96-well microplate, each well was coated with 100 μl of anti-TfR mAb OKT9 (2 μg/ml in Dulbecco's PBS) for 90 min and subsequently blocked with 175 μl of 10% (v/v) fetal calf serum, 3% (w/v) bovine serum albumin in PBS for 30 min. All incubation steps were carried out under gentle agitation at room temperature. Cell culture supernatant was centrifuged (20,800 × *g*, 4 °C, 20 min) to remove cellular debris and 100-μl aliquots pipetted in triplicate and incubated for 2.5 h. The plate was washed with PBS containing 0.05% (v/v) Tween 20 (PBST) and bound TfR detected with 100 μl of anti-TfR polyclonal antibody pAB063 (1:2000 in PBST) and 100 μl of peroxidase-labeled swine anti-rabbit IgG (1:2000 in PBST). The purification of placental TfR, which served as internal standards for the quantitation of sTfR, and colorimetric reaction were performed as previously described (46).

**Protein Determination**—Protein concentrations were determined in duplicate in microplates using the BCA protein assay (no. 23225, Pierce) and the appropriate microscale protocol of the manufacturer.

## RESULTS

**HL-60 Cells Release sTfR Cleaved at Arg-100**—Because the promyelocytic cell line HL-60 release relatively high amounts of sTfR (19), we chose this cell line for further investigations. First we isolated sTfR from the culture cell medium by ferritin-Sepharose affinity chromatography and analyzed the site of cleavage by N-terminal sequencing. The N terminus of the sequenced sTfR was constituted by LAG(T)ES, revealing the physiological cleavage site at Arg-100 as described by Shih *et al.* (18). Because Thr-104 is reported to be modified by O-glycosylation (2, 3), only a weak signal was detected for this amino acid.

**Detection of TfR fragments**—HL-60 cells were washed once in PBS and subsequently lysed in PBS containing 1% Triton X-100. The lysate was separated by 10% SDS-PAGE under nonreducing conditions and TfR detected by Western blotting using OKT9 mAb directed against the extracellular domain or H68.4 mAb directed against the intracellular domain of the TfR (Fig. 1A). To determine the TfR shedding activity in membranes of HL-60 cells, membrane preparations were incubated at 4 or 37 °C for 18 h and separated by 12% SDS-PAGE under

either nonreducing or reducing conditions. TfR was detected by Western blotting using OKT9 or H68.4 (Fig. 1B). Altogether six TfR fragments were detected and identified by size and immunological characteristics as follows: ~190-kDa dimer of TfR (TfR·TfR), ~110-kDa dimer of TfR lacking one extracellular domain (TfR·mTfR), ~90-kDa monomeric TfR (TfR), ~80-kDa soluble monomeric TfR (sTfR), ~25-kDa dimer of TfR lacking both extracellular domains (mTfR·mTfR), and ~13-kDa monomeric membrane fragment (mTfR). The TfR fragments are schematically presented in Fig. 1E. The sTfR was not detected by H68.4, indicating that it is N-terminally truncated, whereas the fragments that only consist of small membrane fragments were not detectable by OKT9, revealing that they are C-terminally truncated. The ratios of the detected fragments varied from preparation to preparation. Nevertheless, in most of the blots derived from nonreducing SDS-PAGE, the amount of TfR dimer exceeded the amounts of monomeric TfR and TfR·mTfR. Furthermore, in all cases the levels of fragments containing full-length TfR (TfR·TfR, TfR·mTfR, and TfR) decreased after incubation at 37 °C, whereas the levels of cleaved fragments (sTfR, mTfR·mTfR, and mTfR) increased. Several wash steps of the membrane with detergent-free buffer did not reduce the sTfR releasing activity, indicating that the TfR-shedding protease is tightly bound to the membrane. Just below the sTfR we detected a faint band that coappears in some cases with the sTfR, but in much lower concentrations (see also Figs. 1D, 2A, 5B, and 6D). It remains unclear under which conditions this alternative sTfR is generated, but in general it appears in a linear relationship to the major sTfR. The molecular weight of the alternative sTfR corresponds to the molecular weight of the product of a TfR-cleaving protease recently identified in our lab as neutrophil elastase (19).

**sTfR Release Assay**—After pelleting the incubated membranes at 20,800 × *g*, the supernatant was separated by nonreducing SDS-PAGE and analyzed by Western blotting; OKT9 solely detected the sTfR (Fig. 1C), whereas H68.4 did not detect any TfR fragment in the supernatant (data not shown). The apparent molecular mass of the sTfR did not change under nonreducing conditions, revealing the lack of intermonomeric disulfide bonds (Fig. 1B). This provides evidence that the sTfR lacks the transmembrane domain and that the TfR is cleaved within the stalk region C-terminal of Cys-98. The sTfR-releasing protease is active at 4 °C but the level of cleaved product increased ~5-fold at 37 °C. To confirm the exact site of cleavage, sTfR was purified by affinity chromatography on ferritin-Sepharose. N-terminal sequencing revealed that the fragment released from membranes is indeed generated by cleavage of the TfR C-terminal of Arg-100, as determined for sTfR from the culture medium of HL-60 cells. Because the release of sTfR from isolated HL-60 membranes represents a cell-free system similar to that in living cells, the quantitation of sTfR using OKT9 is a valuable tool for examining the shedding process of TfR. Moreover, the cell-free assay provides a few advantages over a whole cell system. First, it enables the examination of the sTfR-shedding protease without effecting synthesis and intracellular trafficking of TfR so that an observed inhibition can be traced back to a direct inhibition of the sTfR shedding activity. Second, the enzyme system involved is more accessible to inhibitors in the sTfR release assay than in whole cell systems. Finally, the isolated HL-60 membranes may represent a suitable starting material for purifying the TfR-shedding protease. Solubilization of the protease is an absolute requirement for purification; however, solubilization of the membranes with various detergents (Triton X-100, CHAPS, Brij 58, 8-POE, or *n*-octylglucoside) almost completely destroyed the sTfR releasing activity (Fig. 1D). Interestingly

