TACE/ADAM-17 maturation and activation of sheddase activity require proprotein convertase activity

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Received 5 September 2003; accepted 25 September 2003

First published online 16 October 2003

Edited by Veli-Pekka Lehto

Abstract Proprotein convertases (PCs) have been proposed to play a role in tumor necrosis factor-α converting enzyme (TACE) processing/activation. Using the furin-deficient LoVo cells, as well as the furin-proficient synoviocytes and HT1080 cells expressing the furin inhibitor α_1-PDX, we demonstrate that furin activity alone is not sufficient for effective maturation and activation of the TACE enzyme. Data from in vitro and in vivo cleavage assays indicate that PACE-4, PC5/PC6, PC1 and PC2 can directly cleave the TACE protein and/or peptide. PC inhibition in macrophages reduced the release of soluble TNF-α from transmembrane pro-TNF-α. We therefore conclude that furin, in addition to other candidate PCs, is involved in TACE maturation and activation.
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Key words: Tumor necrosis factor-α converting enzyme; Tumor necrosis factor-α; Proprotein convertase; Furin; LoVo cell; AT-PDX

1. Introduction

As implied by its acronym, TACE (tumor necrosis factor-α converting enzyme) or ADAM-17 was originally cloned and named for its ability to cleave and convert tumor necrosis factor-α (TNF-α) into a soluble form [1,2]. Since then, TACE has been demonstrated to solubilize a variety of substrates including transforming growth factor-α (TGF-α) [3], members of the membrane-bound epidermal growth factor (EGF) family ligand, the Notch receptor [4], the chemokine fractalkine [5], both TNF receptors, TNFR-I [6] and TNFR-II [3], macrophage/colony-stimulating factor receptor [7], L-selectin [3], and the β-amyloid precursor protein [8]. As predicted by the wide variety of TACE substrates, in vivo inhibition of TACE or disruption of the TACE gene resulted in the death of mice between embryonic day 17.5 and the first day after birth [3] due to a number of developmental defects reminiscent of those seen in mice lacking TGF-α or EGF receptor [9]. In addition, TACE inhibition alone or in combination with matrix metalloproteinases (MMPs) has been demonstrated to have therapeutic potential in a variety of pathological conditions including polycystic kidney disease [10], airway inflammation [11] and arthritis [12,13]. Because of the wide physiological and pathological importance of TACE, much attention has been paid to its structure and mechanism of activation.

Similar to other members of the ADAM family, the structure of TACE is characterized by distinct domains which include a prodomain, a metalloprotease and a disintegrin domain, followed by a cysteine-rich domain containing an EGF-like repeat, a transmembrane domain and a cytoplasmic tail. The prodomain of catalytically active TACE is now known to act as an inhibitor of the protease via a cysteine switch mechanism consisting of an intramolecular complex between an unpaired cysteine residue of the prodomain and the zinc atom in the active catalytic site [14]. TACE can be activated by nitrosation, alkylation, and oxidation, resulting in the dissociation of the cysteine thiol–zinc linkage and thereby release of the prodomain inhibitory function [14,15]. Also, prodomain removal is likely to be a prerequisite for the activation of the TACE protease. This activation pathway is predominant for the activation of several MMPs and appears to be mediated by furin and related proprotein convertases (PCs) [16,17]. Despite the potential importance of this activation mechanism for the TACE enzyme, little is still known about the identity of the convertase(s) involved in this processing. Members of the PC family are potential candidates as a TACE convertase as first predicted by the identification of a putative furin-like recognition site (^{212}RVKR) at the junction between the TACE prodomain and the catalytic domain [1,2]. Also, co-expression of pro-TACE and furin in insect cells results in efficient maturation of TACE [17]. TACE prodomain removal has been reported to occur in a late Golgi compartment, a feature also consistent with a role for furin-like PCs in this process [18].

The mammalian PCs comprise a complex family of enzymes that includes furin, PC1, PC2, PC4, PC5/PC6 and PC7 [19]. While most studies have focussed on substrate cleavage by furin, it is now becoming more evident that the other convertases may perform redundant cleavage functions due to recognition of similar basic cleavage sites (e.g. KR or RR) [20,21]. In the case of furin, this convertase has been demonstrated to mediate the activation of various proenzymes involved in matrix remodeling such as the membrane type forms of the MMPs and most proteolytic active ADAMs (e.g. ADAM-9 [22], ADAM-10 [23], ADAM-12 [24], ADAM-15 [25], ADAM-19 [26], and ADAMTS4 [27]). However, some metalloproteases such as ADAM-10 have also been shown to

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0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)01159-1

be activated by other furin-like PCs [23]. The sum of these data leads us to conclude that while furin plays an important role in MMP and ADAM activation, it is likely that other convertases are also involved. In this report, we used several gene complementation and enzyme inhibition systems to present evidence that furin is a convertase involved, in part, in the processing and activation of TACE. This result confirms very recent studies indicating the requirement of furin in TACE maturation [28,29]. We also uncovered the existence of candidate PCs including PACE-4, PC5/PC6, PC1 and PC2 with the ability to specifically process the TACE enzyme.

2. Materials and methods

2.1. Cell cultures

Adenocarcinoma LoVo cells (ATCC, Rockville, MD, USA) were maintained in F-12 nutrient mixture (Gibco BRL, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS; Bio Media, Drummondville, QC, Canada). HT1080 cells (human fibrosarcoma; ATCC) were cultured in minimum essential medium (Gibco BRL) with 10% FBS. Rat synovial cells were isolated and cultured as previously described [30]. Jurkat cells (human acute T leukemia cells; ATCC) were cultured in RPMI 1640 medium (Gibco BRL) with 10% FBS. Human THP-1 cells (human acute monocytic leukemia from ATCC) were maintained in RPMI 1640 medium with 10% FBS, 2.5 g/l D-glucose, 1 mM sodium pyruvate (Gibco BRL) and 10 μM β-mercaptoethanol (Sigma, Oakville, ON, Canada). MonoMac-1 cells (human acute monocytic leukemia from DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 medium with 5% FBS, 1 mM sodium pyruvate and 100 μM non-essential amino acids (Gibco BRL).

2.2. Cell transfectants

LoVo cells transfected with furin were a kind gift of Dr. N. Kitamura (Institute for Liver Research, Kansai Medical University, Osaka, Japan) [31]. The control clone (LoVo Neo) and two clones transfected with furin (LoVo Fur1 and LoVo Fur2) were maintained in Ham F-12 medium supplemented with 10% FBS and 600 μg/ml geneticin (G-418, Gibco BRL).

The AT-PDX gene, kindly provided by Gary Thomas (University of Oregon, Portland, OR, USA), was inserted into the *Eco*RI/*Apa*I cloning site of a pcDNA3 plasmid vector containing a geneticin resistance cassette. pcDNA3-AT-PDX or the empty control vector was transfected into HT1080 cells and plasmid-expressing cells were selected using 600 μg/ml geneticin. One control clone (HT1080 Neo) and one clone expressing the highest level of HT1080-AT-PDX (out of nine obtained), as determined by Western blotting, were retained.

