Biochemical and Pharmacological Criteria Define Two Shedding Activities for TRANCE/OPGL That Are Distinct from the Tumor Necrosis Factor α Convertase*

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A number of structurally and functionally diverse membrane proteins are released from the plasma membrane in a process termed protein ectodomain shedding. Ectodomain shedding may activate or inactivate a substrate or change its properties, such as converting a juxtaerine into a paracrine signaling molecule. Here we have characterized the activities involved in protein ectodomain shedding of the tumor necrosis factor family member TRANCE/OPGL in different cell types. The criteria used to evaluate these activities include (a) cleavage site usage, (b) response to activators and inhibitors of intracellular signaling pathways, and (c) sensitivity to tissue inhibitors of metalloproteases (TIMPs). At least two TRANCE shedding activities emerged, both of which are distinct from the tumor necrosis factor α convertase. One of the TRANCE sheddases is induced by the tyrosine phosphatase inhibitor pervanadate but not by phorbol esters, whereas the other is refractory to both of these stimuli. Furthermore, the pervanadate-regulated sheddase activity is sensitive to TIMP-2 but not TIMP-1, which is consistent with the properties of a membrane type metalloproteinase. This study provides insights into the properties of different activities involved in protein ectodomain shedding and has implications for the functional regulation of TRANCE by potentially more than one protease.

A diverse set of transmembrane proteins is known to undergo proteolysis in their juxtamembrane region leading to the release of their extracellular domains into the surrounding milieu (reviewed in Refs. 1–4). This process, which has been termed ectodomain shedding, affects a wide variety of proteins, including growth factors and cytokines, growth factor receptors, adhesion molecules, and ectoenzymes. Ectodomain shedding has been shown to profoundly affect the biological activity of its target proteins and plays a critical role in the development and physiology of multicellular organisms. Ectodomain shedding regulates signaling via epidermal growth factor receptor ligands (5, 6), axonal guidance (7–9), and Notch-mediated lateral inhibition (10–17). Discovering the molecular identity of sheddases should lead to a better understanding of the mechanisms that regulate their function and may provide targets for therapeutic interventions in diseases that are caused by misregulated ectodomain shedding.

Based on inhibitor studies, ectodomain shedding is predominantly mediated by metalloproteases (5). Specifically, several members of the ADAM (α disintegrin and metalloprotease; also known as metalloprotease-disintegrins and MDC proteins) family of metalloproteases have been implicated as ectodomain sheddases (reviewed in Refs. 18–20). TNFα convertase (TACE, ADAM 17) has a role in the shedding of TNFα (21, 22), TGFA, l-selectin, both TNF receptors (5), interleukin-1 receptor II (23), HER4 (24), and Notch (17) and can act as a phorbol ester-responsive APP ζ-secretase (25). Kuzbanian (KUZ, ADAM 10, MADM, SUP-17) is required for Notch signaling (10–13), can cleave the Notch ligand Delta (15), as well as ephrin A2 (9), and has been shown to have APP ζ-secretase activity (26). ADAM 9 (MDC 9) has been implicated in the phorbol ester-stimulated shedding of heparin binding epidermal growth factor-like growth factor (27). Finally, ADAM 19 has been linked to shedding of the epidermal growth factor receptor-ligand neuregulin-β1 (28). In addition to the ADAM proteases, at least one matrix metalloprotease, MMP-7 (matrilysin) has a functionally relevant role in shedding TNFα (29) and FasL (30). For the majority of proteins subjected to ectodomain shedding, the proteases responsible have not been identified. Furthermore, in most cases it is not clear whether one or several proteases can target the same protein or whether the same protease mediates shedding in response to distinct stimuli.

In this study, we used the TNF family member TRANCE (TNF-related activation-induced cytokine; also known as osteoprotegrin ligand, OPGL, RANK ligand, RANKL, and osteoclast differentiation factor) as a model to ask whether one or more sheddases are involved in its release from the membrane and to...
define the properties of this sheddase or these sheddas. TRANCE is required in osteoclast differentiation and activation, in T and B cell maturation and dendritic cell survival, and in mammary gland development during pregnancy (31–38). We have previously shown that TRANCE is proteolytically released from the plasma membrane in a number of cell lines and that the solubilized ligand is functional in in vitro osteoclast differentiation and dendritic cell survival assays (39). We have now further characterized the TRANCE sheddase activities in CHO cells and embryonic fibroblasts using several biochemical and pharmacological criteria. These include cleavage site selection, response to activators and inhibitors of intracellular signaling pathways, and the inhibition by tissue inhibitors of matrix metalloproteases (TIMPs). Based on these results we have identified two distinct TRANCE sheddase activities that differ markedly from the TNFα convertase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phorbol-12-myristate 13-acetate, hydrogen peroxide, sodium vanadate, and anti-FLAG M2 monoclonal antibody were purchased from Sigma. Pervanadate was generated immediately prior to each experiment by mixing sodium orthovanadate with hydrogen peroxide to a final concentration of 100 mM of each. Protein A- and protein G-Sepharose were purchased from Amersham Pharmacia Biotech. Restriction enzymes and Taq polymerase were purchased from Roche Molecular Biochemicals. Fyu Turbo DNA polymerase was obtained from Stratagene. The MEK 1 and 2 inhibitor U0126 and p38 MAP kinase inhibitor SB202190 were purchased from CalBiochem. SulfO-NHS-biotin was purchased from Pierce. Anti-huTNFα polyclonal antibody was obtained from Endogen. The recombinant TRANCE-receptor Fc fusion protein, TR-Fc (30), was the generous gift of Y.-W. Choi, B. Wong, and J. Arron. The metalloprotease inhibitor BB-94 was kindly provided by J. D. Becherer (Glaxo-Wellcome, Research Triangle Park, North Carolina). Recombinant human TIMP-1 and -2 were the kind gift of Gillian Murphy. The MT1-MMP cDNA was provided by M. Tischer and M. Muller.