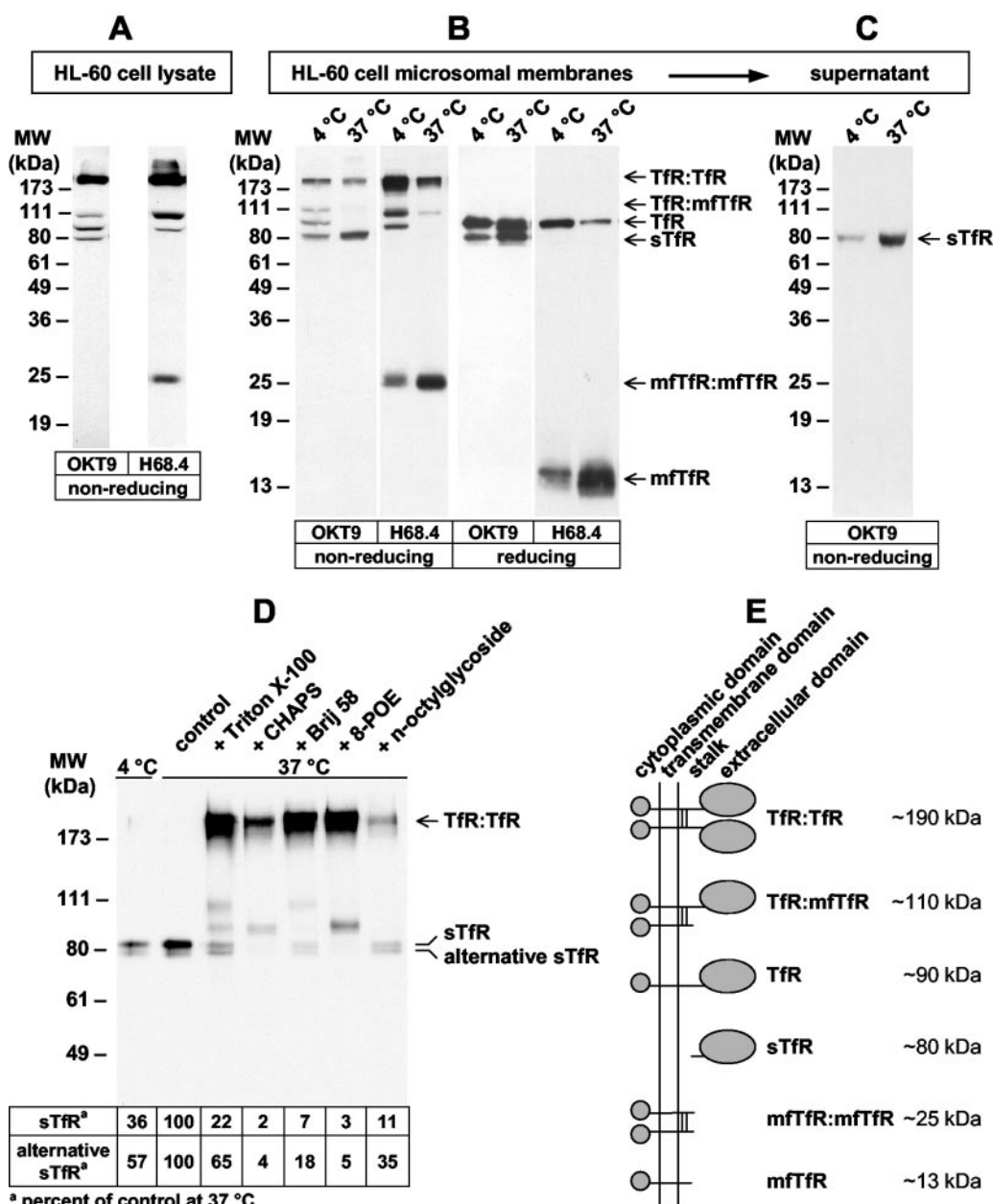
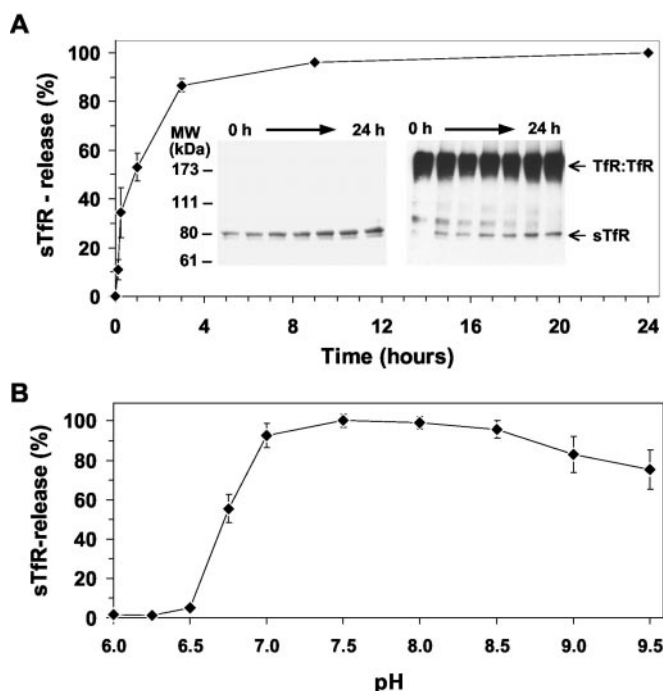