2.3. Western blot analysis

Cells (2×10^6) were seeded onto 60 mm Petri dishes in culture medium containing 1% FBS and treated with varying concentrations of dec-RVKR-cmk (Bachem, Torrance, CA, USA). For experiments not involving inhibitors, 2.5×10^6 cells were seeded onto 10 cm Petri dishes in culture medium containing 10% FBS. After 24 h incubation, Western blot analysis was performed as previously described [30] using 1:1000 rabbit anti-TACE polyclonal antibodies (Chemicon). As previously described, LoVo cells were infected with vaccinia recombinant for PC1/PC3, PACE-4, PC5/PC6, furin or PC7 or an unrelated control vaccinia recombinant both used at a multiplicity of infection (MOI) of 5 [21]. Eighteen hours after infection, cells were lysed, aliquots were separated on reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and immunoblotted using rabbit anti-TACE polyclonal antibodies.

2.4. Adenoviral vector construction and cell infection

The gene encoding full length AT-PDX was inserted into the multiple cloning site of the transfer vector pAd-TR5F-DC-GFP and placed under the control of a modified cytomegalovirus (CMV) promoter containing a tetracycline-regulated expression cassette [32,33] and expressed together with the green fluorescent protein (GFP) tracer. The production of adenoviral vectors was performed as described [33] and titered by flow cytometry using GFP fluorescence as a marker of infection. AdCMVtTA, expressing the transactivator tTA under control of a constitutive CMV promoter, was obtained from Dr. Bernard Massie (Biotechnology Research Institute of Montreal, Montreal, QC, Canada).

2.5. TNF-α release

MonoMac-1 and THP-1 cells (1×10^6) were preincubated for 22 h with varying concentrations of dec-RVKR-cmk. Then, 500 ng/ml of *Escherichia coli* lipopolysaccharide (LPS) O127:B8 and 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) were added. After 3 h, cells and supernatants were harvested and lysates were prepared as described above. An enzyme-linked immunosorbent assay (Quantikine® from R&D System, Minneapolis, MN, USA) was used to measure supernatant and cell-associated TNF-α concentrations.

2.6. TNFR p75 shedding

LoVo Neo and LoVo Fur2 cells (2×10^6) were seeded onto 60 mm Petri dishes in culture medium containing 1% FBS and treated with 100 μM dec-RVKR-cmk. After 24 h of incubation, cells were fixed in 2% paraformaldehyde and permeabilized with 0.1% saponin. For immunolabeling, cells were incubated overnight with R-phycoerythrin-conjugated anti-TNFR-II antibody (1:20; Caltag Laboratories, Burlingame, CA, USA), R-phycoerythrin-conjugated anti-IgM (1:100; Jackson ImmunoResearch, West Grove, PA, USA), or rabbit anti-TACE antibody (1:100; Chemicon). Cells were washed and incubated with fluorescein-conjugated anti-rabbit IgG (1:1000; Jackson ImmunoResearch) in phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% saponin for 30 min. A FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) was used for fluorescence detection. Results were analyzed using the CellQuest™ software (Becton Dickinson).

2.7. In vitro cleavage assays

Recombinant PCs were expressed in Schneider 2 cells and purified as described [34]. Peptide cleavage assays were performed with two TACE peptides (long and short form) spanning the activation site in the N-terminal region. The sequences of the TACE peptides are: PEELVHRVKRRADPDPMK (18-mer TACE peptide) and LVDREPPEELVHRVKRRADPDP (22-mer TACE peptide). The peptides were synthesized by the Sheldon Biotechnology Institute (Montreal, QC, Canada). In vitro cleavage assays consisted of 5 U enzyme (1 U = 1 pmol of AMC released/h) and 5 nmol of each peptide. Analysis of cleavage was carried out either by direct injection of the reaction mixture on high performance liquid chromatography (HPLC) or through mass spectrometry (MS) analysis. Laser desorption/ionization MS analysis was performed with a Applied Biosystems (Framingham, MA, USA) voyager-DE STR time of flight mass spectrometer with delayer extraction, operating with a pulsed nitrogen laser at 337 nm S.

3. Results and discussion

3.1. Role of furin in the processing of TACE

We first investigated the proportion of unprocessed pro-TACE and mature TACE in various hematopoietic and non-hematopoietic cell types (Fig. 1). The cells used include the furin-positive cell lines THP-1, Jurkat, HT1080 and rat synoviocytes as well as the furin-deficient LoVo cells. Cell lysates were assessed by immunoblotting for the production of TACE proteolytic fragments using TACE-specific antibodies directed against the cytoplasmic domain of the enzyme, so both the precursor (pro-TACE) and the mature forms (TACE) would be detected. As demonstrated in Fig. 1, TACE is predominantly expressed as a 100 kDa mature form in all of these cell lines except in LoVo cells where an additional 110 kDa band (pro-TACE) is observed. This suggests that the TACE enzyme is completely processed in all the furin-positive cell lines tested so far.

To investigate the role of furin in TACE maturation, we next carried out complementation studies in LoVo cells (Fig.