**Constructs and Mutagenesis**—The TRANCE-FLAG cDNA (40) was provided by B. Wong and Y.-W. Choi. The human TNFα cDNA was kindly provided by M. Milli. The coding region of TNFα was amplified by polymerase chain reaction using primers containing NotI and XbaI sites in the 5′ and 3′ primes, respectively. The resulting fragment was subcloned in frame into the NotI and XbaI sites of pFLAG CMV2 to allow the expression of N-terminally tagged TNFα. All chimeras were generated by polymerase chain reaction using standard techniques (41). The sequence of all polymerase chain reaction-generated constructs were confirmed by DNA sequencing (The BioResource Center, Cornell University, Ithaca, NY). A full-length murine TACE cDNA cloned into the eukaryotic expression vector pE12 (42) was the generous gift of G. Murphy. The M1-TIMP cDNA was provided by M. Tischer and M. Muller.

**Cell Culture, Transfection, and Metabolic Labeling**—CHO cells were grown in Ham’s F-12 medium supplemented with 5% fetal calf serum and 1% penicillin/streptomycin and glutamine. COS-7 cells were grown in DME with identical supplements. CHO and COS-7 cells were transfected using a standard calcium phosphate precipitation technique. Cells were allowed to recover and metabolically labeled overnight in DME without cytochalasin B and 10% dialyzed fetal calf serum, nonessential amino acids, and 1% penicillin/streptomycin and glutamine. Transfected CHO and COS-7 fibroblasts were cultured in DME supplemented with 10% fetal calf serum, nonessential amino acids, and 1% penicillin/streptomycin and glutamine. Recombinant human TIMP-1 and -2 were the kind gift of Y.-W. Choi, B. Wong, and J. Arron. Protein A- and protein G-Sepharose beads overnight at 4 °C were used to isolate FLAG-tagged TRANCE, and the other was used to immunoprecipitate TACE utilizing an anti-TACE monoclonal antibody. In all cases, immunoprecipitations were incubated at 4 °C for at least 6 h following by washing as above. Samples were then analyzed by SDS-PAGE and autoradiography as described above.

**TRANCE Cleavage Site Determination**—15-cm-diameter plates of CHO cells were transfected with LipofectAMINE and the TRANCE-FLAG plasmid or control vector. COS-7 cells were electroporated with the TRANCE-FLAG or control plasmid using a 0.4-cm cuvette in a Bio-Rad GenePulser II with a capacitance extender and 0.4-cm cuvettes at 270 V, 960 microfarads, or transfected using a standard calcium phosphate precipitation technique. Cells were allowed to recover and metabolically labeled overnight in DME without cytochalasin B and 10% dialyzed fetal calf serum, nonessential amino acids, and 1% penicillin/streptomycin and glutamine. Pervanadate was generated immediately prior to each experiment by mixing sodium orthovanadate and hydrogen peroxide to a final concentration of 100 mM of each. Protein A- and protein G-Sepharose were purchased from Amersham Pharmacia Biotech. Restriction enzymes and Taq polymerase were purchased from Roche Molecular Biochemicals. Fyu Turbo DNA polymerase was obtained from Stratagene. The MEK 1 and 2 inhibitor U0126 and p38 MAP kinase inhibitor SB202190 were purchased from CalBiochem. SulfO-NHS-biotin was purchased from Pierce. Anti-huTNFα polyclonal antibody was obtained from Endogen. The recombinant TRANCE-receptor Fc fusion protein, TR-Fc (30), was the generous gift of Y.-W. Choi, B. Wong, and J. Arron. The metalloprotease inhibitor BB-94 was kindly provided by J. D. Becherer (Glaxo-Wellcome, Research Triangle Park, North Carolina). Recombinant human TIMP-1 and -2 were the kind gift of Gillian Murphy. The MT1-MMP cDNA was provided by M. Tischer and M. Muller.

**Cell Culture, Transfection, and Metabolic Labeling**—CHO cells were grown in Ham’s F-12 medium supplemented with 5% fetal calf serum and 1% penicillin/streptomycin and glutamine. COS-7 cells were grown in DME with identical supplements. CHO and COS-7 cells were transfected in six-well tissue culture plates with LipofectAMINE (Life Technologies, Inc.) following the manufacturer’s recommendations. Cells were allowed to recover for at least 4 h prior to metabolic labeling. For metabolic labeling, cells were washed twice with PBS and then incubated in DME lacking methionine and supplemented with 10% dialyzed calf serum and 200 μCi/ml of [35S] Pro-mix (Amersham Pharmacia Biotech). Cells were harvested for 10–14 h after labeling, the cells were washed twice with PBS, pH 7.4, and chased for one hour in OptiMEM (Life Technologies, Inc.) with the indicated additives. The conditioned medium was collected. The cells were washed twice with PBS and lysed in lysis buffer (TBS, pH 7.4), 1% (v/v) Nonidet P-40, 10 mM NaF, 1 mM Na3VO4, 1 mM 1,10-phenanthroline and protease inhibitors (2 μg/ml leupeptin, 0.4 mM benzamidine, 10 μg/ml soybean trypsin inhibitor, 0.5 mM iodoacetamide). In experiments examining the MAP kinase inhibitors U0126 and SB202190, the cells were preincubated in OptiMEM with the indicated concentration of inhibitor for 2 h. The cells were then washed twice in PBS and chased in OptiMEM with additives for 1 h. The medium and lysate were then processed as above. All lysates and supernatants were plated on a top centrifuge (Sorvall) for 15 min at 13,000 rpm to remove nuclei and cellular debris.