FIG. 1. Six TfR fragments were detected in HL-60 membranes. **A**, HL-60 cell lysate was separated by 10% SDS-PAGE under nonreducing conditions and analyzed by Western blotting with anti-TfR mAb directed against the extracellular domain (OKT9) or intracellular domain (H68.4). **B**, a membrane fraction from HL-60 cells was incubated for 18 h at 4 or 37 °C, separated by 12% SDS-PAGE under either nonreducing or reducing conditions, and analyzed by Western blotting with OKT9 or H68.4 mAb. The proposed fragment compositions are indicated. **C**, incubated HL-60 membranes were centrifuged at  $20,800 \times g$ , and the supernatant separated by 12% SDS-PAGE under nonreducing conditions and analyzed by Western blotting with OKT9. Only the sTfR could be detected, indicating the lack of the transmembrane domain. **D**, HL-60 membrane fractions were solubilized with indicated detergents and incubated and analyzed as described above, but using a 7.5% SDS-PAGE. The table shows the relative amounts of sTfR and alternative sTfR quantified by chemiluminescence imaging on a FujiFilm LAS-1000 system. **E**, schematic presentation of the six TfR fragments detected in HL-60 cell membranes. Dimers are linked by two disulfide bonds in the stalk region. MW, molecular weight.

the appearance of the alternative sTfR is not affected as much by solubilization with Triton X-100, Brij 58, and *n*-octylglycoside as compared with the major sTfR. This suggests that the protease that processes the alternative sTfR may not require membrane localization for TfR cleavage.

**Rate of sTfR Release**—Quantitation of the time-dependent release of sTfR from HL-60 membranes showed that, within 1 h, ~50% of the total generated sTfR was released (Fig. 2A). Because the amount of full-length TfR is not significantly altered over a 24-h period at 37 °C (Fig. 2A, right inset), the limited sTfR release is not the result of substrate deprivation but rather of a limited stability of the sTfR releasing activity.

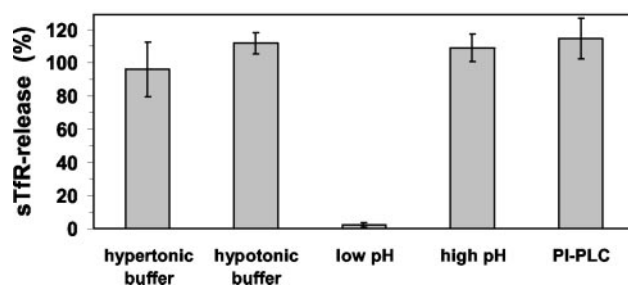
**pH Dependence of sTfR Release**—Incubating the membranes at different pH showed that cleavage activity was completely abolished at less than pH 6.5, increased substantially within the pH range from 6.5 to 7.0, reached a maximum at 7.5 and decreased slightly at more basic pH (Fig. 2B). Maximum activity at neutral pH provides evidence that the shedding process occurs in a neutral cell compartment or at the cell surface and indicates the catalytic type of the protease. Aspartic proteases are generally active at acidic pH, metalloproteases are restricted to neutral or basic pH, and the serine and cysteine proteases are active over a more broad pH range (for a comprehensive review, see Ref. 49).



**FIG. 2. sTfR release assay.** Generation of sTfR (a typical result is shown in Fig. 1C) upon incubation at 37 °C was quantified by chemiluminescence imaging on a FujiFilm LAS-1000 system. Data represent mean values for at least two independent experiments with S.E. A, rate of sTfR release at 37 °C over a 24-h period. A representative result is shown (left inset). Right inset shows a similar experiment without centrifugation of the incubated membranes. Samples were separated by SDS-PAGE under nonreducing conditions and detected by Western blotting using OKT9. B, pH dependence of sTfR release at 37 °C analyzed and quantified as described above.

**Integral Membrane Anchoring**—To determine how the protease is anchored in the membrane, we incubated the membranes under various conditions resulting in the release of either peripheric membrane proteins (hypertonic buffer, hypotonic buffer, low or high pH) or glycosylphosphatidylinositol (GPI)-anchored membrane proteins (by phosphatidylinositol-specific phospholipase C). Only the preincubation of membranes at low pH significantly lowered the sTfR releasing activity of the membranes (Fig. 3). This is, however, the result of the instability of the protease at low pH (Fig. 2B), activity of which cannot be restored at neutral pH, rather than a result of the release of the protease from the membrane. Thus, it can be concluded that the TfR-shedding protease and potential cofactors are integral membrane proteins.