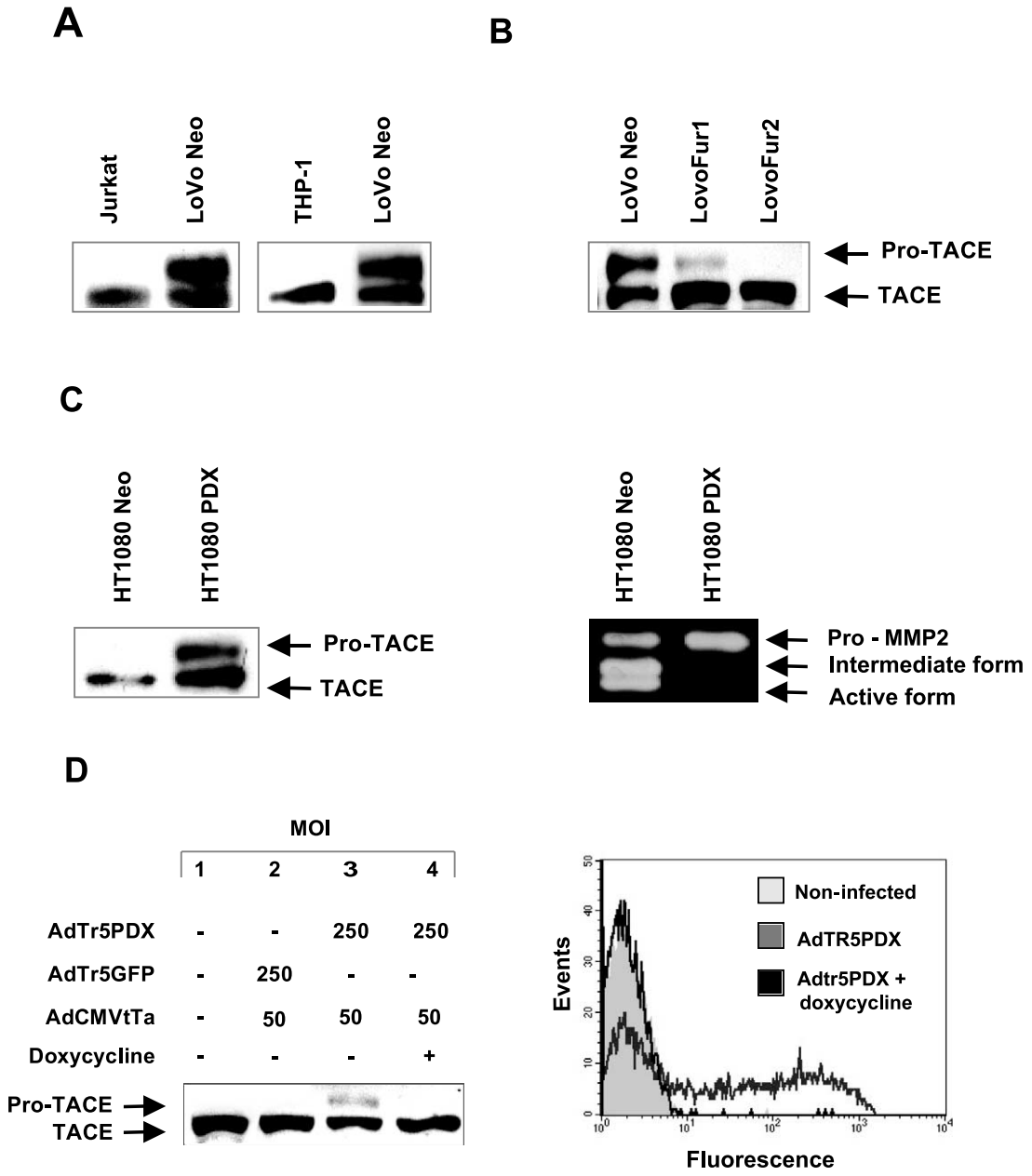


Fig. 1. Maturation of TACE by furin. A: Western blot analysis of TACE expression in cell lysates of Jurkat, THP-1 or LoVo Neo cells. B: LoVo Neo cells transfected with either an empty vector (Neo) or the *fur* gene (Fur1, Fur2). C: HT1080 cells transfected with either an empty vector (Neo) or AT-PDX (PDX) (left panel); and zymography of HT1080 supernatants showing MMP-2 maturation (right panel). D: Primary cultures of rat synovial cells were infected for 40 h with AdTr5PDX, AdTr5GFP, or AdCMVtTa at the indicated MOIs. The infection was carried out in the presence of doxycycline (1 μg/ml). Western blotting of cell lysates using anti-TACE antibodies (left panel); and flow cytometry analysis of GFP-AT-PDX-positive cells (right panel).

1B). These are human colon carcinoma cells which have a point mutation in both alleles of the *fur* gene leading to production of a defective enzyme [35]. Immunoblotting of cell lysates from control LoVo Neo cells revealed that these cells expressed approximately 50% of processed TACE as seen by the relative intensity of the mature TACE and the pro-TACE immunoreactive bands (Fig. 1B). Genetic complementation of these cells with furin resulted in a strong diminution of the immunoreactive pro-TACE band with concomitant increase in the intensity of the 100 kDa mature species. Thus transfection of the *fur* gene restored the normal processing pattern observed in furin-positive cell lines, indicating that furin was responsible for the discrepancy between LoVo cells and the other wild-type cells. To provide further evidence for the role of furin in TACE maturation, we verified the impact of furin inhibition in furin-positive cell lines (Fig. 1C,D). Stable transfectants (HT1080 PDX) were generated from human fibrosarcoma HT1080 cells using a vector encoding AT-PDX (Fig. 1C), a potent furin inhibitor, which also inhibits PC6 to some extent with a K_i of 0.6 and 2.3 nM respectively [36]. As expected, AT-PDX expression resulted in the reappearance of the pro-TACE band, which was absent in control HT1080 Neo cells (Fig. 1C, left panel). As observed in LoVo cell lysates, the processed pro-TACE band was also immunode-

tected in furin-inhibited HT1080 cells. As a control, the maturation/activation of MMP-2, an event known to be under the control of furin through the activation of MT1-MMP, was completely blocked in AT-PDX-expressing HT1080 cells (Fig. 1C, right panel), indicating that the serpin was expressed at levels sufficient to completely block furin activity in these cells.

Next, we also inhibited furin in rat synoviocytes (Fig. 1D), a cell culture relevant to rheumatoid arthritis. Because of low transfection rates and difficulty in producing stable transfectants with primary synovial cell cultures, a viral gene delivery system was chosen to introduce AT-PDX into these cells. For this, we used a modified adenovirus vector (AdTR5) in which the α1-AT-PDX gene is placed under the negative control of a tetracycline/doxycycline-regulated promoter that is sensitive to low concentrations of doxycycline in cell cultures [37]. As illustrated in Fig. 1D (left panel), lane 3, we observed about 20% accumulation of the pro-TACE form in synoviocytes co-infected with the adenoviral vector encoding for AT-PDX and with AdCMVtTA, while this does not occur in either non-infected (lane 1) or control adenovirus-infected synoviocytes (lane 2). In addition, when the tTA blocker doxycycline (1 μg/ml) is added to the culture medium (lane 4), only the mature form of TACE is present indicating specificity to the AT-PDX inhibitor. The observed 20% accumulation of the pro-TACE form in synoviocytes may seems low compared to the 40–50% accumulation in HT1080 PDX cells (Fig. 1C). However, this likely reflects the efficiency of PDX delivery using the adenoviral system that is estimated to be 60% (Fig. 1D, right panel).

The observed role of furin in TACE maturation is consistent with the already described or proposed role of furin in the activation of other metalloproteases including MT1-MMP [17], and several proteolytically active ADAMs (e.g. ADAM-9 [22], ADAM-10 [23], ADAM-12 [24], ADAM-15 [25], ADAM-19 [26], and ADAMTS4 [27]).

3.2. Role of PCs other than furin in TACE maturation

Because complete inhibition of pro-TACE processing could not be achieved by AT-PDX or in furin-deficient LoVo Neo cells, our results further suggest that other enzymes, present in cells and not inhibited by the AT-PDX serpin, participate in the TACE maturation process. Since the TACE processing site (RVKR↓) could also be recognized by other members of the PC family, it is possible that PCs other than furin also participate in the TACE maturation process. To assess this possibility, we used dec-RVKR-cmk, a synthetic peptide that mimics the proprotein recognition site, and that has been demonstrated to inhibit the enzymatic activity of most PCs, including furin, PC5/PC6, PC1, PC2, PACE-4 and PC7 [36]. LoVo Neo and LoVo Fur2 cells were incubated for 24 h with several concentrations of that PC inhibitor, and cell lysates were assessed for TACE maturation by Western blotting as described above. The addition of dec-RVKR-cmk to LoVo Neo cells resulted in a concentration-dependent inhibition of TACE maturation, with maximal effect observed at 100 μM dec-RVKR-cmk (Fig. 2A), while a scrambled CMK peptide had no inhibitory effect (data not shown). As illustrated in Fig. 2B dec-RVKR-cmk inhibition is statistically significant at $P < 0.01$. Similar but more dramatic results were obtained in LoVo Fur2 cells, given that TACE maturation is complete in untreated LoVo Fur2 cells. No additional effect was observed when using a concentration of 200 μM in either LoVo Neo or Fur2 cells (data not shown). These results indicated that PCs other than furin are involved in the processing of TACE. Further, using a combination of the hydroxamate-based metalloprotease inhibitor BB-3103 and dec-RVKR-cmk used at optimal concentrations (0.5 μM and 100 μM, respectively) we did not observe additional inhibitory effects (data not shown), suggesting that metalloproteases, including potential catalysis or autocatalysis by TACE itself, are not involved in the TACE maturation process.