For immunoprecipitations, the TRANCE receptor Fc fusion protein (TR-Fc) or a rabbit TNFα antiserum, together with protein A-Sepharose, was added to the medium. FLAG M2 monoclonal antibody and protein G-Sepharose were used to immunoprecipitate full-length TRANCE and TNFα from the lysates. The resulting suspensions were incubated at 4 °C for 2 h to overnight, and the beads were washed three times with PBS, pH 7.4, 0.5% (v/v) Nonidet P-40 (for supernatants). 2× sample loading buffer supplemented with 5 mM dithiothreitol was added, and the samples were incubated at 95 °C for 5 min prior to SDS-PAGE analysis. Gels were fixed in 50% (v/v) methanol, 10% (v/v) acetic acid for 15 min, rehydrated in water for 15 min, dried, and exposed to Kodak BioMax MR film. Quantification was performed using a Fuji BAS200 Bio Imaging analyzer.

TACE-deficient embryonic fibroblasts immortalized by infection with a retrovirus encoding ras and myc (E3 clone) (5, 23) were kindly provided by P. Reddy, J. Peschon, and R. Black (Immunex, Seattle, WA) and cultured in DME supplemented with 2% fetal calf serum and 1% penicillin/streptomycin and glutamine. SV40 transformed embryonic fibroblasts from ADAM 10-deficient mice and their wild type littermates were the generous gift of D. J. Pan (16). These cells were maintained in DME supplemented with 10% fetal calf serum and 1% penicillin/streptomycin and glutamine. Primary fibroblasts were isolated from day E13.5 murine embryos homozygous for both ADAM 9 and ADAM 15 gene disruptions2 or ADAM 9, ADAM 12, and ADAM 15 gene disruptions,3 homozygous for a gene trap insertion in the ADAM 19 gene. CHO and COS-7 cell lines were transfected using a FujiF BAMI electroporation device.

1. G. Weskamp, L. Lum, and C. Blobel, manuscript in preparation.
2. G. Weskamp, A. Fujisawa-Sehara, and C. P. Blobel, manuscript in preparation.
3. H. Zhou, G. Weskamp, B. Skarnes, and C. P. Blobel, manuscript in preparation.
Evaluation of TRANCE/OPGL Sheddases

TRANCE and TNFα shedding activities, the release of soluble TRANCE and TNFα ectodomains was examined in CHO cells. Cells were transiently transfected with N-terminally FLAG-tagged TNFα or TRANCE and metabolically labeled. The cells were chased for 1 h with or without the addition of several potential modulators of shedding. Labeled TNFα and TRANCE in the conditioned medium and lysate was then isolated by immunoprecipitation and analyzed by SDS-PAGE.

In agreement with previously published observations (39, 44–47), both TRANCE and TNFα ectodomains are released into the medium by a mechanism sensitive to the hydroxamate-based metalloprotease inhibitor BB-94 (Fig. 1, A and B). The release of TRANCE is not further enhanced by treating CHO cells with PMA, a phorbol ester, but is significantly enhanced in the presence of pervanadate, a tyrosine phosphatase inhibitor (Fig. 1A, top panel). Pervanadate-stimulated shedding is inhibited by the presence of BB-94. In contrast, TNFα ectodomain release is increased in CHO cells treated with PMA, as previously described (Fig. 1B, top panel). In addition, we found that pervanadate is also effective at stimulating TNFα processing. In all cases, TNFα release is sensitive to inhibition by BB-94.

The inability of PMA to affect the rate of ectodomain shedding of TRANCE is at odds with our previous findings in COS-7 cells (39). We therefore examined the shedding of TNFα and TRANCE in COS-7 cells under conditions identical to those used above (Fig. 1, A and B, lower panels). The release of TNFα in COS-7 cells resembles that seen in CHO cells, with both PMA and pervanadate increasing ectodomain shedding (Fig. 1B, compare upper and lower panels). As in CHO cells, constitutive and stimulated shedding is inhibited by BB-94. TRANCE processing in COS-7 is also a BB-94-sensitive process, but, in contrast to our previous study, is minimally affected by PMA (Fig. 1A, lower panel). We cannot at present explain this discrepancy, which may be due to previous experimental error or may reflect a difference in the COS-7 cells used in this study.
compared with the previous one. Pervanadate treatment leads to only a marginal increase in TRANCE ectodomain shedding in COS-7 cells, in sharp contrast to its effect on CHO cells. The difference in pervanadate-mediated TRANCE shedding in CHO and COS-7 cells was confirmed by quantifying the TRANCE released from cells treated with either PMA, pervanadate, or carrier as control (Fig. 1C). Although pervanadate-treated COS-7 cells release more TRANCE than control treated COS-7 cells (172% of control), it was considerably lower than the amount released by CHO cells in response to pervanadate (484% of control). In contrast, PMA and pervanadate induced a similar increase in the release of TNFα in both COS-7 and CHO cells (Fig. 1D).

To control for the specificity of pervanadate stimulation, the effect of equal concentrations of sodium vanadate, hydrogen peroxide, and pervanadate on both TNFα and TRANCE shedding was evaluated (Fig. 1E). Neither sodium vanadate nor hydrogen peroxide is able to enhance shedding above that seen in unstimulated cells, whereas pervanadate enhances the shedding of both proteins. Thus, although both TNFα and TRANCE ectodomain shedding is sensitive to BB-94, TNFα processing is stimulated by both PMA and pervanadate, whereas TRANCE shedding is only enhanced in the presence of pervanadate.

