Biochem. J. (2000) 347, 131–138 (Printed in Great Britain)

Intracellular maturation and localization of the tumour necrosis factor α convertase (TACE)

Johannes SCHLÖNDORFF*†, J. David BECHERER‡ and Carl P. BLOBEL*[1]

*Cellular Biochemistry and Biophysics Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, Box 368, 1275 York Avenue, New York, NY 10021, U.S.A., †Tri-Institutional (Cornell University/Rockefeller University/Sloan-Kettering Cancer Center) M.D.-Ph.D. Training Program, 1300 York Avenue, New York, NY 10021, U.S.A., and ‡Department of Molecular Biochemistry, Glaxo Wellcome Research and Development Inc., 2512 S. Tri-Center Blvd, Research Triangle Park, NC 27713, U.S.A.

Tumour necrosis factor α convertase (TACE) is a metalloprotease/disintegrin involved in the ectodomain shedding of several proteins, a process thought to be important in inflammation, rheumatoid arthritis and murine development. The characterization of the intracellular maturation and subcellular localization of endogenous TACE is decribed in the present study. Similarly to other proteolytically active metalloprotease/disintegrins, two forms of TACE are found in cells; a full-length precursor and a mature form lacking the prodomain. Prodomain removal occurs in a late Golgi compartment, consistent with the proposed role of a furin type proprotein convertase in this process. An additional form of TACE, lacking the pro and cytoplasmic domains, is detected when cell lysates are prepared in the presence of EDTA instead of a hydroxamate-based metalloprotease inhibitor or 1,10-phenanthroline. This form appears to be generated by mature TACE cleaving its own cytoplasmic tail and may explain why little mature TACE has been detected in previous studies. In cell-surface labelling experiments, mature TACE was detected on the cell surface but immunofluorescence data indicate that TACE is predominantly localized to a perinuclear compartment similar to that described for tumour necrosis factor (TNF)α. This raises the possibility that TACE-mediated ectodomain shedding may occur in an intracellular compartment in addition to the cell surface.

Key words: ADAM, MDC, metalloproteinases, protein ectodomain shedding.

INTRODUCTION

The metalloprotease/disintegrin family of proteins [also referred to as ADAMs (a disintegrin and metalloprotease) and MDC (metalloprotease, disintegrin, cysteine rich proteins)] has been implicated in a variety of cellular processes, including sperm-egg binding, sperm migration, muscle cell fusion, protein ectodomain shedding and Notch-mediated signalling events (for recent reviews see [1–4]). This family of transmembrane proteins is defined by a distinct domain structure, which includes a signal sequence, a prodomain, a metalloprotease domain and a disintegrin domain, followed by a cysteine-rich region, often containing an epidermal growth factor-like repeat, a transmembrane domain and a cytoplasmic tail. Sixteen of the thirty ADAM proteins identified to date are predicted to be catalytically active, based on the presence of a conserved zinc binding sequence (HEXXH) in the protease domain, whereas the other members are not likely to be active proteases. The purification and identification of tumour necrosis factor (TNF) α convertase (TACE/ADAM17) as a metalloprotease/disintegrin marked the first instance in which a proteolytically active ADAM was paired with a bona fide substrate [5,6].

In addition to cleaving TNFα, TACE has been shown to be necessary for the release of transforming growth factor (TGF)α, p75 TNF receptor (p75 TNFR), L-selectin [7] and β-amyloid precursor protein [8], but not of angiotensin converting enzyme [9]. Furthermore, TACE-deficient mice have been generated and show a number of developmental defects reminiscent of those seen in animals lacking TGFα or epidermal growth factor receptor, most likely indicating that shed TGFα plays a critical role which cannot be compensated for by the membrane-bound form of the protein [7]. Thus TACE appears to be a sheddase with a wide range of substrates and an important role in development.

The prodomain of catalytically active ADAMs is thought to act as an inhibitor of the protease via a cysteine-switch mechanism, in which a free cysteine residue from the prodomain coordinates with the zinc ion in the active site of the protease [10–12]. Prodomain removal is therefore thought to be a prerequisite for TACE protease activity [6,11,13]. The prodomains of several ADAM proteins have been shown to be constitutively cleaved by a furin-type proprotein convertase as they progress through the secretory pathway [11,14,15]. TACE has also been shown to be processed when overexpressed, but little mature TACE appears to accumulate [13,16,24,25].