**Inhibition of sTfR Release**—To further determine the nature of the sTfR releasing activity, we tested various protease inhibitors (summarized in Table I). The protease inhibitors specific for aspartic, serine, or cysteine proteases showed no significant inhibitory effect at the concentrations tested, with the exception of the serine protease inhibitor PefablocSC. This will be discussed below in context with inhibition by the furin inhibitor FCI. In contrast, the general metalloprotease inhibitor 1,10-phenanthroline inhibits cleavage dose-dependently (Fig. 4A). Cleavage could also be inhibited by the chelating reagents EDTA and EGTA, but only at concentrations exceeding the concentration of  $\text{Ca}^{2+}$  (0.9 mM) and  $\text{Mg}^{2+}$  (0.5 mM) cations in the reaction buffer. Because binding of divalent cations to chelators may lower the pH of a solution and thus inactivate the sTfR releasing activity, we tested the pH of the incubation buffer and found no significant change. We observed that preincubation of the membranes with 1,10-phenanthroline in the absence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  destroyed the sTfR releasing activity irreversibly, possibly because of its alkaline



**FIG. 3. sTfR releasing activity is membrane-anchored by an integral domain.** HL-60 membrane fractions were preincubated under conditions releasing peripheric membrane proteins (hypertonic buffer (1 M NaCl), hypotonic buffer (0.015 M), pH 3.3 and 9.5 for 30 min at 4 °C) or in the presence of GPI-specific phospholipase C to release GPI-anchored membrane proteins (30 min at 37 °C). Subsequently the membranes were pelleted by centrifugation at  $20,800 \times g$ , washed once, and incubated at 37 °C for 18 h. The sTfR was quantified using the sTfR release assay.

earth metal-chelating ability. The activity, however, remained unaffected in the presence of 0.9 mM  $\text{Ca}^{2+}$  and 0.5 mM  $\text{Mg}^{2+}$ . Addition of  $\text{Zn}^{2+}$  to membranes preincubated with 1 mM 1,10-phenanthroline, in the presence of 0.9 mM  $\text{Ca}^{2+}$  and 0.5 mM  $\text{Mg}^{2+}$ , restored the sTfR releasing activity almost completely, whereas additional  $\text{Ca}^{2+}$  in the same concentrations did not (Fig. 4B). It could be concluded that the inhibition by 1,10-phenanthroline is specific because of its chelating activity toward transition metals. We found that the decrease in sTfR releasing activity in the presence of high  $\text{Zn}^{2+}$  concentrations is caused by an inhibitory effect of transition metals on sTfR release (Fig. 4C). The fact that metalloproteases are inhibited by millimolar concentrations of  $\text{Zn}^{2+}$  has been shown, and the structural features of the inhibition effect were determined for carboxypeptidase A (50).  $\text{Ca}^{2+}$  reduced sTfR release only at high concentrations, whereas  $\text{Mg}^{2+}$  resulted in a slight increase in sTfR release at high concentrations. To further characterize the metalloprotease, we tested more specific metalloprotease inhibitors. An inhibitor developed for the specific inhibition of matrix metalloproteinases (MMP inhibitor 1) showed no significant effect, whereas MMP inhibitor 2 and an inhibitor that blocks shedding of  $\text{TNF}\alpha$  (TAPI-2) specifically decreased sTfR release (Fig. 5, A and B). MMP inhibitor 2 and TAPI-2 are hydroxamic acid-based inhibitors of metalloproteases belonging to the MMP and ADAM families (51, 52). Members of these families of metalloproteases are often activated by furin-type pro-protein convertases; we therefore tested the furin inhibitor FCI (Fig. 5A). This inhibitor decreased the amount of sTfR release, indicating that the sTfR releasing activity is indeed activated by a furin-type pro-protein convertase. The serine protease inhibitor PefablocSC, tested within the same concentration range, reduced sTfR release only at high concentrations (Fig. 5A). This could be a result of the moderate inhibitory effect of serine protease inhibitors on furin (53). The most specific inhibitors tested, the TIMPs, are naturally occurring proteins of ~20–28 kDa, which bind to metalloproteases of the MMP or ADAM families. The inhibitors were tested at concentrations up to 1  $\mu\text{M}$  in the sTfR release assay and 20 nM in HL-60 cell culture. It has been reported that TIMPs in these concentrations inhibit the shedding of TRANCE (54), L-selectin (55), and HER-2 (56). No clear inhibitory effect for TIMP-2 or TIMP-3 could be detected in the sTfR release assay (Table I), or in HL-60 cell culture (data not shown). TIMP-1 showed a moderate inhibitory effect; however, this appeared to be batch-dependent. TIMP-1 and TIMP-2 are supplied in a detergent-containing buffer (0.05% Brij 35), which appeared to enhance sTfR release from HL-60 membranes, when added in low concentrations. Because different