To further investigate the difference between TRANCE shedding in CHO and COS-7 cells, we compared the soluble TRANCE ectodomain released from these cells as well as the membrane stubs generated by shedding. Interestingly, CHO cells produce at least two forms of TRANCE ectodomain, whereas only the slower migrating form is seen in the medium from COS-7 cells (Fig. 2A). Immunoprecipitation of cell lysates with an anti-FLAG antibody recovers a small amount of both cleaved ectodomain as well as membrane stub in addition to the full-length TRANCE protein (Fig. 2B). Although COS-7 cells contain only one form of TRANCE ectodomain and a corresponding membrane stub, in CHO cell lysates a slightly smaller form of ectodomain and a correspondingly larger membrane stub is also detected. To confirm that the differently migrating forms of TRANCE ectodomain are generated by proteolysis at distinct sites, TRANCE was purified from the conditioned medium of either CHO or COS-7 cells overexpressing FLAG-tagged TRANCE and subjected to N-terminal sequencing. Only a single N terminus was detected in TRANCE from COS-7 cells, which corresponded to cleavage at the previously reported site between Arg138 and Phe139 (33). In contrast, three forms of soluble TRANCE differing at their N terminus were isolated from CHO conditioned medium, corresponding to cleavage between Gln137 and Arg138, Arg138 and Phe139, and Met145 and Met146 (Fig. 2C). Therefore, CHO cells shed TRANCE by cleaving the membrane bound protein at three distinct sites, one of which corresponds to the previously reported site used in 293 and COS-7 cells.

Because CHO cells produce several distinct forms of TRANCE, we wished to determine whether this is due to the activity of a single protease or of different proteases. We therefore examined the ability of the metalloprotease inhibitors TIMP-1 and TIMP-2 to inhibit the production of the distinct forms of TRANCE (Fig. 3A). TIMP-2, but not TIMP-1, was able to inhibit the production of the low molecular weight form of TRANCE and the high molecular weight form but did not effect the release of the intermediate form. In addition, pervanadate stimulation appears to specifically enhance the production of the high and low molecular weight forms of soluble TRANCE but not the intermediate form (Fig. 1A, top panel). In the presence of TIMP-2, constitutive and pervanadate-stimulated shedding are indistinguishable, suggesting that pervanadate specifically enhances the activity of the TIMP-2-sensitive protease.

TIMP-2 was able to efficiently inhibit pervanadate-induced TRANCE shedding at concentrations in the low nanomolar range (Fig. 3B) with maximum inhibition seen by 18 nM TIMP-2. No further inhibition of the remaining constitutive sheddase activity was seen with concentrations of TIMP-2 up to 90 nM. TIMP-1 had no effect on either constitutive or stimu-
overnight and then chased for 1 h in medium with 100 cells overexpressing Flag-tagged TRANCE were metabolically labeled with MMP-7, implicated as a TNF receptor activator (39), a site used in COS-7, 293 fibroblasts, and CHO cells (Fig. 3). trimming of TNF receptor activator (39), a site used in COS-7, 293 fibroblasts, and CHO cells (Fig. 3). TIMP-2 inhibition, although it is possible that the protease acts in a subcellular compartment that is inaccessible to the TIMPs. The TRANCE sheddase in COS-7 cells shares the characteristics of this second protease, because it is not stimulated by pervanadate and is not inhibited by either TIMP-1 or TIMP-2.

As a comparison with the shedding of TRANCE, the effect of TIMP-1 and -2 on TNFα shedding was assessed (Fig. 3C). Neither constitutive nor PMA or pervanadate-stimulated shedding of TNFα was altered by TIMP-1 or -2 in CHO cells. These results are consistent with the likely role of TACE, an ADAM metalloprotease, as a TNFα sheddase (21, 22). TACE has previously been shown to be sensitive to TIMP-3, but not to TIMP-1 or -2 (42). Furthermore, these results indicate that MMP-7, implicated as a TNFα sheddase in macrophages (29), is likely not involved in TNFα shedding in CHO cells.

The ectodomain shedding of several proteins has been shown to involve the MEK/ERK and p38 MAP kinase pathways. In particular, constitutive shedding of TGFα, a TACE substrate, requires the p38 MAP kinase pathway, whereas the MEK/ERK MAP kinase pathway mediates the increase in TGFα shedding in response to phorbol ester and epidermal growth factor and platelet-derived growth factor receptor activation (48). To determine whether these signaling pathways are also required for TRANCE shedding, the effect of MAP kinase pathway inhibitors on constitutive and pervanadate-stimulated shedding was assessed in CHO cells (Fig. 4). Neither the MEK1/2 inhibitor U0126 nor the p38 MAP kinase inhibitor SB202190 affected the shedding of TRANCE. In contrast, under similar conditions the metalloprotease mediated shedding of interleukin-2 receptor α, and the low affinity p75 NGFR was impaired by these inhibitors, confirming that the inhibitors are active.5 These results further emphasize that the shedding of TRANCE is distinct from previously described shedding events.

Through the use of cells generated from various mouse “knock-out” mice, including embryonic fibroblasts, it has been possible to examine the importance of several proteases as potential sheddases (5, 16, 17, 21, 23–25, 29, 30). We therefore assessed whether murine embryonic fibroblasts might offer a system for analyzing TRANCE shedding (Fig. 5). TRANCE shedding in fibroblasts from wild type embryos closely resembles that seen in CHO cells. Shredding is enhanced by pervanadate but not PMA (Fig. 5A). In addition, TIMP-2, but not TIMP-1, inhibits the pervanadate-stimulated sheddase (Fig. 5B). Thus, identical or very similar proteases are likely active in TRANCE shedding in both CHO cells and embryonic fibroblasts. 