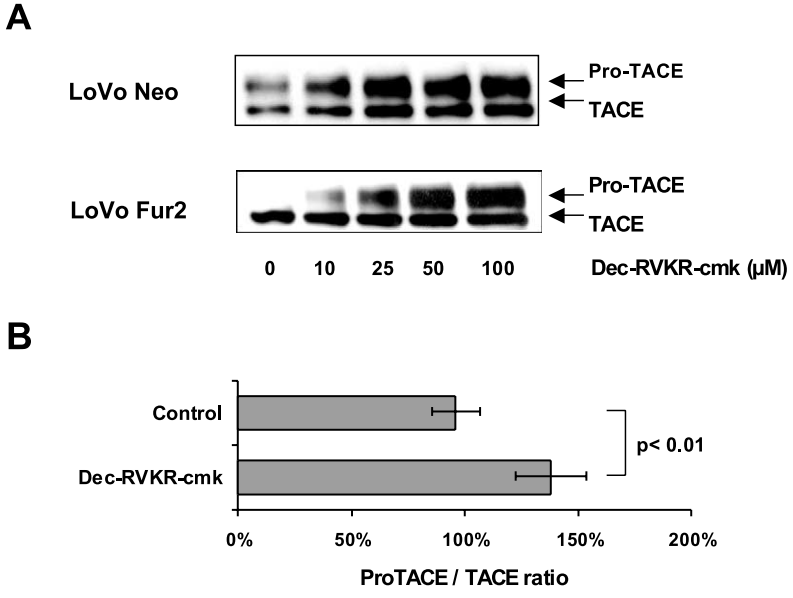


Fig. 2. Maturation of TACE PCs. A: LoVo Neo and LoVo Fur2 cells were incubated for 24 h with various concentrations of dec-RVKR-cmk, lysed and subjected to Western blot analysis as described in Section 2. B: The relative densities of the pro-TACE and TACE bands from LoVo Neo cells treated or not with dec-RVKR-cmk (100 μM) were determined using NIH Image software. The paired *t*-test was used for statistical analysis. $n = 4$.

3.3. In vitro cleavage of TACE peptides by PCs

To determine if PCs cleave TACE directly or act through an intermediate, we resorted to an in vitro cleavage assay. An oligopeptide that encompasses the furin-like consensus recognition motif PEELVH**RVKR**↓RADPDPMK (18-mer TACE peptide) found at the junction between the proregion and the active enzyme sequence and a N-terminally extended peptide that encompasses both the consensus furin recognition sequence and a second potential PC recognition site (mono-R) [38] LVD**R**↓EPPEELVH**RVKR**↓RADPD (22-mer TACE peptide) were synthesized and incubated with purified recombinant PCs. As demonstrated in Fig. 3A (top right panel), the analysis of the control unprocessed 18-mer TACE peptide revealed a retention time of 29 min. When the 18-mer TACE peptide was incubated with furin for 4 h (Fig. 3A, middle panel), the unprocessed peptide peak (29 min) was diminished while two peaks, corresponding to cleavage fragments of the original peptide, appeared at retention times of 16 and 22 min. This indicates that furin has the ability to directly cleave TACE at the junction between the mature enzyme and the proregion. Moreover, when incubation was conducted overnight (Fig. 3A, middle right panel), the 29 min peak disappeared, indicating that all of the original peptide had been processed. A similar pattern was observed when PC2 was used instead of furin, at both incubation times (Fig. 3A, bottom panel). In addition, PC1 and PC5/PC6 were also able to cleave the 18-mer TACE peptide (data not shown).

In Fig. 3B, MS analysis confirms the expected furin cleavage site for the 18-mer TACE peptide with a fragment with a predicted mass of 1262.48. Similar results were observed for all cleavage-competent PCs including PC1, PC2 and PC5/PC6. HPLC and MS analysis were also performed with the 22-mer TACE peptide. HPLC analysis resulted in the same cleavage profile of the 22-mer peptide as previously observed with the 18-mer, i.e. the same cleavage-competent PCs were observed (data not shown). MS analysis of the obtained fragments resulted in a mass of 1972.33 (predicted mass 1972.28) that corresponds to cleavage at the RVRR↓ cleavage site and not the monobasic DR↓ site. Therefore, the MS data confirm that furin, PC1, PC2 and PC5/PC6 cleave each of the TACE peptides at the expected RVRR↓ furin-like cleavage site. It has been shown that overexpression of TACE in CHO cells results in the secretion of the processed enzyme with two N-termini at Val^{212} and Arg^{215} [39]. Our MS results support the notion that the Val^{212} N-terminus is unlikely to be a result of a PC cleavage event, possibly because of the presence of a valine at the P1′ position of monobasic sites, which is not favored by this type of enzyme [38].