In addition to having its prodomain removed, mature TACE must ultimately be present in the same subcellular compartment as its potential substrate in order for ectodomain shedding to take place. The mature form of some ADAMs, such as MDC15 [14] and ADAM10/KUZ [17], are apparently mainly localized in an intracellular compartment, whereas the mature form of others, such as ADAM28, are localized mainly on the cell surface (L. Howard, R. A. Maciewicz and C. P. Blobel, unpublished work). Previous studies indicate that TNFα is processed in a post-endoplasmic reticulum compartment and that this processing is tightly coupled with transport to the cell surface [18], and that surface TGFα is also rapidly released from stimulated cells [19,20]. Together, these observations raise the question whether

Abbreviations used: ADAM, a disintegrin and metalloprotease; BB-94, batimastat, a metalloprotease inhibitor; ConA, concanavalin A; endoH, endoglycosidase H; GST, glutathione S-transferase; MDC, metalloprotease, disintegrin, cysteine-rich protein; PNGase F, peptide:*N*-glycosidase F; TACE, tumour necrosis factor α convertase; TACE ΔMP, TACE lacking the pro and metalloprotease domains; TGF, transforming growth factor; TNF, tumour necrosis factor; TNFR, TNF receptor.

[1] To whom correspondence should be addressed (e-mail c-blobel@ski.mskcc.org).

© 2000 Biochemical Society

mature, and thus presumably catalytically active, TACE is localized mainly on the cell surface or resides mainly in the lumen of an intracellular compartment.

In order to examine the intracellular maturation and subcellular localization of TACE, we have raised domain-specific antibodies against TACE and have used them in pulse–chase and Western-blot experiments. The steady-state population of TACE, analysed by Western blot using untransfected cells, consists of both full-length protein and a mature form lacking the prodomain. Similar to other ADAMs, including MDC9 [11] and MDC15 [14], the prodomain of TACE is removed in a late Golgi compartment, most likely by a furin-type protease. We found that the cytoplasmic tail of mature TACE is rapidly removed after cells are lysed in non-ionic detergent, perhaps explaining why little, if any, mature TACE has been reported in previous publications. Furthermore, whereas TACE can be detected on the cell surface by biotinylation, it appears to be predominantly localized to a perinuclear compartment when examined by immunofluorescence. This localization is not significantly affected by the addition of phorbol ester, a stimulus known to increase ectodomain shedding. The results of the present study are compared with the maturation of other ADAM proteins and discussed in terms of potential mechanisms of TACE regulation.

EXPERIMENTAL

Reagents and cell lines

All reagents were obtained from Sigma unless otherwise indicated. Redivue Pro-mix *in vitro* cell-labelling mix was purchased from Amersham Pharmacia Biotech. Synthesis of BB-94 (batimastat), a metalloprotease inhibitor, has been described previously [6,21]. THP-1, a human monocytic cell line, and COS-7, an SV40 transformed kidney cell line from the African green monkey, were obtained from the American Type Culture Collection (A.T.C.C.).

Antibody production

A cDNA fragment coding for the prodomain of human TACE (amino acids 18–214) was generated from a human TACE cDNA clone [6] utilizing PCR. Primers were designed with appropriate restriction sites at both the 5′ and 3′ ends and with a 3′ stop codon. The resulting fragment was ligated, in frame, into a pGEX plasmid vector (Amersham Pharmacia Biotech, Uppsala, Sweden), sequenced to rule out mutations introduced by PCR (The BioResource Center, Cornell University, Ithaca, NY, U.S.A.), and the construct was transfected into XL1-Blue *Escherichia coli* cells (Stratagene). The bacterially-expressed glutathione S-transferase (GST) fusion protein was purified as described previously [22]. A truncated form of TACE, consisting of the extracellular domains (amino acids 215–651) expressed and purified from a baculoviral expression system, was generated as described previously [6]. Both antigens were used to immunize New Zealand White rabbits following established protocols (Covance Research Products, Denver, PA, U.S.A.; [23]). Generation of the anti-TACE cytoplasmic tail antibody has been described elsewhere [24,25]. A mouse monoclonal antibody (Tc3-7.49) recognizing the metalloprotease domain of human TACE has been described previously [6,13]. Polyclonal antisera and conditioned hybridoma media were used directly for Western blotting and for most immunoprecipitations (see below). For use in pulse–chase experiments and immunofluorescence, IgG was purified from anti-TACE extracellular pre-immune and immune serum. Briefly, serum was passed over a 1 ml HiTrap Protein A column (Pharmacia) and the column was then extensively washed with PBS. Bound IgG was eluted with 0.1 M glycine, pH 3.2, and immediately neutralized by the addition of Tris/HCl, pH 7.4 to a final concentration of 100 mM. The concentrations of immune and pre-immune IgG were equalized. The purified antibodies are referred to as Protein-A-purified anti-TACE extracellular IgG.