5 J. Schlöndorff, L. Lum, and C. P. Blobel, unpublished results.

![Fig. 3. Sensitivity of TRANCE and TNFα shedding to inhibition by TIMP-1 and -2. A, CHO (upper panel) and COS-7 (lower panel) cells overexpressing Flag-tagged TRANCE were metabolically labeled overnight and then chased for 1 h in medium with 100 μM pervanadate (−). TIMP-1 (15.2 nM) or TIMP-2 (17.8 nM) was present during the chase period as indicated. Shed protein was recovered from the conditioned supernatant by immunoprecipitation. B, TRANCE overexpressing CHO cells were metabolically labeled and chased as above in the presence (PV) or absence (−) of 100 μM pervanadate and the indicated concentration of TIMP-2. Soluble TRANCE ectodomain was recovered from the medium and detected by autoradiography. C, CHO cells were transiently transfected with FLAG-tagged TNFα and labeled as above. Cells were then chased for 1 h in OptiMEM with carrier (−), 25 ng/ml PMA, or 100 μM pervanadate. TIMP-1 (15.2 nM) or TIMP-2 (18.9 nM) was present in the chase medium as indicated. Soluble TNFα ectodomain was recovered from the medium by immunoprecipitation.

![Fig. 4. Effect of MEK/ERK and p38 MAP kinase inhibitors on the shedding of TRANCE. CHO cells were transfected with FLAG-tagged TRANCE. Cells were metabolically labeled overnight and then chased for 2 h in medium containing either carrier, 5 μM U0126 (a MEK 1 and 2 inhibitor), 10 μM SB202190 (a p38 MAP kinase inhibitor), or both inhibitors. Cells were then placed in fresh medium containing the same inhibitors with no further additions (−) or 100 μM PV and chased for an additional hour. Shed TRANCE was recovered from the conditioned supernatant by immunoprecipitation and analyzed by SDS-PAGE. The amount of shed protein recovered from the medium was assessed using a PhosphorImager and compared relative to the amount in the untreated cells. Values represent the averages of two experiments; error bars indicate one standard deviation.

![Image 60x432 to 287x730](http://www.jbc.org/Downloaded from)
TACE-deficient mice, either alone or together with full-length murine TACE (Fig. 6A). TRANCE shedding by cells lacking or expressing TACE is indistinguishable. Similar to COS-7 cells, the TACE-deficient fibroblasts show only a small increase in TRANCE shedding in response to pervanadate stimulation; coexpression of TACE does not alter this response. Thus, although TACE is capable of cleaving TRANCE in vitro, it is not required for the constitutive shedding of TRANCE in fibroblasts. The inability of TACE overexpression to induce pervanadate-stimulated shedding does not in itself rule out a role for TACE in this process. However, two aspects of pervanadate-stimulated shedding of TRANCE argue against a role for TACE. First, the pervanadate-responsive protease in CHO cells cleaves TRANCE at a site distinct from that targeted by TACE in vitro. Secondly, the pervanadate-stimulated sheddase is inhibited by TIMP-2 (Fig. 3A), whereas TACE is not (42).

Kuzbanian (KUZ, ADAM 10, MADM) has been reported as a candidate sheddase for several of the same proteins as TACE, particularly TNFα and APP (26, 49, 50). The potential role of KUZ as a TRANCE sheddase was assessed by comparing the release of TRANCE ectodomain from fibroblasts derived from KUZ-deficient murine embryos and their wild type littermates (Fig. 6B). No appreciable difference in TRANCE shedding is observed in these two cell lines. Similar to results using the TACE knock-out cells and COS-7 cells, TRANCE shedding was not significantly enhanced in response to pervanadate in either cell type. Furthermore, based on the different TIMP sensitivities (KUZ is inhibited by TIMP-1 and -3 (51), and the pervanadate-stimulated TRANCE sheddase is inhibited by TIMP-2 but not TIMP-1), it is unlikely that KUZ is involved in pervanadate-stimulated TRANCE shedding. Thus, neither TACE nor KUZ are required for the constitutive or pervanadate-stimulated release of TRANCE in fibroblasts.

In addition to TACE and KUZ, several other ADAM metalloproteases with a catalytic site consensus sequence (ADAM 1, 8, 9, 12, 15, and 19) are currently known to be widely expressed. Because the TIMP inhibition profile of most of these potential TRANCE sheddases has not yet been established, we addressed the role of ADAM 9, 12, 15, and 19 in TRANCE shedding using primary embryonic fibroblasts lacking one or more of these ADAMs. Fig. 6C shows a representative experiment where TRANCE shedding is compared between fibroblasts from wild type embryos, embryos homozygous for a gene trap insertion in the ADAM 19 gene, and ADAM 9-, 12-, and 15-deficient animals. TRANCE shedding in the absence of stimuli and in response to pervanadate is very similar in all three cell populations. Similar observations were made using embryonic fibroblasts derived from ADAM 9/15-deficient mice (data not shown). In the case of ADAM 19, the gene trap gives rise to a truncated and thus most likely nonfunctional ADAM 19 protein lacking parts of the ectodomain and all of its cytoplasmic domain. These results argue against a role for ADAM 9, 12, 15, and 19 as constitutive or inducible TRANCE sheddases.