3.4. In vivo cleavage of TACE by PCs

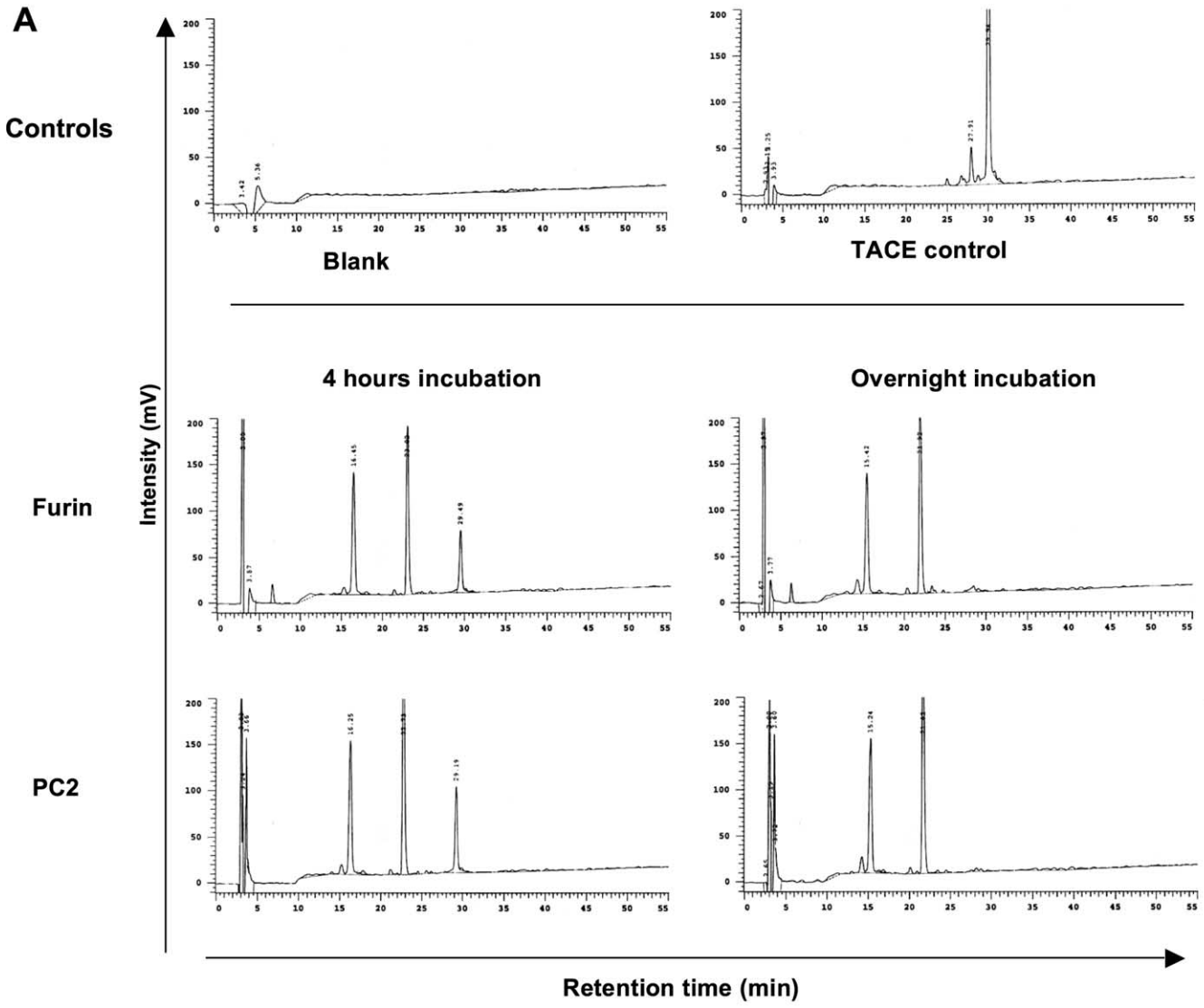
To define if PCs cleave TACE effectively in an intact cell system, we used recombinant vaccinia virus for cell delivery of PC1/PC3, PACE-4 and PC5/6 in LoVo cells. After 18 h infection, cell lysates were analyzed for TACE maturation by immunoblotting. As illustrated in Fig. 3C, LoVo cells infected with control recombinant virus (vaccinia virus:pro-opiomelanocortin (POMC)) exhibited about 50% cleavage of pro-TACE as evidenced by the detection of the pro-TACE and the mature TACE bands (Fig. 3C). In contrast, infection with vaccinia virus encoding PC1, PACE-4 or PC5/PC6 resulted in a significant increase in the relative proportion of the proteolytically processed mature TACE form. Taken together with the results indicating that furin is required, in part, for in vivo TACE processing, our findings suggest furin PACE-4, PC5/PC6 and PC1 are able to process TACE in intact cells. We have not assessed the ability of PC2 to process TACE in LoVo cells since these cells lacks the 7B2 chaperone needed for proper PC2 routing stability and activity [40].

3.5. Impact of the inhibition of TACE maturation on TACE shedding activities

TACE has been shown to mediate cleavage of TNF-α as well as a variety of ectodomains including the TNF p75 receptor (TNFR-II) [3]. To investigate whether the levels of TACE maturation observed in LoVo transfectants impact TACE-related sheddase activity, we first measured cell surface p75 TNFR expression as a marker for TACE cell surface activity. Briefly, LoVo Neo and LoVo Fur2 cell samples were labeled with anti-TACE or anti-TNF p75 receptor antibodies and cell fluorescence was analyzed on a FACScan. Results expressed in Fig. 4A indicated that furin complementation of LoVo cells reduces cell surface p75 TNF receptor expression by about 50% (from 60% to 32%) without significant impact on the levels of cell surface TACE. Similar results have also been observed in MonoMac-1 and THP-1 cell lines (data not shown), in this case we used dec-RVKR-cmk-induced inhibition of TACE maturation. To ensure that the discrepancy observed in TNFR p75 cell surface expression between LoVo Neo and LoVo Fur2 cells was not due to a difference in the levels of TACE expression between clones, LoVo Fur2 cells were incubated with dec-RVKR-cmk and samples were processed for cell surface TNFR p75 by immunodetection. As predicted, inhibition of PCs by dec-RVKR-cmk led to higher levels of TNFR p75 cell surface expression (Fig. 4B), which correlates with lower levels of TACE maturation in these conditions. Taken together, our results also indicate that furin activity increases TNFR p75 shedding. Therefore removal of the TACE prodomain by furin increases its activity, as postulated.

Next, we measured the ability of dec-RVKR-CH_2Cl to block TNF-α release from the human monocytic cells THP-1 and MonoMac-1, which naturally produce TNF-α. For this, cells were preincubated for 22 h with varying concentrations of dec-RVKR-CH_2Cl and TNF-α production was induced by LPS and PMA. Results expressed in Fig. 5A indicated that treatment of these cells with a PC inhibitor blocked about 50% of TNF-α release with an ED_{50} around 20 μM. To ensure that the inhibition of TNF-α was due to changes in sheddase activity, we next measured TNF-α shedding rate as currently defined by ratios of supernatant to cell-associated TNF-α. The results indicate that increasing doses of dec-RVKR-CH_2Cl resulted in a gradual decrease in supernatant to cell-associated TNF-α ratios (Fig. 5B). These results suggest that inhibition of TACE processing leads to an impairment in TACE sheddase activities.

