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Review

Structure and functions of tumor necrosis factor-α converting enzyme[★✪]

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Tumor necrosis factor-α converting enzyme (TACE) is the first described and best characterized secretase. In this review the structure and the possible roles for TACE are summarized. The substrate specificity and the regulation of TACE activity as well as redundancy and possible cooperations of distinct secretases are also discussed.

Ectodomain shedding is the process in which transmembrane proteins are proteolytically cleaved to release their extracellular domains from the cell membrane. Ectodomain shedding is typical for numerous growth factors, cytokines, growth factor- and cytokine receptors, and adhesion molecules. This process may have a significant impact on the biological functions of these proteins by converting them from juxtacrine into paracrine acting

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Abbreviations: ADAM, a disintegrin and metalloproteinase domain; APP, amyloid precursor protein; BACE, β-acting cleaving enzyme; EC, endothelial cells; EGF, epidermal growth factor; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PMA, phorbol myristate acetate; TACE, tumor necrosis factor-α converting enzyme; TIMP, tissue inhibitor of metalloproteinases; TGFα, transforming growth factor-α; TNFα, tumor necrosis factor-α; TNF-RI, TNF-RII, tumor necrosis factor-α receptor I and II; TRANCE, TNF-related activation-induced cytokine.

molecules. Moreover, the release of receptors from the cell membrane may diminish the responsiveness of the cells to corresponding ligands. The putative enzymes responsible for ectodomain shedding were named sheddases or secretases (Arribas & Borroto, 2002; Kiessling & Gordon, 1998).

The first secretase, the one responsible for releasing soluble TNFα from the cell membrane and thus designated as TACE (**t**umor necrosis factor-α **c**onverting **e**nzyme), was identified, characterized and cloned in 1997 by two independent groups (Black *et al.*, 1997; Moss *et al.*, 1997).

ADAM FAMILY OF METALLOPROTEINASES

TACE (ADAM17, CD156b, EC 3.4.24.-) belongs to the ADAM (**a** **d**isintegrin **a**nd **m**etalloproteinase domain) family of proteins that together with snake venom metalloproteinases (SVMPs) constitutes the reprolysin family of zinc metalloproteinases. The archetypical ADAM protein has structural similarity and 30% sequence identity to SVMPs. The disintegrin domain of SVMPs containing the RGD motif inhibits platelet aggregation by blocking essential procoagulant integrins on platelets, whereas the metalloproteinase domain digests type IV collagen. Currently, the ADAM family comprises more than 30 members, although the function of most of them is scarcely known. Despite the striking similarity to SVMPs only four of ADAMs have been shown to act as metalloproteinases on identified substrates and only some of ADAMs show an ability to interact with integrins. At least half of ADAMs are expressed predominantly in testes and play a role in spermatogenesis and fertilization and others are involved in neurogenesis, myogenesis, osteogenesis and in the regulation of immune responses. Table 1 presents some of the known ADAMs functions (Primakoff & Myles, 2000; Turner & Hooper, 1999; Yamamoto *et al.*, 1999).

THE STRUCTURE OF TACE

The gene encoding TACE comprising approximately 50 kb consists of 19 exons and is localized in human chromosome 2p25 and in murine chromosome 12. Alternative splicing may generate two mature transcripts; the shorter one encodes a protein that lacks the cytoplasmic domain (Cerretti *et al.*, 1999). TACE mRNA was found in many tissues which suggests ubiquitous expression of this protein (Black *et al.*, 1997). Although the amino-acid sequence of TACE shows relatively low homology to other members of the ADAM family, the structure of TACE closely resembles that of other ADAM proteins and contains all the domains characteristic for the whole family (Black *et al.*, 1997) (Fig. 1).

Table 1. Distinct functions of selected ADAM family members

Protein	Function
ADAM1 – 7	spermatogenesis, fertilization
ADAM8, 28	immune functions
ADAM9, 12, 19	myogenesis, osteogenesis
ADAM10, 11, 13	neurogenesis
ADAM17	processing of TNFα

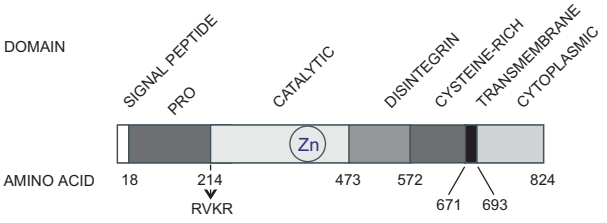

Figure 1. The structure of TACE

TACE is a type I transmembrane protein synthesized as a zymogen. The prodomain, similarly to other ADAMs and also to matrix metalloproteinases is thought to act as an inhibitor of the protease activity *via* the cysteine

switch mechanism (Van Wart & Birkedal-Hansen, 1990). The free cysteine residue from the sequence PKV**C**GVPD present in the prodomain coordinates with the zinc in the active site of the protease thus preventing enzymatic activity before TACE maturation. Prodomain removal is therefore regarded as a prerequisite for TACE activity (Milla *et al.*, 1999). The potential furin cleavage site (RVKR) localized between the pro- and the catalytic domains is responsible for the generation of biologically active TACE by removal of the prodomain by furin and possibly other proprotein convertases (Peiretti *et al.*, 2003a).

The metalloproteinase domain contains the zinc-binding consensus motif **HE**X**GH**X-X**G**XX**HD** (in TACE: **HE**L**GH**NF**G**A**E**HD**)** involved in coordinating zinc with histidine residues and creating the active site of the enzyme (Black *et al.*, 1997; Moss *et al.*, 1997). X-Ray crystal structure analysis of the catalytic domain of TACE shows its substantial topological similarity to the catalytic domains of other ADAMs but also some unique features (Maskos *et al.*, 1998).