The TIMP inhibition profile of the pervanadate-stimulated TRANCE sheddase is similar to that of several of the MT-MMPs. Furthermore, based on Northern blot analysis, CHO cells express MT1-MMP, whereas COS-7 cells express little or no MT1-MMP (data not shown). To assess whether MT1-MMP is capable of acting as a TRANCE sheddase, MT1-MMP was coexpressed with TRANCE in both COS-7 and CHO cells (Fig. 7). Overexpression of MT1-MMP led to a large increase in the amount of TRANCE ectodomain released from both CHO and COS-7 cells. At least some increase in TRANCE release was observed when cells were treated with pervanadate. Furthermore, the TRANCE ectodomain released from cells overexpressing MT1-MMP appears to migrate with the lower molecular weight forms of TRANCE released by CHO cells.

To assess whether MT1-MMP targets the same peptide bonds as the pervanadate-induced sheddase in CHO cells, soluble TRANCE ectodomain was purified from CHO cells overexpressing and metabolically labeled in the presence of MT1-MMP and subjected to N-terminal sequencing as above. Although N-terminal sequencing of ecto-TRANCE released from control COS-7 cells confirmed the result shown in Fig. 2C, a second N-terminal sequence was seen in ecto-TRANCE released from COS-7 cells that had been cotransfected with MT1-MMP. This sequence (MEGXXLD) matches one of the sequences of ecto-TRANCE released from CHO cells exactly (Fig. 2C). The results from this analysis are consistent with a role of MT1-MMP or of another MT-MMP with a similar cleavage site specificity in PV-dependent TRANCE shedding in CHO cells.

The finding that two type II transmembrane proteins belonging to the TNF protein family, TNFα and TRANCE, are shed by different metalloproteases raises the question of which determinant in each substrate is necessary for recognition and cleavage by a specific protease. For several type I transmembrane proteins, including TGFα, the juxtamembrane sequence surrounding the cleavage site has been shown to be sufficient to target the protein for regulated shedding (24, 52–54). To assess whether this is also true for TRANCE and TNFα, several chimeric proteins were generated. Specifically, the constructs were designed to test the importance of the cytoplasmic domain and transmembrane sequence, the juxtamembrane region, and the TNF domain. The TNF-TRANCE chimeras (see schematic in Fig. 8A) were overexpressed and metabolically labeled in CHO cells, and the release of ectodomain under constitutive and PMA or pervanadate stimulation was examined (Fig. 8B). In all cases, the origin of the juxtamembrane region determines the shedding profile of the chimeric protein. The shedding of proteins containing the juxtamembrane sequence of TNFα (chimeras 1 and 4) is increased by both PMA and pervanadate treatment, whereas chimeras containing the TRANCE juxtamembrane region (chimeras 2 and 3) underwent enhanced shedding in the presence of pervanadate only. Furthermore, for chimeras containing the TRANCE juxtamembrane region, pervanadate specifically enhances the production of the high and low molecular weight forms of ecto-TRANCE.

FIG. 5. TRANCE shedding in murine embryonic fibroblasts. A, FLAG-tagged TRANCE was overexpressed in primary embryonic fibroblasts from wild type embryonic day 13.5 mice. The cells were metabolically labeled overnight followed by a one hour chase in OptiMEM with no additions (−), 25 ng/ml PMA, or 100 µM PV. Soluble TRANCE ectodomain was recovered from the conditioned medium and analyzed by SDS-PAGE followed by autoradiography. B, primary embryonic fibroblasts were transfected and metabolically labeled as above and chased for 1 h in OptiMEM either with (PV) or without (−) 100 µM pervanadate. In addition, 10 ng of TIMP-1 or TIMP-2 was added to the medium as indicated. Soluble TRANCE was isolated and visualized as above.
low molecular weight forms of the soluble ectodomain but not the intermediate form (e.g. chimera 2), similar to its effect on TRANCE (Figs. 1A and 2A). Therefore, the juxtamembrane sequences of TNFα and TRANCE appear to specify their distinct shedding profiles. Furthermore, the cytoplasmic domain of TRANCE, which is tyrosine phosphorylated upon pervanadate stimulation (data not shown), does not appear to be required for ectodomain shedding of TRANCE.

In the case of several shed proteins, including TNFα, it requires the intact juxtamembrane sequence to target shedding, and whether the protease relies on particular sequences deleted in TRANCE for proteolysis. However, whether the protease remains to be determined.

Fig. 6. Requirement of ADAM proteases for TRANCE shedding. A, immortalized embryonic fibroblasts from TACE-deficient mice were electroporated with plasmids encoding for full-length murine TACE and FLAG-tagged TRANCE as indicated. Cells were metabolically labeled overnight followed by a 1-h chase in OptiMEM with or without 100 μM pervanadate, as indicated. Soluble TRANCE ectodomain (ecto-TRANCE) was recovered from the conditioned medium and analyzed by SDS-PAGE followed by autoradiography. B, SV40 transformed embryonic fibroblasts from KUZ-deficient mice (ADAM 10−/−) or their wild type littermates (wild type) were electroporated either with control vector (V) or a plasmid encoding for FLAG-tagged TRANCE. Cells were then treated as above (A), and soluble TRANCE ectodomain (ecto-TRANCE) was recovered from the conditioned medium (ecto-TRANCE) was analyzed by SDS-PAGE followed by autoradiography.

Fig. 7. Effect of MT1-MMP expression on TRANCE shedding. CHO and COS-7 cells transiently overexpressing FLAG-tagged TRANCE alone (TRANCE) or together with MT1-MMP (TRANCE/MT1-MMP) were metabolically labeled overnight and then chased for 1 h in OptiMEM with or without 100 μM PV as indicated. TRANCE ectodomain was recovered from the medium and analyzed by SDS-PAGE followed by autoradiography. Immunoprecipitation of full-length TRANCE from the cell lysates revealed considerably more TRANCE in cells expressing TRANCE alone compared with cells cotransfected with the MT1-MMP plasmid, indicating that overall expression levels can not account for the difference in TRANCE shedding (data not shown).