TABLE I  
Inhibition of sTfR releasing activity

	Concentration	Inhibition <sup>a</sup>	Target proteases
		% of control	
Pepstatin A	1 mM	Not detectable <sup>b</sup>	Various aspartatic proteases
E64	85 μM	Not detectable	General inhibitor of cysteine proteases
Antipain	1 mM	Not detectable	Cysteine and to a lesser extent serine proteases
Chymostatin	1 mM	Not detectable	Serine proteases of the chymotrypsin family and lysosomal cysteine proteases
PefablocSC	1 mM	31 ± 4	General inhibitor of serine proteases
Leupeptin	1 mM	Not detectable	Serine proteases of trypsin family
FCI	1 mM	87 ± 2	Furin-type pro-protein convertases
1,10-Phenanthroline	3 mM	94 ± 4	Metalloproteases
EDTA	3 mM	99 ± 0	Metalloproteases
EGTA	3 mM	88 ± 7	Metalloproteases
Phosphoramidon	100 μM	Not detectable	Thermolysin and other bacterial metalloproteases, as well as collagenase
MMP inhibitor 1	1 mM	Not detectable	MMPs (MMP-1, MMP-3, MMP-8, and MMP-9)
MMP inhibitor 2	1 mM	92 ± 4	MMPs (MMP-1, MMP-3, MMP-7, and MMP-9)
TAPI-2	1 mM	87 ± 2	TNFα-shedding proteases
TIMP-1	1 μM	55 ± 3	Various MMPs and ADAMs
TIMP-2	1 μM	15 ± 2	Various MMPs and ADAMs
TIMP-3	1 μM	19 ± 5	Various MMPs and ADAMs

<sup>a</sup> Average values ± S.E. are shown.

<sup>b</sup> Not detectable, no significant inhibition in sTfR release could be observed.

batches of TIMP-1 and TIMP-2 seemed to contain different amounts of Brij 35, the results were not consistent between batches.

**TfR Shedding from HL-60, Jurkat, and U937 Cells**—To assess the level of sTfR released into the cell culture medium, we developed an ELISA specific for TfR (for details see “Experimental Procedures”). The basal sTfR levels measured correspond well to the sTfR detected by immunoprecipitation as reported earlier by our group (19). Moreover, for HL-60 cells we determined the correlation between cell growth and rate of sTfR release into the cell culture medium (Fig. 6A). The rate of sTfR release correlated with the growth rate with a maximal release during the log phase. This indicates that both events are in a close relationship. The common effector of ectodomain shedding, PMA, exhibited no strong effect on TfR shedding in the leukocytic cell lines HL-60 and Jurkat (Fig. 6B). PMA stimulated sTfR release solely in U937 cells, which display a very low basal level of sTfR release, to a level equivalent to the basal level of HL-60 cells. Because PMA induces endocytosis of TfR, one could argue that internalization withdraws the TfR from the protease. PMA also did not reduce TfR shedding; thus, it may be concluded that TfR shedding is mediated independent of PKC-activation as well as of TfR localization in the cell. The phosphatase inhibitor pervanadate was the only reagent we found that was suitable to significantly stimulate TfR shedding. However, this effect was dependent on the cell line. Pervanadate increased TfR shedding severalfold in all cell lines examined, with the greatest effect observed in Jurkat cells (Fig. 6B). The inhibitors shown to be active in the sTfR release assay and exhibiting no effect on cell growth (see “Discussion”), namely TAPI-2, MMP inhibitor 2, FCI, and PefablocSC, were utilized to examine constitutive TfR shedding into the medium by HL-60 cells. sTfR was quantified using the sTfR-ELISA (Fig. 6C). The cells were harvested in the log phase, fresh culture medium added, and grown for 20 h in the absence and presence of the inhibitors (50 μM concentration). In comparison to the untreated cells, the hydroxamic acid-based inhibitors TAPI-2 and MMP inhibitor 2 decreased sTfR release by 40–50%, the furin inhibitor by 60%, whereas the serine protease inhibitor PefablocSC inhibited sTfR release by only 10%. To prove the specificity of the sTfR-ELISA for the major sTfR, we precipitated sTfR from the HL-60 inhibition experiment (Fig. 6C) with ferri-transferrin-Sepharose, separated the precipitate by SDS-PAGE, and detected TfR by Western blotting using OKT9 mAb

(Fig. 6D). We found that the levels of precipitated sTfR were in good agreement with the sTfR levels measured by the sTfR-ELISA. Furthermore, only very small amounts of alternative sTfR were detectable in the HL-60 culture medium, confirming that the alternative sTfR did not significantly contribute to the total amount of released sTfR. Moreover, we compared the gel mobility of sTfR isolated from HL-60 cell culture medium and from supernatant of incubated HL-60 cell membranes by SDS-PAGE (Fig. 6D). We found that sTfR from both assays revealed the same molecular weight, as expected because N-terminal sequencing revealed that they possessed the same N termini.

#### DISCUSSION

In the present study we could show for the first time, in a microsomal membrane fraction of HL-60 cells, the existence of all theoretical TfR fragments that are generated during shedding of TfR. As the question regarding the location of the TfR shedding process is unresolved, the membrane-bound remnants that remain after shedding of at least one extracellular domain are of particular interest. In previous studies a 105-kDa TfR fragment was detected under nonreducing conditions in cell membranes of K562 and HL-60 cells. This fragment was proposed to be consistent with the truncation of one extracellular domain of the transferrin receptor (57). A small TfR fragment, lacking both extracellular domains, could not be observed in the cell membranes, but was detected in the exosomes obtained from cell culture supernatant (57). An 18-kDa peptide was also detected in sheep exosomes but not in plasma membranes of human red blood cells, indicating that the sTfR originating from maturing red blood cells may be cleaved by a leukocyte protease after the release of exosomes containing full-length TfR (13). In contrast, in HL-60 cell membrane preparations, we were able to detect small TfR fragments, levels of which increased after incubation at 37 °C. Furthermore, we could also show the existence of TfR fragments of the same sizes in HL-60 cell lysates, as detected in membrane preparations. This provides strong evidence that TfR shedding occurs at the cell membrane independent of exosome formation.