Unlike for some other members of the ADAM family an interaction of the disintegrin domain of TACE with integrins has not yet been documented. Analysis of the amino-acid sequence of TACE indicates the presence of an EGF-like domain and a crambin-like domain, unique for TACE, within the untypical cysteine-rich domain (Black *et al.*, 1997), but their roles are not well determined. The cysteine-rich domain may be important for some substrate recognition (Reddy *et al.*, 2000) as well as for the process of TACE maturation (Milla *et al.*, 1999).

The cytoplasmic tail of TACE contains potential sites for interaction with Src-homology 2 (SH2) and Src-homology 3 (SH3) domains and a potential tyrosine phosphorylation site (Moss *et al.*, 1997) as well as potential MAPK phosphorylation site (Diaz-Rodriguez *et al.*, 2002), three putative sites of phosphorylation by PKC and some potential sites of phosphorylation by casein kinase II (ProfileScan database).

TACE is a glycoprotein and this feature has been exploited for its purification using concanavalin A chromatography (Moss *et al.*, 1997). Mature TACE molecule has six putative N-glycosylation sites. Two of them localized in the catalytic domain were shown to undergo glycosylation in the *Saccharomyces cerevisiae* expression system (Clarke *et al.*, 1998). The glycosylation pattern of TACE occurring in mammalian cells has not been determined yet.

Table 2. Putative substrates of TACE

Development and differentiation	Immune system	Other
TGFα	TNFα	APP
HB-EGF	TNF-RI	PrP^c*
amphiregulin	TNF-RII	MUC1
neuregulins	IL1-RII	
TrkA	L-selectin	
HER4 JM-a	fractalkine	
GH-R*	IL6-Rα*	
Notch	M-CSF-R*	
TRANCE*	TRANCE*	

*TACE involved only in stimulated shedding

PUTATIVE PHYSIOLOGICAL ROLES OF TACE

TNFα is not the only substrate of TACE. Generation of transgenic mice with a deletion of the zinc-binding sequence within the catalytic domain of TACE that is necessary for its enzymatic activity ($TACE^{\Delta Zn/\Delta Zn}$) (Black *et al.*, 1997) allowed the identification of other TACE substrates, that might be arbitrarily divided into two major groups of proteins: (i) those involved in development and differentiation and (ii) those involved in the regulation of immune responses (Table 2). Some of the TACE substrates cannot be categorized into either group.

TACE AND DEVELOPMENT

Most TACE$^{\Delta Zn/\Delta Zn}$ mice die at birth due to TACE deficiency that affects development of skin, muscles, lungs, neuronal system and probably other tissues and organs (Peschon *et al.*, 1998; Zhao *et al.*, 2001). Some of the developmental defects such as the failure of eyelids to fuse and specific hair and skin defects observed in the embryos and in the few mice that survive 2–3 weeks closely resemble the defects characteristic for the transforming growth factor-α (TGFα) deficiency. Moreover, it has been shown that the release of TGFα from the cell membrane of TACE$^{\Delta Zn/\Delta Zn}$ cells is inhibited. These observations strongly suggest that TACE-mediated shedding of TGFα is needed for normal mice development (Peschon *et al.*, 1998). These findings were confirmed by recent studies comparing EGFR activation by soluble- *versus* membrane-bound TGFα (Borrell-Pages *et al.*, 2003). The authors examined the activation of EGFR by measuring its phosphorylation state in cells exposed to cells expressing proTGFα but not capable of releasing its ectodomain. The shedding of TGFα was prevented by three approaches (i) use of metalloprotease inhibitors, (ii) use of shedding-defective mutant cells transfected with proTGFα cDNA and, (iii) use of cells transfected with cDNAs of uncleavable proTGFα mutants. Despite the observed interactions between the cells expressing proTGFα and the cells expressing its receptor, no significant EGFR activation occurred. This finding challanges the view of the possibility of juxtacrine activation of cells by transmembrane TGFα (Wong *et al.*, 1989) and stresses the requirement of TGFα shedding for its biological activity. Interestingly, the authors found significant overexpression of TACE in the majority of human breast tumor samples and a correlation between the levels of TACE expression and the levels of EGFR activation (Borrell-Pages *et al.*, 2003).

Since TGFα-deficient mice do not die prematurely, it has been postulated that TACE processes additional substrates important in development. The additional epithelial defects observed in fetuses of TACE$^{\Delta Zn/\Delta Zn}$ mice resemble those reported for mice lacking the EGFR. This receptor generates intracellular signals in response to various growth factors as TGFα, EGF, amphiregulin, heparin-binding EGF (HB-EGF) and others. Many of the ligands that interact with receptors of the EGFR family are synthesized as transmembrane precursors, thus it was postulated that TACE might be essential for the shedding of at least some of them (Peschon *et al.*, 1998). Indeed, Montero *et al.* (2000) showed that cleavage of one of neuregulins – proNRGα 2c was defective in fibroblasts derived from TACE$^{\Delta Zn/\Delta Zn}$ animals and Sunnarborg *et al.* (2002) demonstrated that TACE might shed amphiregulin and heparin-binding EGF (HB-EGF).

The involvement of TACE in the release of the extracellular domain of TRANCE, a TNFα family member participating in osteoclastogenesis was suggested by studies of Lum *et al.* (1999).

TACE may affect developmental processes not only by the shedding of growth factor molecules but also by releasing extracellular domains of growth factor receptors, such as growth hormone receptor (GHR) and one of the isoforms of HER4 as well as TrkA.

Shedding of the ectodomain of GHR by TACE (Zhang *et al.*, 2000) generates growth hormone binding protein (GHRB) that interacts with growth hormone (GH) with high affinity and therefore down-regulates the availability of the ligand. This process together with decreased membrane levels of GHR significantly impairs GH signaling.