Membrane preparation

Membranes were prepared from THP-1 or COS-7 cells using a protocol modified from van't Hof and Resh [26]. THP-1 cells were washed in ice-cold STE buffer [100 mM NaCl, 10 mM Tris/HCl (pH 7.4), 1 mM EDTA] and COS-7 cells were washed once in STE buffer and then scraped from the tissue-culture plates into STE buffer. Cells were centrifuged at 1000 ***g*** for 5 min and resuspended in ice-cold, hypotonic lysis buffer A [10 mM Tris/HCl (pH 7.4), 0.2 mM $MgCl_2$, containing protease inhibitors (2 μg/ml leupeptin, 0.4 mM benzamidine, 10 μg/ml soy-bean trypsin inhibitor, 0.5 mM iodoactamide [27]) and 1 μM BB-94]. After incubating on ice for 15 min, the cells were homogenized with 30 strokes of a Dounce homogenizer with a tight pestle. The lysate was adjusted to 250 mM sucrose, 1 mM EDTA, and centrifuged at 1000 ***g*** for 10 min. The supernatant was retained and the pellet was resuspended in 250 mM sucrose, 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, protease inhibitors (as above) and 1 μM BB-94, homogenized with 10 strokes of a Dounce homogenizer and the homogenate was centrifuged at 1000 ***g*** for 10 min. The supernatants from the first and second centrifugation procedures were combined and re-centrifuged at 10000 ***g*** for 10 min, to remove mitochondria, and then at 100000 ***g*** for 60 min in an SW40Ti rotor (Beckman). The pellet, representing total cellular membranes, was either resuspended in sample loading buffer or homogenized in Tris-buffered saline (TBS) (25 mM Tris base,135 mM NaCl, 2.6 mM KCl, pH 7.4) supplemented with protease inhibitors (as above). The final protein concentration was determined with the BCA protein assay (Pierce) following the supplier's recommendations.

Glycoprotein preparation for Western-blot analysis

THP-1 cells were pelleted at 700 ***g*** for 5 min, washed twice in PBS, and resuspended in lysis buffer B [1 % (v/v) Nonidet P-40, TBS, protease inhibitors (as above), and 1 μM BB-94 unless otherwise indicated]. COS-7 cells were grown to confluence on 15-cm diam. plates, washed with PBS and lysed with 5 mls of lysis buffer B per plate. All lysates were centrifuged at 13000 ***g*** for 20 min, and the resulting supernantants were incubated with 10 μl of concanavalin A (ConA)–Sepharose beads (Pharmacia)/ml of lysate for 2 h at 4 °C. The beads were then washed three times with lysis buffer B and bound material was boiled in sample loading buffer [125 mM Tris/HCl, 15 % (w/v) sucrose, 4 % (w/v) SDS, 10 mM EDTA, 0.1 mg/ml Bromophenol Blue], either with or without the addition of 10 mM dithiothreitol, for 5 min at 95 °C. Sample proteins were separated by SDS/PAGE, transferred to nitrocellulose (Schleicher & Schuell) and Western blots were performed as decribed previously [14]. Polyclonal antisera were used at a 1:500 dilution; the anti-TACE metalloprotease monoclonal-antibody-conditioned media was used at a 1:20 dilution.

Detergent partitioning with Triton X-114

COS-7 cells were washed once with TBS and removed from the plate into ice-cold TBS with the protease inhibitor cocktail but without metalloprotease inhibitors. Detergent partitioning was performed following standard protocols [28]. Precondensed Triton X-114 was added to a final concentration of 2 % (v/v) and

© 2000 Biochemical Society

the cells were incubated on ice for 15 min. Insoluble material was removed by centrifugation at 10000 ***g*** for 10 min at 4 °C. The supernatant was warmed to 37 °C for 15 min and then centrifuged at 1000 ***g*** for 10 min at room temperature. The aqueous and detergent phases were isolated. Ice-cold Triton X-114 and TBS were added to the aqueous and detergent phases to yield a final concentration of 2 % (v/v) Triton X-114. Each sample was mixed with an equal volume of cold lysis buffer B and incubated with ConA–Sepharose beads for 2 h at 4 °C. The beads were washed three times with lysis buffer B, and bound material was mixed with sample loading buffer, separated by SDS/PAGE and the gels were analysed by Western blotting, as desribed above.