To address the importance of this region in TRANCE shedding, a deletion mutant was constructed that lacks the first 44 amino acids of the juxtamembrane domain, including both cysteine residues (TRANCE JM), placing the cleavage sites at the same distance from the membrane as for TNFα. This deletion mutant undergoes ectodomain shedding, but pervanadate stimulation is not observed (Fig. 8B). Furthermore, only one form of TRANCE ectodomain is visualized by SDS-PAGE for proteolysis. However, whether the protease relies on the overall length of the juxtamembrane region or whether particular sequences deleted in TRANCE JM recruit the protease remains to be determined.
Several members of the TNF family of cytokines undergo metalloprotease-mediated ectodomain shedding, including TNFα (44–47), FasL (58, 59), and TRANCE (33, 39). We have previously shown that TACE is capable of cleaving the osteoclast differentiation and dendritic cell survival factor TRANCE/OPGL in vitro at the major cleavage site that is used in COS-7 and human 293 cells (33, 39). Here we have extended our studies by examining TRANCE shedding in CHO cells and in fibroblasts, both cell types that are commonly used for studies of protein ectodomain shedding. We found that at least two distinct metalloproteases are involved in shedding TRANCE in these cells. These enzymes are distinct from TACE and differ from one another in their responsiveness to known stimulators of ectodomain shedding, their cleavage site selection, and their sensitivity to TIMPs.

At the outset of the present study, we tested the hypothesis that TACE may have a role in shedding TRANCE by directly comparing several properties of TRANCE and TNFα sheddases. We found several lines of evidence indicating that TACE is in fact not a TRANCE sheddase. In primary embryonic fibroblasts and CHO cells, TRANCE shedding is stimulated by pervanadate but not PMA. In contrast, in most cases where TACE has been shown to play a role as a sheddase, it has been in the context of phorbol ester-stimulated shedding (5, 23–25). In addition, pervanadate stimulation of CHO cells appears to enhance a protease activity that cleaves TRANCE at sites distinct from those targeted by TACE in vitro. Finally, the pervanadate-stimulated sheddase is inhibited by TIMP-2, but not TIMP-1, whereas TACE is sensitive to TIMP-3, but not TIMP-1 or -2 (42). These results strongly argue against a role for TACE in pervanadate-activated TRANCE shedding.

The matrix metalloprotease inhibitors TIMP-1 and -2 not only helped distinguish between TACE and TRANCE sheddases but also revealed the presence of a constitutive TRANCE sheddase that is distinct from the pervanadate-stimulated activity. (It should be noted that although both shedding activities are present in unstimulated CHO cells, we use the term constitutive TRANCE sheddase to refer to the protease(s) that does not respond to PMA or pervanadate stimulation.) The constitutive activity depends on a metalloprotease, as it is sensitive to BB-94 but, unlike the pervanadate-stimulated activity, is not inhibited by TIMP-1 or -2. Furthermore, the constitutive and pervanadate-stimulated sheddases cleave TRANCE at distinct sites within the juxtamembrane region, generating slightly different forms of soluble TRANCE. Only the constitutive TRANCE sheddase in COS-7 and CHO cells targets the same site that is cleaved by TACE in vitro. Yet TACE is not necessary for this activity either, because embryonic fibroblasts lacking TACE still shed TRANCE constitutively. These findings demonstrate the presence of two distinct shedding activities for TRANCE in the same cell type.

With respect to the existence of the two distinct TRANCE sheddases observed here, it is interesting to point out that TNFα can be released by at least three different proteases in a functionally relevant manner, albeit in different cells and under different conditions. TACE is the major TNFα converting enzyme in T cells and most likely also in several other cell types (21, 23). Yet matrilysin (MMP-7) can also release TNFα from its membrane anchor, and this activity has been shown to be physiologically relevant in a herniated disc resolution model (29). Finally, the serine proteinase 3 can produce functional soluble TNFα, at least in inflammatory foci (60). In addition to these three proteases, ADAM 10 (49) and MT4-MMP (61) are capable of acting as TNFα sheddases when overexpressed in cells.

For the majority of shed proteins, the protease or proteases responsible for their release are still unknown. To date, only five metalloproteases, TACE, KUZ, ADAM 9, ADAM 19, and MMP-7 have been implicated as sheddases. With respect to the activities that are involved in TRANCE shedding in CHO cells and primary fibroblasts, our results have excluded these five sheddases and have substantially narrowed down the remaining list of candidate metalloproteases.

All soluble matrix metalloproteases examined to date are sensitive to both TIMP-1 and TIMP-2, ruling out the currently known soluble MMPs, including MMP-7 (62, 63), as constitutive or stimulated TRANCE sheddases. Furthermore, the TIMP inhibition profile of the pervanadate-stimulated TRANCE sheddase not only rules out TACE (see above), but also rules out KUZ (ADAM 10), which is sensitive to TIMP-1 and -3 (51). Constitutive shedding of TRANCE is intact in fibroblasts lacking TACE and in cells lacking KUZ, indicating that these ADAMs are not required as constitutive TRANCE sheddases.

Besides TACE and KUZ, only five currently known ADAMs have a catalytic site consensus sequence and are expressed in a number of somatic tissues outside of the testis (ADAM 1, 9, 12, 15, 19). Of these, ADAMs 9, 12, and 15 can be excluded as TRANCE sheddases because the pervanadate-stimulated and constitutive shedding activities are still present in primary embryonic fibroblasts lacking these ADAMs. ADAM 19 can most likely also be ruled out because embryonic fibroblasts homozygous for a gene trap insertion in the ADAM 19 gene still have both TRANCE shedding activities. Thus, the majority of the currently known ADAMs predicted to be catalytically active and widely expressed have been excluded as candidate TRANCE sheddase.