HER4/erbB4, a member of the EGFR family, is a receptor for neuregulins, a family of growth and differentiation factors. HER4 can be also activated by some EGF family members. The involvement of HER4 is implicated in the development of a variety of organs and cell types and is essential for heart development. Two isoforms of HER4 exist that differ in their extracellular juxtamembrane region

and susceptibility to shedding (the JM-a isoform undergoes shedding whereas JM-b does not) (Elenius *et al.*, 1997). It has been demonstrated that TACE is essential for releasing the ectodomain of HER4 JM-a from the plasma membrane (Rio *et al.*, 2000). However, the physiological significance of this process remains unknown. It is unclear whether it constitutes a mechanism for receptor down-regulation or whether the HER4 JM-a ectodomain can bind neuregulins and inhibit their effect.

TrkA, the receptor for nerve growth factor (NGF) is involved in the development and survival of neural cells. It has been shown that phorbol esters, known PKC activators, up-regulate cleavage of TrkA generating two fragments: a soluble ectodomain and cell-bound truncated fragments that contain the transmembrane and cytosolic domains. These membrane-associated fragments were found to be phosphorylated and associated with intracellular signaling molecules, which suggested the possibility of ligand-independent activation (Diaz-Rodriguez *et al.*, 1999). Recently, the involvement of TACE in PMA-induced shedding of TrkA was demonstrated (Diaz-Rodriguez *et al.*, 2002) indicating that in this particular case TACE activity would promote stimulation of the processed receptor, TrkA. It is worth mentioning that ectodomain shedding of TrkA was also observed in response to its physiological ligand, NGF (Cabrera *et al.*, 1996), but it remains unknown whether TACE is involved in this process.

A role for TACE in the complex proteolytic processing of Notch has also been proposed. Notch signaling initiated by Notch receptors-Notch ligands interactions that occur between adjacent cells is crucial for regulation of many cell fate decisions at all stages of development of a multi-cellular organism. Signals generated by Notch receptor influence proliferation, differentiation and apoptotic events (Artavanis-Tsakonas *et al.*, 1999).

During export to the cell surface Notch receptor (Notch) undergoes its first proteolytic cleavage by furin that creates a heterodimer of two noncovalently linked domains, the extracellular (ECD) and the intracellular (ICD) one. Ligand binding is thought to induce conformational changes in ECD that expose a proteolytic site for α-secretase responsible for Notch ectodomain shedding. Removal of the large ECD is believed to eliminate steric hindrance for presenilin/γ-secretase, which is responsible for the final intramembrane cleavage of Notch. This process releases Notch intracellular domain that translocates to the nucleus and participates in the activation of target genes, encoding proteins involved in cell differentiation (Bray, 2000). TACE was shown to play the role of α-secretase, whose action is prerequisite for the final proteolytic step of Notch activation (Brou *et al.*, 2000). Interestingly, LaVoie and Selkoe (2003) reported recently that TACE and presenilin/γ-secretase might also cooperate in the processing of Notch ligands.

TACE AND IMMUNE SYSTEM

TACE also participates in the shedding of a number of proteins involved in the functioning of the immune system, such as: cytokines, cytokine receptors and cell adhesion molecules (Table 2). The fact that TACE activity may be regulated at different levels (see further chapters) creates the possibility of its involvement in the complex regulation of immune responses.

However, it is difficult to study *in vivo* the consequences of TACE activity towards elements of the immune system due to premature death of $TACE^{\Delta Zn/\Delta Zn}$ mice. Thus, TACE substrates were identified mostly using appropriate $TACE^{\Delta Zn/\Delta Zn}$ cells. The requirement of TACE for the release of ectodomains of some of them was confirmed using experimental system in which $TACE^{\Delta Zn/\Delta Zn}$ fibroblasts were transfected with a plasmid containing cDNA encoding the protein in question together with a plasmid containing TACE

cDNA or with control plasmid (Althoff *et al.*, 2000; Reddy *et al.*, 2000). The potential roles of TACE in the regulation of immune responses are proposed based on the results of *in vitro* experiments as well as on indirect studies on the differences in activities of membrane-bound *versus* soluble forms of particular TACE substrates.

Originally TACE was discovered as a result of searching for the secretase of TNFα (Black *et al.*, 1997; Moss *et al.*, 1997), the major pro-inflammatory cytokine with broad and diverse immune effects (Aggarwal *et al.*, 2001). Biological activity is attributed to both soluble and transmembrane TNFα, although the effects of the two forms are not always identical (Mueller *et al.*, 1999). There are two TNFα receptors that differ in their intracellular domains and stimulate signal transduction pathways that only partially overlap (MacEwan, 2002; Hehlgans & Mannel, 2002). It has been suggested that membrane-bound rather than soluble TNFα is the prime physiological activator of TNF-RII which may explain some differences in the effects of the two forms of TNFα (Grell *et al.*, 1995). The release of soluble TNFα by TACE promotes systemic effects of this cytokine. This process may be responsible for TNFα-mediated septic shock, since mice with a mutated, uncleavable form of TNFα are not sensitive to otherwise lethal doses of LPS injected in the presence of D-galactosamine (Mueller *et al.*, 1999). Additionally, because of the slightly different activities of the soluble *versus* the transmembrane form of the cytokine, TACE might to some extent modulate the overall outcome of TNFα effects.

The finding that TACE may also release the ectodomains of both TNFα receptors (Peschon *et al.*, 1998; Reddy *et al.*, 2000) adds another level of complexity to the regulation of TNFα activities by TACE. The shedding of TNFα receptors might diminish their levels on the cell surface, thus limiting the cell's responsiveness to the cytokine. Moreover, an increased plasma concentration of soluble TNFα receptors may weaken the systemic effects of the cytokine since soluble forms of both receptors are capable of binding TNFα and reducing its effects (Aderka, 1996). This hypothesis was confirmed by partial elucidation of the etiology of periodic fever syndrome, a genetic disease characterized by unexplained episodes of fever and severe localized inflammation. It was determined that the disease is connected with a mutation in the extracellular part of TNF-RI that impairs its shedding. The levels of soluble TNF-RI in the plasma of patients were half normal and leukocytes showed increased membrane content of TNF-RI and reduced receptor cleavage following stimulation (McDermott *et al.*, 1999). This observation stresses the physiological importance of the shedding processes in the immune system.