Therefore, we demonstrate in this study that among the PC family of enzymes, furin, PC5/PC6, PC1 and PC2 are all TACE-competent convertases and that cleavage at the furin site results in an increased sheddase activity. Redundancy between members of the PC family is well established and a recent example is the ability of furin, PC5/PC6, and PC7 to cleave with similar efficacy a peptide containing the processing site of an Ebola virus surface glycoprotein [41]. Other examples include pro-insulin-like growth factor-1 receptor process-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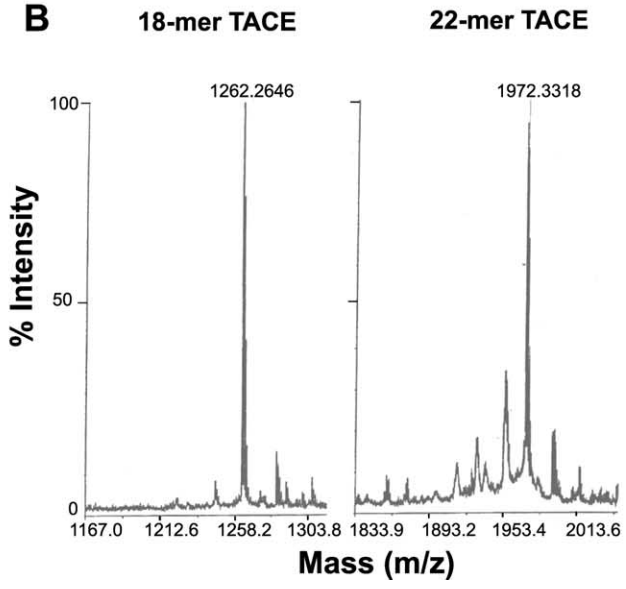
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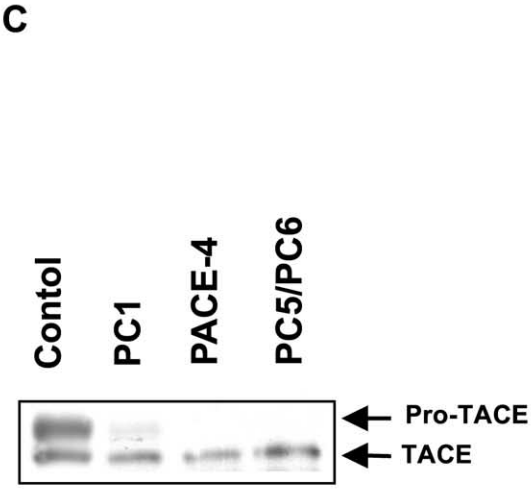
C
Contol
PC1
PACE-4
PC5/PC6
Pro-TACE
TACE

Fig. 3. In vitro and in vivo maturation of TACE-derived peptides by PCs. A: An 18-mer TACE was incubated for 4 h or overnight with purified recombinant furin or PC2. Represented are the results of HPLC analysis. The two top panels represent the analysis of a blank control (left) or the TACE peptide incubated without PCs (right). B: MS analysis of the cleaved (A) TACE short (18-mer) and (B) TACE long (22-mer) peptides. Predicted molecular masses after cleavage are 1262.48 for the TACE short peptide and 1972.28 for the TACE long peptide. C: LoVo cells were infected with vaccinia recombinant for control protein (POMC), or the convertases PC1, PACE-4 or PC5/PC6. Eighteen hours after infection, cells were lysed, aliquots were separated on reducing SDS–PAGE gels and immunoblotted using rabbit anti-TACE polyclonal antibodies (1:1000).
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ing by furin and PC5A, but not by PACE-4 or PC7 [42] and pro-endothelin by furin and PC7 [20].

Just prior to the submission of this article, Peiretti et al. [28,] and Endres et al. [29] published results indicating that furin was responsible, in part, for TACE processing in LoVo cells as well as various cell types including HT29 and HEK293 cells. In addition, an increased amount of processed TACE was observed in cells overexpressing PC7 [29]. Since LoVo cells are known to express PACE-4 and PC7, they concluded that PC7 was responsible, in part, for basal levels of TACE processing in these cells [29]. Our results indicating that PACE-4 can process the TACE enzyme in vitro and in cells further suggest that both PACE-4 and PC7 convertases are involved in TACE maturation by LoVo cells.

PC5/PC6 is known to be expressed in many endocrine and non-endocrine tissues, with a particularly high expression in the gut [43,44]. In the present study, we observed that PC6 is a TACE-competent convertase both in vitro and in vivo. Interestingly, high levels of TACE have been detected in normal gut and the median TACE level was increased in patients with active ulcerative colitis [45]. This raises the possibility that the activation of TACE by PC6 is one of the mechanisms that

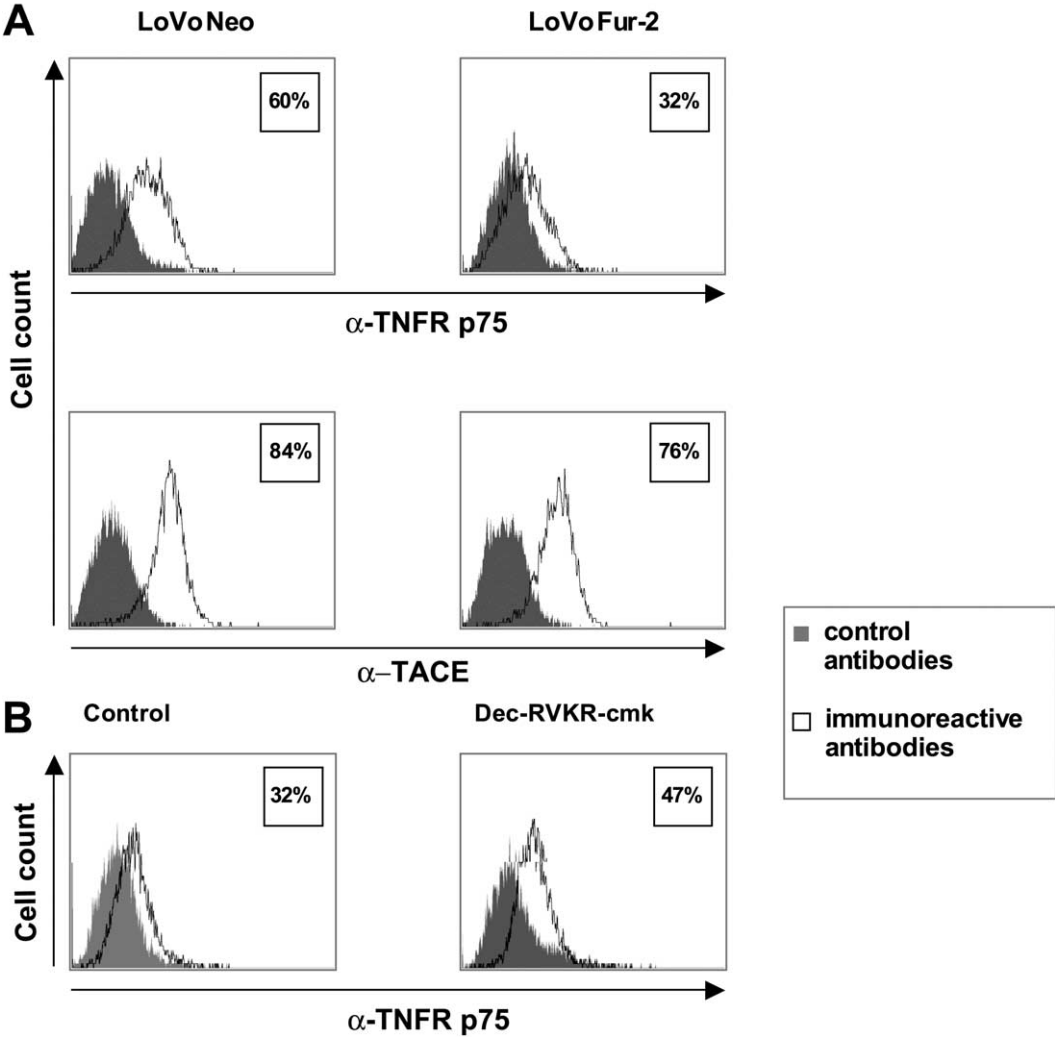

Fig. 4. Impact of the inhibition of TACE maturation by a PC inhibitor on p75 TNFR cell surface expression. A: Flow cytometry analysis of LoVo Neo and LoVo Fur2 stained with control (shaded), anti-TNFR p75 (unshaded, top panels) or anti-TACE (unshaded, middle panels) antibodies. A representative experiment out of two is illustrated. B: LoVo Fur2 cells were treated or not with 100 μM dec-RVKR-cmk and stained using anti-TNFR p75 antibody. Indicated in the top right corner of each panel is the percentage of cells positive for TNFR p75 or TACE.