Interestingly, the sensitivity of the pervanadate-stimulated TRANCE sheddase to TIMP-2 but not TIMP-1 matches the TIMP inhibitor profile of several MT-MMPs (63–66), which thus emerge as good candidate TRANCE sheddases in CHO cells and primary fibroblasts. In fact, overexpression of MT1-MMP in both CHO and COS-7 cells leads to a dramatic increase in TRANCE shedding, and this effect is further enhanced by pervanadate treatment. In addition, although CHO cells clearly express MT1-MMP, COS-7 cells express little if any detectable MT1-MMP mRNA (data not shown). Finally, at least one of the TRANCE cleavage sites that is used in CHO cells but not COS-7 cells is cleaved in COS-7 cells when MT1-MMP is coexpressed. These results are consistent with a role of MT1-MMP in pervanadate-stimulated TRANCE shedding. Nevertheless, further studies will be necessary to address whether or not other MT-MMPs may also meet the same set of criteria for candidate pervanadate-dependent TRANCE sheddases.

The ability to shed TRANCE in response to pervanadate stimulation appears to be limited to certain cell types. Although CHO cells and primary embryonic fibroblasts show a robust increase in TRANCE shedding in response to pervanadate, COS-7 cells and three independently derived immortalized embryonic fibroblast cell lines (TACE<sup><sup>Δ22</sup></sup>, ADAM 10<sup>Δ1</sup>–/–, and ADAM 10 wt) do not. It is likely that the difference between CHO and COS-7 cells is due to a lack of a protease, such as an MT-MMP, or alternatively due to a lack of a signaling pathway that is necessary to activate the protease. Our results also do not rule out that the inability of COS-7 cells to process TRANCE at the two additional sites used in CHO cells is due to a species specificity. The different TRANCE shedding seen in the primary and immortalized embryonic fibroblasts is intriguing. The primary fibroblast cultures likely represent a more diverse population of cell types compared with the immortalized cell lines. The expression of the pervanadate-stimulated
sheddase may be limited to only some of the cells in a preparation of embryonic fibroblasts. Alternatively, the process of immortalization could interfere with the pathway responsible for pervanadate-induced TRANCE shedding.

TRANCE is only one of a few proteins whose shedding is not enhanced by phorbol ester. For the majority of shed proteins, phorbol ester treatment induces a rapid increase in ectodomain processing. Indeed, it has been proposed that a general shedding mechanism exists that is responsible for the proteolysis of numerous structurally and functionally distinct proteins in response to PMA (67). Although pervanadate is also able to enhance the shedding of various proteins, in most of these cases PMA has a similar effect. Furthermore, in the case of erbB-4/HER4 shedding, a single protease, TACE, has been shown to be necessary for both phorbol ester and pervanadate-stimulated shedding (24). Thus, although PMA and pervanadate may initially activate distinct signaling pathways, they both can lead to the activation of a common sheddase(s). The finding that the ectodomain shedding of TRANCE (this study) and HER2/neu/erbB-2 (68) is enhanced by pervanadate but not by phorbol ester implies that in addition to activating a pathway targeted by phorbol ester, pervanadate also activates shedding events not amenable to phorbol ester stimulation. Furthermore, the distinct TIMP inhibition profiles of the TRANCE and HER2 sheddases (HER2 shedding is inhibited by TIMP-1, but not TIMP-2 (68)) suggest that at least two distinct proteases must be activated in response to pervanadate and not PMA.

Finally, given the clear differences in the inducible shedding of TRANCE and TNFα, both of which are TNF family members, we decided to evaluate which part of the substrates are necessary to determine specific targeting by one or the other sheddase. Previous studies have addressed this question for several type I integral membrane proteins that are shed, including TGFα, APP, and erbB-4 (24). In all three cases, the juxtamembrane region is sufficient for stimulated shedding. Our results demonstrate that this is also the case for the type II transmembrane proteins TRANCE and TNFα. For both proteins, the juxtamembrane region is necessary and sufficient to govern their characteristic shedding behavior. Interestingly, the juxtamembrane sequence of TRANCE is significantly longer than that of TNFα, and TRANCE is cleaved at least 66 amino acids from the membrane, whereas the predominant TNFα cleavage site is 23 amino acids from the predicted transmembrane domain. For TNFα, the distance from the membrane has been suggested to play a critical role in determining cleavage site selection (55). Similarly, when the juxtamembrane region of TRANCE is shortened, the pervanadate-responsive cleavage is lost. However, constitutive shedding remains intact. Thus, the length of the juxtamembrane stalk region as well as specific sequences within this domain may be determinants in regulating the shedding of TRANCE and TNFα.

In summary, biochemical and pharmacological studies have revealed different endogenous TRANCE and TNFα shedding activities in the same cell type. In CHO cells and embryonic fibroblasts, a constitutive and an inducible metalloprotease, the latter most likely an MT-MMP, have emerged as TRANCE sheddases. Both of these sheddases have properties that clearly distinguish them from the TNFα convertase. Criteria such as those applied here toward TRANCE shedding should also be useful in defining the properties of relevant sheddases for other substrates of interest. These could then be compared with the properties of the expanding list of potential sheddases. Because TRANCE is shed by at least one metalloprotease in all cells analyzed in this study, we predict that ectodomain shedding is likely to occur in cells and tissues where TRANCE is active as a signaling molecule. If so, shedding is likely to have a role in regulating TRANCE function, either by activating or inactivating it or by producing a soluble growth factor that can function in a paracrine or autocrine manner.

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