Type II IL-1 receptor acts as a decoy receptor by binding IL-1β with high affinity and neutralizing its activity. The soluble form of the receptor that possesses high affinity for IL-1β, but only low affinity for IL-1α, and virtually no affinity for IL-1 receptor antagonist (IL-1ra) is considered an ideal antagonist of the IL-1 system (Symons *et al.*, 1995). TACE might limit the proinflammatory activity of IL-1 by releasing soluble type II IL-1R (Reddy *et al.*, 2000).

In contrast to the majority of soluble receptors that inhibit the actions of their ligands, soluble IL-6 receptor-α (IL-6Rα) acts agonistically upon IL-6 binding. The receptor for IL-6 consists of two chains, IL-6Rα that specifically recognizes and binds IL-6 and gp130 that is a signal-transducing subunit shared by receptors for the IL-6 family of cytokines. Whereas expression of gp130 is ubiquitous, IL-6Rα is expressed on a limited number of cell types (hepatocytes, immune cells) rendering other cells unable to respond to IL-6. However, the complex of IL-6 and soluble IL-6Rα (sIL-6Rα) may bind gp130 on cell membranes (Rose-John & Heinrich, 1994) and through this mechanism called transsignaling virtually all cells expressing gp130, including hemopoietic progenitor cells, endothelial cells, and

neuronal cells may respond to IL-6. Thus, the TACE-mediated generation of sIL-6Rα (Althoff *et al.*, 2000) may result in broadening of the set of cell types that are activated in response to IL-6. For example, it has been shown that endothelial cells express chemokines and cell adhesion molecules in response to IL-6/sIL-6Rα complex (Modur *et al.*, 1997; Romano *et al.*, 1997).

L-Selectin is a glycoprotein expressed on most leukocyte subsets. It mediates the attachment of leukocytes to high endothelial venules of lymphoid tissue as well as the earliest interactions between leukocytes and activated endothelium in nonlymphoid organs (Stamenkovic, 1995). TACE was shown to be involved in L-selectin shedding (Peschon *et al.*, 1998) and thus may affect homing and migration of leukocytes to the sites of inflammation. Indeed, it has been shown that soluble L-selectin decreases local inflammation-mediated leukocyte adherence and vascular leakage *in vivo* (Ferri *et al.*, 2002). Moreover, the increased concentrations of soluble L-selectin in sepsis correlate with the increased survival, and may represent a protective mechanism by which the host attempts to diminish the deleterious systemic effects of activated leukocytes (Seidelin *et al.*, 2002).

Fractalkine (FK, CX3CL1) is a novel multidomain protein expressed on the surface of endothelial cells and neurons (Cotter *et al.*, 2002; Cybulsky & Hegele, 2003). The transmembrane form of FK plays a role of a cell adhesion molecule that tightly interacts with its receptor (CX3CR1) expressed on T-cells, NK cells, monocytes and microglia. Proteolytic cleavage of FK releases a soluble form that is a potent chemoattractant for these cells (Greaves & Schall, 2001). TACE was recognized as a major protease responsible for the inducible cleavage of FK resulting in the conversion of fractalkine from a membrane-bound adhesion molecule to a soluble chemoattractant (Garton *et al.*, 2001; Tsou *et al.*, 2001).

TACE has also been shown to release the ectodomain of macrophage colony-stimulating factor receptor (M-CSFR) in macrophages activated with phorbol esters or LPS. However, the physiological role of this rapid M-CSFR down-modulation requires further investigation (Rovida *et al.*, 2001).

TRANCE is involved not only in osteoclastogenesis but also in regulation of immune responses (Wong *et al.*, 1999). Membrane-bound TRANCE expressed on activated T-cells has the potential to activate dendritic cells (DC) and enhance their survival (Josien *et al.*, 1999). The same is true for the soluble form of TRANCE (Josien *et al.*, 2000) thus the significance of TRANCE shedding for the interaction of DC with T-cells remains mostly unknown at this time.

These few examples demonstrate that TACE is an important element in the network of immunoregulatory interactions.

OTHER ROLES OF TACE

The amyloid-β peptide (Aβ) that accumulates in the brains of Alzheimer's patients is derived by proteolytic cleavage of amyloid precursor protein (APP) by aspartyl β- and γ-secretases. However, under physiological conditions the major part of cellular APP is cleaved by α-secretase within the Aβ domain preventing the generation of the pathogenic peptide. This constitutive process occurring in the Golgi apparatus or at the cell surface can be further stimulated by a number of factors, e.g. activators of protein kinase C (Buxbaum *et al.*, 1993). There is some evidence indicating that TACE is involved in this regulated α-secretase-mediated cleavage of APP (Buxbaum *et al.*, 1998). In cultured cells TACE and β-secretase (BACE) compete for cleavage of APP, and an increased cleavage of APP by TACE results in decreased generation of amyloid-β peptide (Skovronsky *et al.*, 2000). Although these results may suggest a role for

TACE in limiting plaque formation in Alzheimer's disease, no differences in TACE levels have been observed between brains of affected and normal subjects. Nevertheless, there is potential for development of new therapeutic approaches based on influencing the balance of TACE *versus* BACE activities (Skovronsky *et al.*, 2001).

Similarly, TACE, identified as an enzyme responsible for inducible cleavage of cellular prion protein (PrP^c) within its "toxic" domain, is considered as a putative cellular target of a therapeutic strategy aimed at depleting cells of the "toxic" domain of PrP^c (Vincent *et al.*, 2001).