Metabolic labelling

COS-7 cells were plated on to 10-cm plates and allowed to recover overnight. Cells were starved for 30 min in growth medium lacking cysteine and methionine and 210 μCi/ml of Redivue Pro-mix ^{35}S-Cys/^{35}S-Met *in vitro* cell-labelling mixture was then added. Cells were incubated for 3 h, washed twice with PBS and incubated in ice-cold growth medium. At 0, 3, 6, 12 and 24 h, cells were washed twice in PBS and lysed in 1.5 ml of lysis buffer B. TACE was immunoprecipitated with either Protein A purified anti-TACE extracellular IgG or pre-immune IgG and Protein A–Sepharose beads (Pharmacia) for 2 h at 4 °C. The beads were washed three times in lysis buffer B and bound material was heated at 95 °C in sample loading buffer for 5 min. In order to reduce the background signal in these samples due to non-specific binding of labelled proteins to the Protein A–Sepharose beads, the samples were diluted 1 : 50 in lysis buffer B and incubated with ConA–Sepharose beads for 2 h at 4 °C to capture glycosylated forms of TACE. The beads were washed three times and bound material was transferred to sample loading buffer, reduced and separated by SDS/PAGE. After electrophoresis, the gels were fixed for 15 min in 10 % (v/v) acetic acid, 50 % (v/v) methanol, rehydrated for 15 min in water, dried and exposed to Kodak XAR autoradiography film for 72 h.

Enzymic deglycosylation

N-Linked carbohydrate residues were removed using either endoglycosidase H (endoH) or peptide:*N*-glycosidase F (PNGase F) (New England Biolabs) as described previously [14]. In brief, membrane preparations were generated as described above from THP-1 and COS-7 cells and resuspended in sample loading buffer containing 10 mM dithiothreitol. After incubation at 95 °C for 5 min, the samples were adjusted to pH 5.5 with sodium citrate for mock and endo H treatment or to pH 7.4 with sodium phosphate and 1 % (v/v) Nonidet P-40 for PNGase treatment. Samples were incubated overnight at 37 °C either without enzyme or with the addition of 500 units of endoH or PNGase F. The samples were then separated by SDS/PAGE and analysed by Western blotting as described above.

Cell-surface labelling

THP-1 or COS-7 cells were washed twice in ice-cold PBS, followed by a 10 min incubation in PBS on ice. The membrane-impermeable biotinylation reagent, NHS-LC-biotin [sulphosuccinimidyl-6-(biotinamido) hexanoate] (Pierce), was added to a final concentration of 0.5 mg/ml and the cells were incubated, on ice, for a further 45 min. The cells were then washed twice with 0.1 M glycine in TBS to quench any unreacted biotinylation reagent and lysed in lysis buffer B containing 1 μM BB-94. After clearing by centrifugation, samples were either precipitated using ConA, as described above, or immunoprecipitated with various anti-TACE antibodies. Immunoprecipitated material was separated by SDS/PAGE, transferred to nitrocellulose and detected with streptavidin coupled to horseradish peroxidase. ConA-precipitated material was analysed on the same gel by Western blotting to determine the electrophoretic mobility of TACE.

Immunofluorescence

To examine the subcellular localization of endogenous TACE, COS-7 cells were plated on to glass coverslips in 6-well tissue-culture plates and allowed to recover overnight. Cells were washed with PBS, fixed in 3 % (w/v) paraformaldehyde in PBS for 30 min, and permeabilized and blocked in PBS containing 0.2 % (v/v) Triton X-100 and 5 % (v/v) goat serum for 30 min. All further incubations and washes were performed with PBS containing 0.2 % (v/v) Triton X-100 and 5 % (v/v) normal goat serum. The fixed cells were probed with Protein A-purified anti-TACE extracellular antibody or pre-immune antibody for 2 h at room temperature and with a Texas-Red-coupled goat anti-rabbit IgG secondary antibody (Molecular Probes) for 30 min. The coverslips were washed five times for 8 min each between the addition of the primary and secondary antibodies and after incubation with the secondary antibody. The coverslips were then mounted on to glass slides with Immuno Floure Mounting Medium (ICN, Costa Mesa, CA, U.S.A.) and allowed to set overnight. For overexpression studies, cells were plated on to coverslips 12–18 h before being transfected with a construct encoding for a truncated form of TACE lacking the pro and metalloprotease domains (TACE ΔMP). This construct consisted of the pcDNA3neo expression vector (Invitrogen) containing a sequence coding for the signal sequence of human MDC9 fused to the sequence coding for amino acids 474–824 of TACE. This form of TACE was efficiently expressed, as determined by Western blot using either the anti-TACE cytoplasmic tail or anti-TACE extracellular antibodies (results not shown). Transfections were carried out using LipofectAMINE (Life Technologies) following the manufacturer's recommendations. After an overnight recovery, the coverslips were processed as described above. Cells were viewed and photographed with a Zeiss Axiophot microscope.