The 80-kDa TfR fragment could only be detected by an antibody directed against the extracellular domain of TfR, the molecular mass remained unchanged under nonreducing and reducing conditions, and it is the only fragment that could be detected in the supernatant of incubated membranes. All these properties are consistent with the identity of sTfR, which could

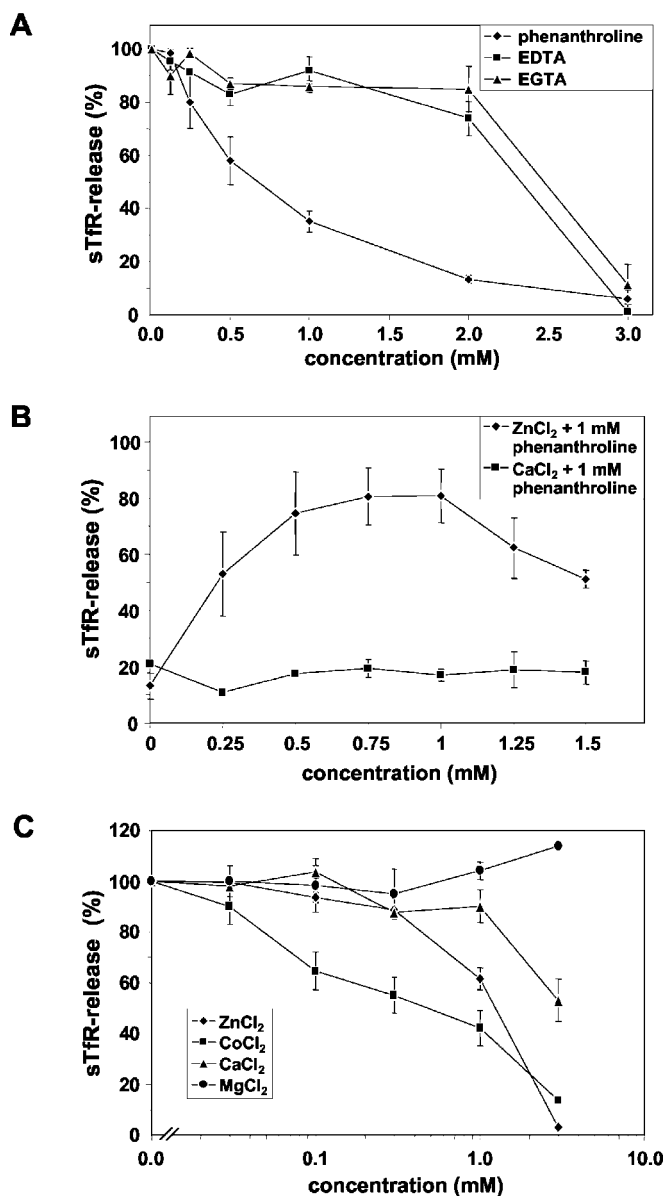


FIG. 4. sTfR releasing activity is inhibited by chelating reagents. Various reagents were added to HL-60 membranes, incubated at 37 °C for 18 h, and cleavage activity analyzed in the sTfR release assay. *A*, 1,10-phenanthroline, EDTA, and EGTA were added in the indicated concentrations. *B*, all samples were preincubated with 1 mM 1,10-phenanthroline for 5 min at 4 °C and ZnCl<sub>2</sub> and CaCl<sub>2</sub> subsequently added in the indicated concentrations. *C*, ZnCl<sub>2</sub>, CoCl<sub>2</sub>, CaCl<sub>2</sub>, and MgCl<sub>2</sub> were added in the indicated concentrations.

be finally proven by N-terminal sequencing. The observation that sTfR derived from HL-60 cell culture, as well as from digested HL-60 membranes, could be purified on Tf-Sepharose illustrates that sTfR retains its Tf binding capacity.

Because sTfR levels increased after incubation at 37 °C, the HL-60 membrane preparations contain a protease that is responsible for TfR shedding. This protease is membrane-bound because membrane fractions washed free of soluble or loosely associated membrane proteins are competent for cleavage. Furthermore, the protease is an integral membrane protein as activity remains membrane-bound under conditions that release peripheral membrane proteins or GPI-anchored membrane proteins. The limited stability of the sTfR releasing activity may be explained by proteolytic degradation or by specific shedding of the sTfR-shedding protease itself, such as described for MT5-MMP (58) or furin (59, 60). This conclusion