TACE may also play a role in shedding of MUC1, the integral cell membrane mucin expressed on epithelial cells including uterine epithelium. MUC1 inhibits the interaction between blastocyst and epithelial adhesion molecules (Aplin *et al.*, 2001) but a local loss of MUC1 was demonstrated at the site of blastocyst attachment to cultured human uterine epithelium (Aplin *et al.*, 2001) and some data suggested the involvement of an ADAM family member in this process (Olson *et al.*, 1998). Recently Thathiah *et al.* (2003) showed that in contrast to normal as well as ADAM9, 12, 15-deficient fibroblasts, human MUC1 was not shed from $TACE^{\Delta Zn/\Delta Zn}$ fibroblasts, however, transfection of these cells with TACE cDNA restored MUC1 ectodomain release. Moreover, the presence of TACE in human uterine endometrium during the receptive phase has been demonstrated by immunocytostaining as well as the interactions between MUC1 and TACE have been shown using co-precipitation experiments. Thus, TACE appears to be a good candidate for the MUC1 secretase that may create an environment promoting embryo implantation, at least in some species (Thathiah *et al.*, 2003). It is very interesting that during the receptive phase the level of MUC1 is elevated throughout human (and rabbit) uterine epithelium and diminished only locally at the site of implantation (Thathiah *et al.*, 2003). If TACE is actually responsible for this effect, its activity has to be locally stimulated by an unknown mechanism.

SUBSTRATE SPECIFICITY

TACE appears to be a secretase with a wide range of substrates and the list of proteins susceptible to its proteolytic activity is still growing. However, the broad substrate specificity of TACE was recently questioned by Mohan *et al.* (2002). Kinetic studies indicated that recombinant TACE as well as a cell membrane extract enriched in TACE were able to efficiently process a synthetic peptide corresponding to the TNFα cleavage site (k_{cat}/K_m = $1.2 \times 10^5\ M^{-1}s^{-1}$), but conversion of peptides derived from APP and TNF-RII occurred only at high enzyme concentrations and prolonged reaction times while peptides derived from other putative substrates (TNF-RI, IL-6R, Notch) were not processed at all (Mohan *et al.*, 2002). It is possible that some of the model peptides are not presented to the enzyme the way the intact substrates are. For example, membrane anchoring of both TACE and TNF-RII is required for efficient proteolysis of TNF-RII. Alternatively, the existence of additional factors that modify substrate presentation and promote their recognition by TACE could explain the discrepancy of these kinetic

Table 3. Cleavage sites in TACE substrates

Substrate	Cleavage site	Distance from membrane*
TNFα	PLAQA ↓ VRSSS	20
TNFR-I	PQIEN ↓ VKGTE	10
TNFR-II	APGAV ↓ HLPQP	43
IL-6Rα	SLPVQ ↓ DSSSV	1
L-selectin	QKLDK ↓ SFSMI	11
APP	VHHQK ↓ LVFFA	12

*Number of amino acids

data with the results of experiments performed in cells. It is also possible that interactions distal to the cleavage site are required for the recognition of at least some substrates as was shown for type II IL-1R (Reddy *et al.*, 2000). The interaction between TACE and TNFα seems to be unique since the catalytic domain of TACE is sufficient for accurate cleavage of TNFα in solution. However, under more physiological conditions, i.e. in the whole cell system TACE must contain at least its transmembrane domain allowing for its membrane anchoring to cleave the substrates (Itai *et al.*, 2001; Reddy *et al.*, 2000). Moreover, co-incubation of cells expressing TACE with cells expressing its substrate does not result in its shedding indicating that TACE acts only on substrates expressed on the same cell (Itai *et al.*, 2001; Reddy *et al.*, 2000).

The mechanism of substrate recognition by TACE (and also by other secretases) remains a mystery. As shown in Table 3 cleavage sites differ between distinct TACE substrates. There are also no similarities in the amino-acid sequences surrounding the cleavage sites.

Moreover, mutagenesis of residues around the cleavage sites (or sometimes even of those creating the scissile bond) did not prevent shedding of some TACE substrates (Althoff *et al.*, 2001; Brakebusch *et al.*, 1994; Migaki *et al.*, 1995; Mullberg *et al.*, 1994) making the existence of a consensus sequence conferring sheddability highly questionable. It has been proposed that the length of the stalk sequence between the membrane and the first extracellular domain of the substrate may be important for its shedding since deletion of more than 10 amino acids from this part of proTNFα abolished the release of soluble TNFα (Tang *et al.*, 1996). However, other features must also be important for this process since an exchange of the proTNFα cleavage domain for the IL-6 cleavable stalk sequence resulted in a chimeric protein resistant to shedding (Althoff *et al.*, 2000). This phenomenon seems to be rather unique since insertion of TGFα or APP cleavage site into betaglycan (type III TGFβ receptor) rendered the latter protein susceptible to shedding (Arribas *et al.*, 1997). Similarly, replacing a short sequence of gp130 (that is not significantly susceptible to shedding) with cleavage site peptides of TNFα, TGFα or IL-6Rα) allowed for the release of its ectodomain. Surprisingly, when the short juxtamembrane part of gp130 was exchanged with the corresponding region of LIF that is also not a substrate for secretases, the resulting chimeric protein was susceptible to shedding (Althoff *et al.*, 2001). Based on all the results of these mutagenetic experiments, it was suggested that the structural integrity of the extracellular domain rather than the amino-acid sequence of the juxtamembrane fragment determines shedding behavior of membrane proteins (Althoff *et al.*, 2001). However, the fact that TACE is able to cleave some of its substrates in their soluble forms at the expected sites (Kiessling & Gordon, 1998; Mohan *et al.*, 2002) suggests some importance of the amino-acid sequences surrounding the cleavage sites.