RESULTS AND DISCUSSION

Western-blot analysis of TACE in THP-1 and COS-7 cells

In order to generate reagents to allow for the characterization of TACE maturation and localization, antibodies against various domains of the protein were raised. Immunoblotting of membrane fractions isolated from THP-1 cells in the presence of 1 μM BB-94 allowed identification of two major forms of TACE (Figure 1A). One migrated as a triplet of approx. 110 kDa under non-reducing conditions and as a single band of 120 kDa under reducing conditions, and the second species migrated as a 80 kDa protein under non-reducing conditions and 100 kDa under reducing conditions. The shift in electrophoretic mobility upon reduction is similar to that of ADAMs and other proteins with cysteine-rich extracellular domains. The antiserum raised against an extracellular domain of TACE, expressed in eukaryotic cells, recognized the non-reduced form of TACE but failed to recognize the reduced protein on Western blots. In contrast, the monoclonal antibody raised against a TACE metalloprotease domain, expressed in bacteria, recognized the reduced forms of TACE but not the non-reduced forms. The antibody raised against the prodomain of TACE failed to recognize the faster migrating form of TACE. As numerous

© 2000 Biochemical Society

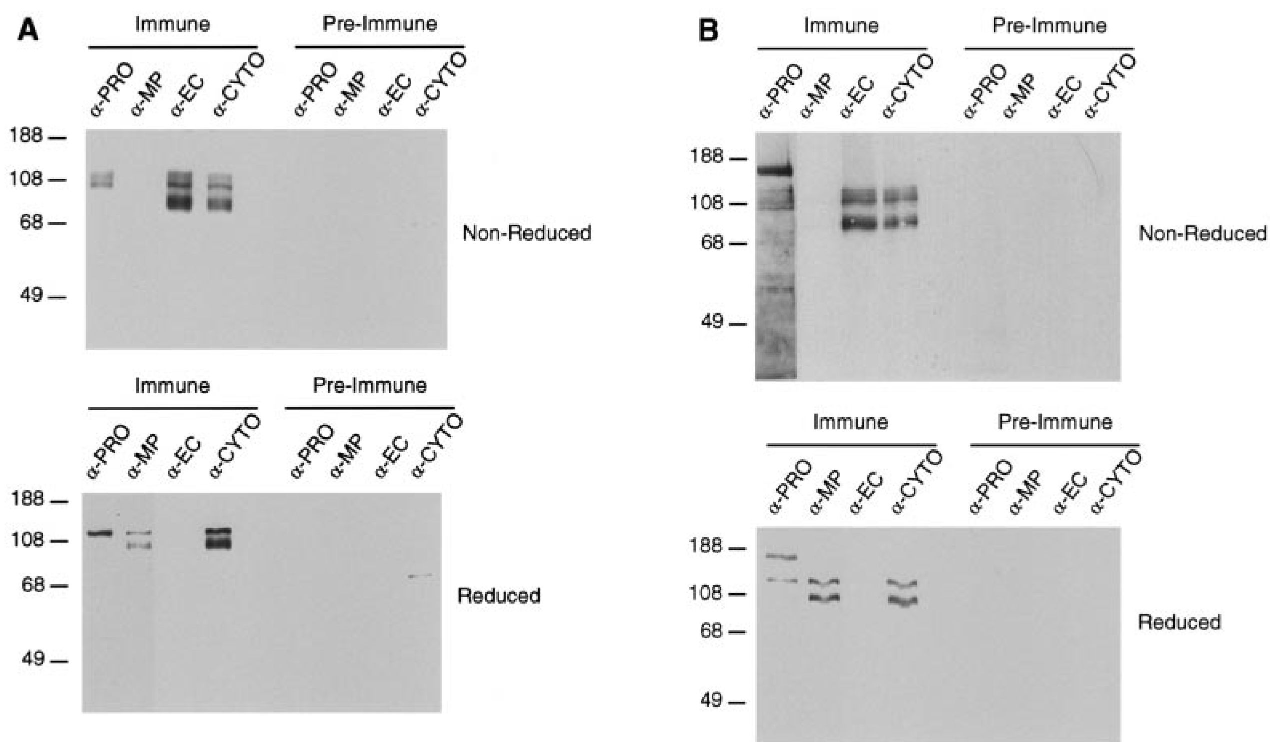

Figure 1 Western blots of TACE expression in cell lines

Membranes were prepared from either THP-1 cells (**A**) or COS-7 cells (**B**), as described in the Experimental section, boiled in sample loading buffer in the absence (top panels) or presence (bottom panels) of dithiothreitol and separated on 8% polyacrylamide gels. Approx. 12.5 μg and 8 μg of protein were loaded per lane in (**A**) and (**B**) respectively. The samples were transferred on to nitrocellulose and analysed by Western blot, using several antibodies raised against different domains of human TACE. Polyclonal antibodies were raised against the prodomain fused to GST (α-PRO), a GST-fusion protein containing the cyotplasmic domain (α-CYTO), and the extracellular portion of TACE lacking the prodomain (α-EC) as described in the Experimental section. The α-MP antibody is a monoclonal antibody raised against a bacterially expressed TACE metalloprotease domain (Tc3-7.49) [6,13]. Lanes were probed with either immune or pre-immune serum in the case of polyclonal antibodies, and with primary and secondary antibodies or secondary antibody alone in the case of the α-MP monoclonal antibody. Molecular-mass markers (in kDa) are shown on the left of the panels