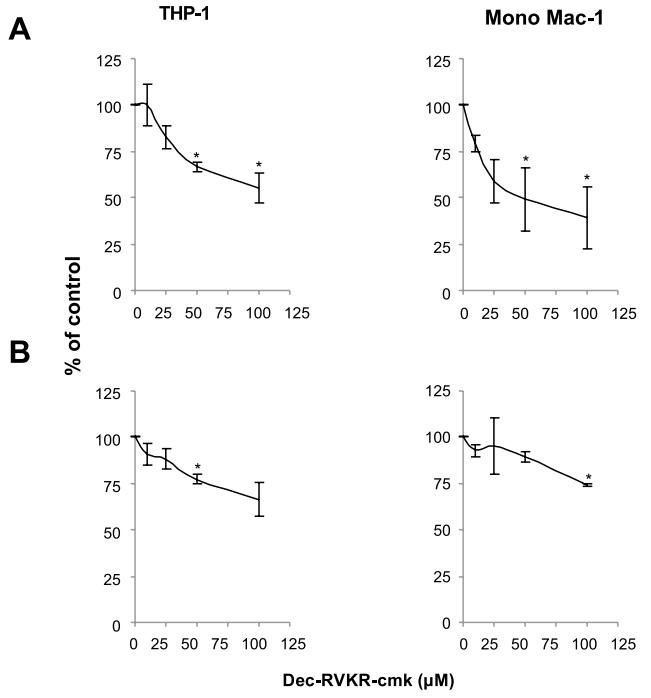

Fig. 5. Inhibition of TNF-α shedding by inhibition of PC-mediated TACE maturation. THP-1 (left) and MonoMac-1 (right) cells were incubated for 22 h with the indicated concentration of dec-RVKR-cmk, and TNFα production was stimulated and measured as indicated in Section 2. A: TNF-α content in supernatants relative to control (untreated with dec-RVKR-cmk). Multiple comparison tests were conducted using analysis of variance. Dunnett's test at the 0.05 significance level was used for follow-up one-to-one comparisons. $n = 3$–4. B: Supernatant to cell-associated TNF-α content ratio relative to control. Comparisons were made using the t-test. $n = 2$. $^*P < 0.05$.

account for elevated expression of TNF-α in this pathological condition.

The observation that PC1 and PC2 also cleaved the TACE peptides suggests potential roles of these enzymes for TACE activation in neuroendocrine tissues and cells as well as in certain types of immune cells. Indeed, TACE expression has been localized in brain tissue, more specifically in distinct neuronal cell populations, including the pyramidal neurons of the cerebral cortex and the granular cell layer neurons of the hippocampus [46]. Since PC1 and PC2 were clearly shown to be expressed in these same neuronal populations [47], it is likely that PC1 and/or PC2 are involved in the cellular activation of TACE within neurons. Regarding the immune system, PC1 and PC2 were also demonstrated to be expressed within polymorphonuclear leukocytes, alveolar macrophages and spleen mononuclear cells [48]. Since macrophages and polymorphonuclear leukocytes are known to produce large quantities of TNF-α, it is conceivable that TACE maturation by PC2 or PC1/PC3 could be an alternative modulation mechanism for TNF-α production in these cells.

PCs are known to be responsible for the maturation of several molecules involved in extracellular matrix-associated diseases. For instance, furin mRNA is upregulated in a rat collagen-induced model of arthritis (A. Lebel et al., unpublished results). Moreover, the increased levels of furin in joints correlate with the intensity of joint inflammation. Furin has been shown to be responsible for the maturation of the TGF-β1 precursor and in turn, TGF-β1 in synovial cells upregulates the furin-encoding *fur* gene, generating a unique enzyme/substrate amplification loop that can be of fundamental importance in inflammatory conditions [30]. For example, the amplified levels of furin and TGF-β can cooperate to increase the levels of ADAMTS4 [49], a newly uncovered furin substrate [27] responsible for the degradation of the matrix protein aggrecan, a major component of cartilage. Beside ADAMTS4, furin can also participate in the maturation of other proteases, which are involved in collagen degradation, including MT1-MMP, a metalloprotease known to activate proMMP2 [50–52] and proMMP13 [53]. Our demonstration that TACE is a PC substrate therefore adds to the possibility that these enzymes are important players in the pathogenesis of inflammatory and matrix-associated diseases such as rheumatoid arthritis. In this context PCs could be a target to prevent the formation of bioactive proteins involved in arthritis, thereby resulting in the prevention or regression of the disease.