REGULATION OF TACE ACTIVITY

The constitutive shedding of membrane proteins is strongly enhanced by various external stimuli with phorbol esters, activators of protein kinase C (PKC), being the most potent and most extensively studied. For some proteins distinct enzymes are involved in their constitutive and stimulated processing. For example the constitutive shedding of fractalkine is mediated by ADAM10, whereas TACE seems to be responsible for the PMA-stimulated proteolysis (Henhouse *et al.*, 2003). The involvement of TACE in the shedding of a number of proteins was shown only for the activated and not for the constitutive process (see Table 2). Numerous studies indicated that the stimulation of shedding involves activation of secretases rather than their substrates (Kiessling & Gordon, 1998). Despite

its obvious importance, the mechanism of TACE activation remains largely unknown. A few possibilities may be considered: (i) activation of TACE by phosphorylation of its cytoplasmic tail, (ii) activation of TACE through interactions between its cytoplasmic tail and proteins involved in intracellular signaling, (iii) activation of TACE through inducing its maturation followed by directing of mature TACE to the cell membrane.

The majority of studies undertaken to elucidate the mechanism of TACE activation were performed using PMA, a non-physiological stimulator of PKC. Although for some TACE substrates the physiological stimulators of their shedding are known (as for example f-MLP, PAF and others in the case of L-selectin or NGF in the case of its receptor TrkA) there are no (as to our knowledge) published, direct proofs of the involvement of TACE in these physiological processes.

Since shedding of some TACE substrates can also be stimulated by growth factors, studies have been conducted to compare the effects and intracellular mediators involved in the PMA- and growth factor-stimulated shedding. Fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) were shown to induce the release of TGFα, TNFα and L-selectin from the cell membrane within 20 min of treatment to an extent similar to that induced by PMA (Fan & Derynck, 1999). The authors also demonstrated an involvement of extracellular signal-regulated kinase (Erk) belonging to MAP kinases in the growth factor- as well as in the PMA-induced shedding. This finding was confirmed by recent studies demonstrating that Erk indeed acts as an intermediate in PMA-regulated TrkA cleavage (Diaz-Rodriguez *et al.*, 2002). Moreover, it was shown that Erk associated with TACE and phosphorylated the enzyme at threonine^{735} and that the mutation Thr^{735} → Ala significantly diminished PMA-induced TrkA cleavage. Moreover, phosphorylation of threonine^{735} also occurred in HEK293 cells exposed to EGF or nerve growth factor (Diaz-Rodriguez *et al.*, 2002). However, the studies by Fan *et al.* (2003) indicated that upon treatment of cells with EGF or FGF, TACE is phosphorylated only on serine and not on threonine or tyrosine residues. Moreover, mutagenetic studies showed that this phosphorylation on serine was not required for the growth factor (serum)-induced TGFα shedding. The activation of TACE by PMA without an involvement of the Erk pathway was also demonstrated (Montero *et al.*, 2002). However, it should be mentioned that the activity of truncated TACE that does not contain its cytoplasmic tail was shown to be stimulated by phorbol esters which suggested that the activation of TACE by PMA is not based on any modification of this domain (Reddy *et al.*, 2000). This finding was unexpected, since activation of another ADAM family member, ADAM9 (MDC9) by PMA required phosphorylation of its cytoplasmic tail by PKCδ (Izumi *et al.*, 1998). Elucidation of these discrepancies requires further studies.

Apart from the possible association of Erk with TACE, other proteins have been shown to interact with the cytoplasmic tail of TACE. One of them is MAD2, a protein participating in the control of mitosis, but the possible role of this interaction in the regulation of shedding is unclear (Nelson *et al.*, 1999). The scaffolding protein synapse associated protein 97 (SAP97) was also identified as a binding partner of the cytoplasmic domain of TACE. This interaction involves the third PDZ domain of SAP97 and the extreme C-terminal amino-acid sequence of TACE. Moreover, overexpression of SAP97 diminished the ability of TACE to process its substrates suggesting an involvement of SAP97 in the regulation of TACE activity (Peiretti *et al.*, 2003b). Another protein that interacts with the C-terminus of TACE *via* a PDZ domain is the protein-tyrosine phosphatase PTPH1, which similarly to SAP97 may play a role as a negative regulator of TACE activity (Zheng *et al.*, 2002).

Studies have been undertaken to examine whether intracellular processing and trafficking might be involved in the regulation of TACE activity. Immunostaining of TACE in cells showed that some mature form of TACE is present on the cell surface but the majority of TACE localizes to the perinuclear area of the cell. Stimulation of cells with PMA for 30 min did not significantly affect TACE distribution, although it cannot be excluded that a small portion of TACE was directed to the cell membrane and mediated increased shedding (Schlondorff *et al.*, 2000). There is also a possibility that TACE-mediated ectodomain shedding may occur not only from the cell membrane but also in intracellular compartments (Schlondorff *et al.*, 2000). Interestingly, it has been shown that treatment of different cells with PMA caused down-regulation of surface expression of TACE resulting from its internalization and degradation (Doedens & Black, 2000). This process was less rapid than the shedding response.

TACE activity was strongly diminished in furin-deficient cells as well as in cells transfected with cDNA coding for selective inhibitor of proprotein convertases indicating that furin is the major proprotein convertase involved in the maturation/activation of TACE (Peiretti *et al.*, 2003a). Although in normal cells maturation of TACE precedes its appearance in the cell membrane, the removal of the prodomain seems not to be a prerequisite for cell-surface expression of TACE, since the immature form of TACE was detected in the cell membrane of cells deprived of active proprotein convertases (Peiretti *et al.*, 2003a).