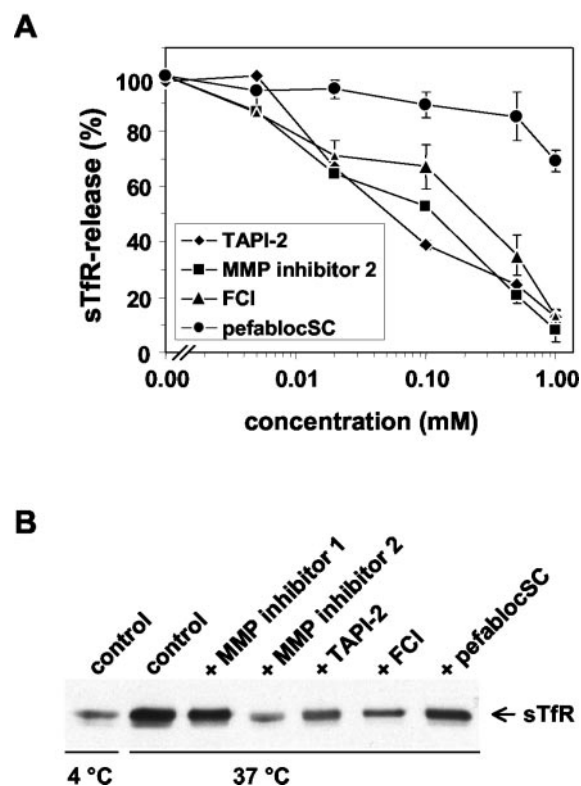
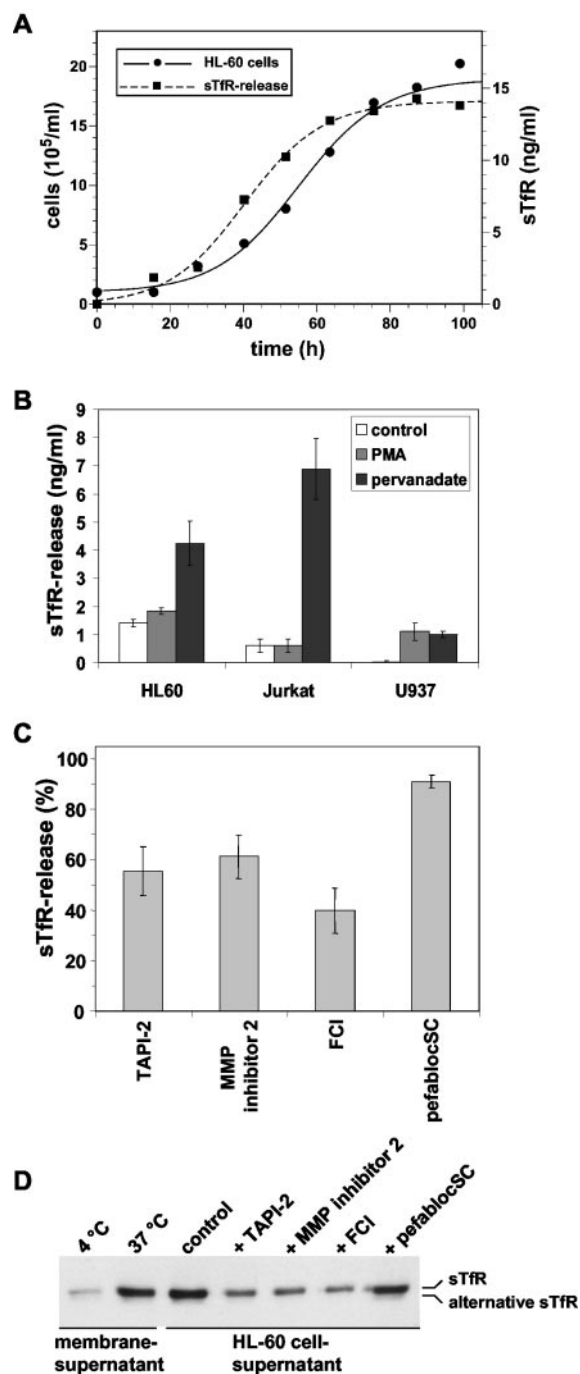


FIG. 5. Inhibition of sTfR releasing activity by specific protease inhibitors. HL-60 membranes were incubated in the presence of four synthetic protease inhibitors and cleavage activity analyzed in the sTfR release assay. *A*, concentration-dependent (0–1 mM range) inhibition of sTfR release. *B*, a representative Western blot showing the effect of 0.1 mM inhibitor.

would imply that the secreted soluble TfR-shedding protease can no longer cleave the membrane-bound TfR. This assumption is supported by our observation that solubilization of the membrane fraction almost completely destroyed sTfR releasing activity, revealing that the TfR-shedding protease and/or its substrate need to be anchored in the membrane. Similarly it has been shown for  $\alpha$ -secretase and angiotensin-converting enzyme secretase that the intact membrane is required to specifically shed APP and angiotensin-converting enzyme (61, 62).

Our studies show that the sTfR releasing activity is caused by a metalloprotease that is membrane-anchored. The sensitivity of the sTfR releasing activity to hydroxamic acid-based inhibitors supplies further evidence for the characterization of the TfR-shedding protease. The hydroxamate group complexes the catalytic zinc and forms two hydrogen bonds to a Glu and an Ala amino acid residue that are located in the catalytic domain. Selectivity can be obtained by varying those groups that fit the S1' and S2' pocket of the target protease. MMP inhibitor 2 has been shown to be a potent inhibitor of MMP-1, MMP-3, MMP-7, and MMP-9 (51), but other related proteases may be inhibited too. TAPI-2 was originally developed to block TNF $\alpha$  release from cells (63). Later it was shown that TAPI-2 is also effective in preventing the cleavage of the 80-kDa TNF $\alpha$  receptor (64), the transmembrane glycoprotein receptor HER2 (56), and the constitutive as well as PMA-activated shedding of transforming growth factor  $\alpha$ , L-selectin, interleukin-6 receptor, and APP (65). Furthermore, TAPI-2 was reported to inhibit the apoptotic induced shedding of the E-cadherin ectodomain in epithelial cells (66). Because TACE and ADAM-10 have been shown to be involved in TNF $\alpha$  release, these enzymes appear to be the main mediators of the above-mentioned shedding pro-