References

[1] Moss, M.L. et al. (1997) Nature 385, 733–736.
[2] Black, R.A. et al. (1997) Nature 385, 729–733.
[3] Peschon, J.J. et al. (1998) Science 282, 1281–1284.
[4] Brou, C. et al. (2000) Mol. Cell 5, 207–216.
[5] Garton, K.J., Gough, P.J., Blobel, C.P., Murphy, G., Greaves, D.R., Dempsey, P.J. and Raines, E.W. (2001) J. Biol. Chem. 276, 37993–38001.
[6] Reddy, P. et al. (2000) J. Biol. Chem. 275, 14608–14614.
[7] Rovida, E., Baccarini, M., Olivotto, M. and Sbarba, P.D. (2002) Oncogene 21, 3670–3676.
[8] Buxbaum, J.D. et al. (1998) J. Biol. Chem. 273, 27765–27767.
[9] Luetteke, N.C., Qiu, T.H., Fenton, S.E., Troyer, K.L., Riedel, R.F., Chang, A. and Lee, D.C. (1999) Development 126, 2739–2750.
[10] Dell, K.M., Nemo, R., Sweeney, W.E.Jr., Levin, J.I., Frost, P. and Avner, E.D. (2001) Kidney Int. 60, 1240–1248.
[11] Trifilieff, A., Walker, C., Keller, T., Kottirsch, G. and Neumann, U. (2002) Br. J. Pharmacol. 135, 1655–1664.
[12] Beck, G. et al. (2002) J. Pharmacol. Exp. Ther. 302, 390–396.
[13] Conway, J.G. et al. (2001) J. Pharmacol. Exp. Ther. 298, 900–908.
[14] Milla, M.E. et al. (1999) J. Biol. Chem. 274, 30563–30570.
[15] Zhang, Z. et al. (2000) J. Leukoc. Biol. 67, 856–862.
[16] Pei, D. and Weiss, S.J. (1995) Nature 375, 244–247.
[17] Yana, I. and Weiss, S.J. (2000) Mol. Biol. Cell 11, 2387–2401.
[18] Schlondorff, J., Becherer, J.D. and Blobel, C.P. (2000) Biochem. J. 347, 131–138.
[19] Steiner, D.F. (1998) Curr. Opin. Chem. Biol. 2, 31–39.
[20] Blais, V., Fugere, M., Denault, J.B., Klarskov, K., Day, R. and Leduc, R. (2002) FEBS Lett. 524, 43–48.
[21] Dubois, C.M., Blanchette, F., Laprise, M.H., Leduc, R., Grondin, F. and Seidah, N.G. (2001) Am. J. Pathol. 158, 305–316.
[22] Roghani, M. et al. (1999) J. Biol. Chem. 274, 3531–3540.
[23] Anders, A., Gilbert, S., Garten, W., Postina, R. and Fahrenholz, F. (2001) FASEB J. 15, 1837–1839.
[24] Loechel, F., Gilpin, B.J., Engvall, E., Albrechtsen, R. and Wewer, U.M. (1998) J. Biol. Chem. 273, 16993–16997.
[25] Lum, L., Reid, M.S. and Blobel, C.P. (1998) J. Biol. Chem. 273, 26236–26247.
[26] Kang, T., Zhao, Y.G., Pei, D., Sucic, J.F. and Sang, Q.X. (2002) J. Biol. Chem. 277, 25583–25591.
[27] Gao, G., Westling, J., Thompson, V.P., Howell, T.D., Gottschall, P.E. and Sandy, J.D. (2002) J. Biol. Chem. 277, 11034–11041.
[28] Peiretti, F., Canault, M., Deprez-Beauclair, P., Berthet, V., Bonardo, B., Juhan-Vague, I. and Nalbone, G. (2003) Exp. Cell Res. 285, 278–285.
[29] Endres, K., Anders, A., Kojro, E., Gilbert, S., Fahrenholz, F. and Postina, R. (2003) Eur. J. Biochem. 270, 2386–2393.

[30] Blanchette, F., Day, R., Dong, W., Laprise, M.H. and Dubois, C.M. (1997) J. Clin. Invest. 99, 1974–1983.
[31] Komada, M., Hatsuzawa, K., Shibamoto, S., Ito, F., Nakayama, K. and Kitamura, N. (1993) FEBS Lett. 328, 25–29.
[32] Mosser, D.D., Caron, A.W., Bourget, L., Jolicoeur, P. and Massie, B. (1997) BioTechniques 22, 150–154.
[33] Petrof, B.J., Acsadi, G., Jani, A., Massie, B., Bourdon, J., Matusiewicz, N., Yang, L., Lochmuller, H. and Karpati, G. (1995) Am. J. Respir. Cell Mol. Biol. 13, 508–517.
[34] Fugere, M., Limperis, P.C., Beaulieu-Audy, V., Gagnon, F., Lavigne, P., Klarskov, K., Leduc, R. and Day, R. (2002) J. Biol. Chem. 277, 7648–7656.
[35] Takahashi, S., Kasai, K., Hatsuzawa, K., Kitamura, N., Misumi, Y., Ikehara, Y., Murakami, K. and Nakayama, K. (1993) Biochem. Biophys. Res. Commun. 195, 1019–1026.
[36] Jean, F., Stella, K., Thomas, L., Liu, G., Xiang, Y., Reason, A.J. and Thomas, G. (1998) Proc. Natl. Acad. Sci. USA 95, 7293–7298.
[37] Yoshida, Y. and Hamada, H. (1997) Biochem. Biophys. Res. Commun. 230, 426–430.
[38] Devi, L. (1991) FEBS Lett. 280, 189–194.
[39] Maskos, K., Fernandez-Catalan, C., Huber, R., Bourenkov, G.P., Bartunik, H., Ellestad, G.A., Reddy, P., Wolfson, M.F., Rauch, C.T., Castner, B.J., Davis, R., Clarke, H.R., Petersen, M., Fitzner, J.N., Cerretti, D.P., March, C.J., Paxton, R.J., Black, R.A. and Bode, W. (1998) Proc. Natl. Acad. Sci. USA 95, 3408–3412.
[40] Mbikay, M., Seidah, N.G. and Chretien, M. (2001) Biochem. J. 357, 329–342.
[41] Basak, A., Zhong, M., Munzer, J.S., Chretien, M. and Seidah, N.G. (2001) Biochem. J. 353, 537–545.
[42] Khatib, A.M., Siegfried, G., Prat, A., Luis, J., Chretien, M., Metrakos, P. and Seidah, N.G. (2001) J. Biol. Chem. 276, 30686–30693.
[43] Lusson, J., Vieau, D., Hamelin, J., Day, R., Chretien, M. and Seidah, N. (1993) Proc. Natl. Acad. Sci. USA 90, 6691–6695.
[44] Nakagawa, T., Hosaka, M., Torii, S., Watanabe, T., Murakami, K. and Nakayama, K. (1993) J. Biochem. 113, 132–135.
[45] Brynskov, J., Foegh, P., Pedersen, G., Ellervik, C., Kirkegaard, T., Bingham, A. and Saermark, T. (2002) Gut 51, 37–43.
[46] Skovronsky, D.M., Fath, S., Lee, V.M. and Milla, M.E. (2001) J. Neurobiol. 49, 40–46.
[47] Schafer, M.K., Day, R., Cullinan, W.E., Chretien, M., Seidah, N.G. and Watson, S.J. (1993) J. Neurosci. 13, 1258–1279.
[48] Vindrola, O., Mayer, A.M., Citera, G., Spitzer, J.A. and Espinoza, L.R. (1994) Neuropeptides 27, 235–244.
[49] Yamanishi, Y., Boyle, D.L., Clark, M., Maki, R.A., Tortorella, M.D., Arner, E.C. and Firestein, G.S. (2002) J. Immunol. 168, 1405–1412.
[50] Yamamoto, M., Mohanam, S., Sawaya, R., Fuller, G.N., Seiki, M., Sato, H., Gokaslan, Z.L., Liotta, L.A., Nicolson, G.L. and Rao, J.S. (1996) Cancer Res. 56, 384–392.
[51] Sato, H., Kinoshita, T., Takino, T., Nakayama, K. and Seiki, M. (1996) FEBS Lett. 393, 101–104.
[52] Strongin, A.Y., Collier, I., Bannikov, G., Marmer, B.L., Grant, G.A. and Goldberg, G.I. (1995) J. Biol. Chem. 270, 5331–5338.
[53] Will, H., Atkinson, S.J., Butler, G.S., Smith, B. and Murphy, G. (1996) J. Biol. Chem. 271, 17119–17123.