Nitric oxide and reactive oxygen species (ROS) were shown to activate TACE-mediated ectodomain shedding (Zhang *et al.*, 2000; Zhang *et al.*, 2001). It has been proposed that the presence of these compounds may lead to nitrosation or oxidation of a cysteine sulphydryl group in the prodomain. This would limit coordination of the catalytic zinc by the cysteine residue and would result in the activation of the latent form of TACE.

Analysis of the biosynthesis of TACE in mutant cell lines that have a general defect in ectodomain shedding showed an insufficient removal of the prodomain of TACE (Borroto *et al.*, 2003). This defect was not due to a deficiency in the activity of proprotein convertases but to a blockade of the exit of TACE from the endoplasmic reticulum. Since the processing of three other ADAMs as well as metalloproteinase MT1-MMP was normal in the mutant cells, the existence of a specific mechanism that directs the proteolytic activation of TACE through the control of its exit from the ER has been suggested (Borroto *et al.*, 2003).

A very interesting mechanism of the regulation of TACE activity was proposed based on studies on L-selectin release from neutrophils (Gomez-Gaviro *et al.*, 2002). The authors examined the effects of 14 different non-steroidal anti-inflammatory drugs (NSAID) on TACE-mediated shedding of L-selectin and found that these compounds differ in their ability to reduce L-selectin surface expression. Diphenylamine-related compounds had the strongest shedding-stimulatory activity and since they failed to down-regulate the surface expression of L-selectin on a $TACE^{\Delta Zn/\Delta Zn}$ monocytic cell line, it was suggested that in normal cells their effects were mediated through stimulation of TACE. Interestingly, the magnitudes of L-selectin down-regulation caused by particular drugs correlated well with their effects on the decrease of the ATP pool in the cells. These results led to the intriguing hypothesis that in resting cells TACE-mediated release of L-selectin is prevented by an ATP-dependent mechanism (Gomez-Gaviro *et al.*, 2002). Stimulation of cells or deprivation of ATP would result in switching off this mechanism allowing activation of TACE. It has not been shown whether this NSAID-induced, TACE-mediated shedding is limited to L-selectin or is a more general phenomenon.

INHIBITION OF TACE ACTIVITY

Tissue inhibitor of metalloproteinases 3 (TIMP-3) is the only known physiological inhibitor of TACE activity (Amour *et al.*, 1998). To date four members of the TIMP family have been identified in mammals. All of them inhibit active forms of all matrix metalloproteinases (MMP) but only TIMP-1 and TIMP-3 show inhibitory activity against some members of the ADAM family. ADAM10 which shares most structure similarity and some substrate specificity with TACE is inhibited by both TIMP-1 and TIMP-3 whereas TACE remains fully active in the presence of TIMP-1 (Baker *et al.*, 2002). These differences in TIMPs specificities make them useful in distinguishing MMP and ADAM10 activities from that of TACE in *in vitro* experiments. Down-regulation of expression of TIMP-3 by cytokines (Bugno *et al.*, 1999) may result in the increase in TACE activity during inflammatory states.

REGULATION OF TACE EXPRESSION

In their pioneering work, Black *et al.* (1997) showed significant differences in TACE mRNA levels in different tissues and in tissues at distinct stages of development (fetal *versus* adult). However, they did not observe differences in the surface expression of TACE in cells stimulated with various reagents as for example in monocytes activated with LPS for 1.5 h. This observation suggested that TACE is not regulated at the transcriptional level. However, in view of later findings (Schlondorff *et al.*, 2000), it seems that the surface expression of TACE might not be an adequate indicator of its cellular abundance nor its activity and susceptibility to activation. There are scarce data demonstrating a lack of changes in TACE mRNA or TACE protein levels in cells exposed to common activators. For example Imaizumi *et al.* (2000) did not detect any increase in TACE mRNA in HUVEC exposed to LPS or IL-1β. There is, however, growing evidence indicating that the expression of TACE can be regulated. Increased levels of TACE mRNA were observed in HL-60 cells stimulated with LPS (Ding *et al.*, 2001) and in murine retinal endothelial cells exposed to vascular endothelial growth factor (VEGF) (Majka *et al.*, 2002). Up-regulation of human TACE mRNA was also demonstrated in *ex vivo* experiments. Patel *et al.* (1998) found elevated levels of TACE mRNA in osteoarthritis- and rheumatoid arthritis-affected cartilage relative to almost undetectable amounts of TACE mRNA in normal cartilage. Since cartilage taken from patients suffering from arthritis produces also TNFα, the authors propose that the increased levels of TACE might promote inflammatory state of tissue (Patel *et al.*, 1998). However, increased levels of TACE may also have the opposite, anti-inflammatory effect on cells that do not secrete TNFα but express its receptors. For example endothelial cells (EC) that do not produce TNFα are highly responsive to this cytokine due to the presence of both TNFα receptors (Madge & Pober, 2001). Stimulation of expression of cell adhesion molecules that promote migration of leukocytes to sites of inflammation and involvement of TNFα in the induction of iNOS (at least in some species) (Bereta *et al.*, 1993; Bereta *et al.*, 1992) are among the most important effects of TNFα on endothelium that may strongly influence the extent and severity of inflammatory reaction. We found that the levels of TACE mRNA are increased in various endothelial cells exposed to TNFα and that this effect was further potentiated by IL-1β and IFNγ. TNFα apparently stimulated transcription of TACE, since its effect was inhibited by actinomycin D. Augmented levels of TACE mRNA were accompanied by up-regulation of TACE protein in murine brain EC as well as by increased shedding of TNF-RI (Bzowska *et al.*, in preparation). These results suggest that TNFα-mediated stimulation of TACE expression in EC might be a part of a novel regulatory loop in

which the action of the ligand leads to a decrease in its receptor level through increased level/activity of TACE.