**FIG. 6. TfR shedding in leukocytic cells.** *A*, HL-60 cells ( $1 \times 10^5/\text{ml}$ ) were cultured for 100 h and cell counts performed every 12 h. The sTfR in the cell culture supernatant was determined by sTfR-ELISA. Data were quantified by using purified human TfR as standard. *B*, HL-60, Jurkat, and U937 cells ( $1 \times 10^6/\text{ml}$ ) were cultured for 5 h in the absence or in the presence of 100 ng/ml PMA or 10  $\mu\text{M}$  pervanadate and sTfR release determined by sTfR-ELISA. Data were quantified from four independent experiments (mean value  $\pm$  S.E.). *C*, HL-60 cells ( $1 \times 10^6/\text{ml}$ ) were cultured for 20 h in fresh medium with or without inhibitors at a final concentration of 50  $\mu\text{M}$  and sTfR levels quantified using an sTfR-ELISA. Data are expressed as percentage of control from four independent experiments (mean value  $\pm$  S.E.). *D*, the supernatant (100  $\mu\text{l}$ ) of incubated HL-60 membrane preparations from Fig. 1C and 5 ml of HL-60 cell culture supernatants from panel C were precipitated with ferri-transferrin-Sepharose, separated by 7.5% SDS-PAGE under reducing conditions, and TfR detected by Western blotting using OKT9 mAb.

cesses. However, the TIMP inhibitor profile observed in our study argue against an involvement of TACE or ADAM-10 in TfR shedding because TACE is inhibited only by TIMP-3 (25)

and ADAM-10 by TIMP-1 and to a lesser extent by TIMP-3 (26). Thus, other proteolytic active ADAMs, which have not been tested to date in their sensitivity to TAPI-2 and TIMPs, are likely to contribute to TfR shedding.

Proteases of the MMP and ADAM families are synthesized as inactive precursors, in which the N-terminal prodomain blocks the catalytic site by coordinating the  $\text{Zn}^{2+}$  by a conserved unpaired Cys residue. Proteolytic cleavage between the prodomain and catalytic domain enables a "cysteine switch" mechanism that leads to the active form of the enzyme (67). The membrane type forms of the MMPs and most proteolytic active ADAMs (e.g. ADAM-9 (68), ADAM-10 (69), ADAM-12 (70), ADAM-15 (71), TACE (28), and ADAM-19 (72)) share the feature that they contain the furin recognition sequence R-X-(K/R)-R between pro- and catalytic domain, which permits their activation by furin-like pro-protein convertases during their intracellular trafficking through the secretory pathway. Our observation that a furin inhibitor reduced the sTfR releasing activity in cell membranes as well as sTfR release in HL-60 cells suggests that the TfR-shedding protease is activated by a furin-like pro-protein convertase. In particular, the processing of ADAM-10 to its active form by furin-type pro-protein convertases, furin and proprotein convertase 7 (PC7), was examined in detail (73). The authors could inhibit the maturation of ADAM-10 to its active form in HEK cells by treatment with FCI.

We observed that the intensity of TfR shedding correlates with cell growth. This is in agreement with the fact that changes in human serum sTfR levels are usually observed in diseases accompanied with altered cell proliferation (7–10). Thus, a decrease in TfR shedding from living cells can be traced back to either direct inhibition of the shedding process or diminished cell growth. The latter was confirmed by the observation that treatment of HL-60 cells with several kinase inhibitors or apoptosis-inducing reagents that reduce cell proliferation resulted in a decreased release of sTfR (data not shown). As protease inhibitors may reduce cell growth, inhibitors like 1,10-phenanthroline that are toxic to cells and lead to an arrest in cell growth must be excluded from interpretation. All inhibitors shown in Fig. 6 neither inhibited proliferation nor showed any detectable toxic effect at the concentrations tested. Our observation that pervanadate stimulates TfR shedding in leukocytic cell lines has also been reported for other shedding processes. The cleavage of HER2 has been shown to be enhanced by pervanadate, but not by PKC activators. This process is efficiently inhibited by TIMP-1 but not TIMP-2 (56). The tumor necrosis factor family member TRANCE is shed by at least two proteolytic activities, which are both distinct from TACE. One proteolytic activity could be induced by pervanadate and is sensitive to TIMP-2 but not TIMP-1 (54).

In summary, we found that the protease(s) that releases sTfR is a transmembrane metalloprotease and its activation is probably controlled by furin or furin-like proteases, which process transmembrane proteases to their active form. The cleavage of TfR could be inhibited by hydroxamic acid-based inhibitors that have been shown to be specific for metalloproteases of the MMP and ADAM families. All synthetic inhibitors that were active in the sTfR release assay also significantly reduced the basal release of sTfR from HL-60 cells. The low constitutive TfR shedding in some cell lines could be enhanced by pervanadate but not by the commonly used ectodomain shedding effector PMA. The above results, especially the inhibition of sTfR release by TAPI-2 and weakly by TIMP-1, but not by TIMP-2 and TIMP-3, suggest that the TfR-shedding protease(s) is a member of the ADAMs family.

**Acknowledgments**—We thank Sabrina Röttger for technical assistance and Anja Fromm for help with the FujiFilm LAS-1000 system. Special thanks go to Dr. Mark Sutherland for critically proofreading this manuscript.

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