Analysis of a DNA fragment representing the promoter and the 5′upstream region of the gene encoding murine TACE revealed the existence of several binding sites for AP2 and Sp1 (Mizui *et al.*, 1999). Although Sp1 binding sites are characteristic mainly for housekeeping genes, the involvement of Sp1 in the stimulation of transcription of some genes has been described (Giraudo *et al.*, 1998; Sanceau *et al.*, 1995; Yan & Ziff, 1997). Moreover, AP2 together with Sp1 have been shown to be involved in activation of VEGF transcription in endothelial cells (Berra *et al.*, 2000). Thus, it is possible that Sp1 or the AP2/Sp1 complex may be involved in regulation of TACE expression. The typical binding sites for transcription factors, such as NF-κB, AP1, STATs, that are commonly activated by cytokines, were not found in the proximity of the transcription start site of the TACE gene. However, the existence of such sequences at a larger distance from the promoter region cannot be excluded. Expression of at least one member of the ADAM family, ADAM8, was shown to be stimulated in response to LPS and IFNγ (Kataoka *et al.*, 1997).

DESIGN OF INHIBITORS OF TACE ACTIVITY AND THEIR POSSIBLE THERAPEUTIC IMPLICATIONS

Since soluble TNFα plays a major role in some acute as well as chronic inflammatory diseases as rheumatoid arthritis or Crohn's disease, TACE represents an important target for the design of specific synthetic inhibitors that might be used in therapies. Inhibition of the processing of TGFα and of other ligands of the EGFR family by blocking TACE activity might also be adopted for treatment of certain tumors, especially that dramatic overexpression of TACE is observed in the majority of mammary tumors (Borrell-Pages *et al.*, 2003). Derivatives of hydroxamic acid that chelate zinc are among the most potent inhibitors of metalloproteinases but they are not sufficiently specific. Attempts to develop synthetic, highly selective inhibitors of TACE have been undertaken by a number of research groups and the efficacy and safety of these putative drugs are being tested in animal models (Beck *et al.*, 2002; Duan *et al.*, 2002; Rabinowitz *et al.*, 2001).

Design of special muteins of tissue metalloproteinase inhibitor 3 (TIMP-3) is another approach to generate effective TACE inhibitors. The N-terminal domain of TIMP-3 (N-TIMP-3) retains its inhibitory activity against TACE and specially designed point mutations resulted in N-TIMP-3 muteins that showed significantly improved potency against TACE (Lee *et al.*, 2002; Lee *et al.*, 2003).

However, the broad substrate specificity of TACE may hamper the therapeutic approaches based on inhibition of TACE activity unless they could be applied locally.

REDUNDANCY OF METALLOPROTEINASES

Although TACE is still regarded as the major secretase responsible for PMA-induced shedding (Moss & Lambert, 2002), recent data indicates the existence of some redundancy in the sheddases' activity as well as possible cooperation between various metalloproteinases.

TACE is responsible for shedding of more than 90% of TNFα from the cell membrane (Black *et al.*, 1997), however, some TNFα release occurs also in fibroblasts derived from $TACE^{\Delta Zn/\Delta Zn}$ mice (Reddy *et al.*, 2000). Another ADAM family member, ADAM10, was shown to be able to cleave proTNFα (Rosendahl *et al.*, 1997) and a role for matrix metalloproteinase 7 (MMP-7) in TNFα release from macrophages has also been suggested (Haro *et al.*, 2000). However, although both enzymes processed a peptide that spans the cleavage

sequence of proTNFα in the correct site, the specificity constants (k_{cat}/K_m) were relatively low: 10-fold lower for ADAM10 and 30-fold lower for MMP-7 comparing to TACE (Mohan *et al.*, 2002; Moss *et al.*, 2001).

Another example of a possible redundancy came from studies on TGFα shedding. Surprisingly, TGFα was shown to be efficiently cleaved from the membrane of TACE$^{\Delta Zn/\Delta Zn}$ cells after their treatment with APMA, an activator of metalloproteinases that contain cysteine-switch (Merlos-Suarez *et al.*, 2001). Apart from TACE, which is an efficient proTGFα N-terminal sheddase, the existence of another convertase that can process proTGFα at the C-terminal site has been recently demonstrated. It is important to stress that both proteolytical reactions are required to yield active TGFα (Hinkle *et al.*, 2003).

The existence of a secretase which, similarly to TACE, is able to release NRGα2c was demonstrated by Montero *et al.* (2002). This secretase seems to recognize a different cleavage site in the NRGα2c molecule and its activity is regulated *via* p38 MAPK. Moreover, some results indicate the possibility of cooperation between TACE and this other secretase (Montero *et al.*, 2002).

It has been shown that, apart from TACE, also ADAM9 may release the ectodomain of TRANCE, suggesting a potential role for this secretase in shedding TRANCE in osteoblasts where both proteins are highly expressed (Chesneau *et al.*, 2003).

Several studies suggested that apart from TACE also ADAM9 and ADAM10 are likely candidate α-secretases for APP (Hotoda *et al.*, 2002; Koike *et al.*, 1999; Lopez-Perez *et al.*, 2001). Recently, it has been demonstrated that indeed all three ADAMs act as APP α-secretases (Asai *et al.*, 2003).

CONCLUDING REMARKS

Although six years of studies on TACE have brought an enormous amount of information, there are still many unanswered questions or even discrepancies of some results. It remains unclear how TACE recognizes its substrates and what is the mechanism that leads to TACE activation. It is possible that TACE activities towards distinct substrates are differently regulated in various cell types. It might also be hypothesized that cooperation with other proteins is required for the recognition of some substrates and/or for the expression of TACE activity. It is still unclear whether TACE may interact with integrins or take part in any adhesion events. To date the roles of EGF- and crambin-like domains remain enigmatic. Further studies using diverse experimental approaches are needed to elucidate these subjects and to verify the created hypotheses.

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