other ADAM proteins have been shown to undergo proteolytic processing to remove their prodomain [11,14,15], the 80 kDa non-reduced/100 kDa reduced form therefore most likely represents mature TACE lacking the prodomain. Furthermore, we found that all four antibodies were capable of recognizing similar proteins in membrane preparations from COS-7 cells (Figure 1B), which presumably represent the African green monkey form of TACE. The prodomain antibody also recognizes a protein of approx. 160 kDa in COS-7 membrane preparations. This is probably a cross-reacting protein, as it is not recognized by any of the other antibodies. In both THP-1 and COS-7 cells, the mature form of TACE represented approx. two-thirds of the total amount of TACE. Similar results were obtained using other human cell lines, including HeLa and MDA-MB-468 cells (results not shown).

Post-lysis autocatalytic removal of the TACE cytoplasmic tail

When the same antibodies were used to detect TACE in ConA-precipitated samples from cell lysates of either COS-7 or THP-1 cells prepared in the presence of protease inhibitors and 10 mM EDTA but without BB-94, the 80 kDa band was detected only weakly (Figures 2A and 2B, and results not shown). Instead, a novel protein of 60 kDa (non-reduced) was recognized by the anti-extracellular antibody but not by the anti-cytoplasmic tail antibody (Figure 2A). However, when the metalloprotease inhibitors 1,10-phenanthroline or BB-94 were present during sample preparation, significantly less of the 60 kDa protein was detected, with a concomitant increase in the amount of 80 kDa protein (Figure 2B). The 80 kDa mature form of TACE but little of the 60 kDa protein were detected in membranes prepared in the absence of non-ionic detergents and metalloprotease inhibitor (results not shown). However, when detergent was added to membrane preparations, the 80 kDa protein was rapidly converted to the 60 kDa form. This conversion was prevented by the addition of 1 μM BB-94 (results not shown). In detergent-partitioning experiments using Triton X-114, the 60 kDa form of TACE was found predominantly in the detergent phase (Figure 2C), indicating that the transmembrane domain was most likely still present. Although most of the 110 kDa proTACE was found in the aqueous phase in the Triton X-114 detergent partitioning (Figure 2C), high-salt or high-pH extraction of membranes clearly showed that proTACE and mature TACE are integral membrane proteins (results not shown). As the 60 kDa protein was not recognized by antibodies raised against the cytoplasmic tail, it appears that this form of TACE is generated after cell lysis through the removal of the cytoplasmic domain by a metalloprotease. The data are consistent with a model whereby cytoplasmic-tail cleavage occurs through an intramolecular reaction in which TACE removes its own cytoplasmic domain. This model is particularly compelling, as no forms of TACE still containing the prodomain but lacking the cytoplasmic tail have been detected. The prodomain of TACE is postulated to act as

© 2000 Biochemical Society

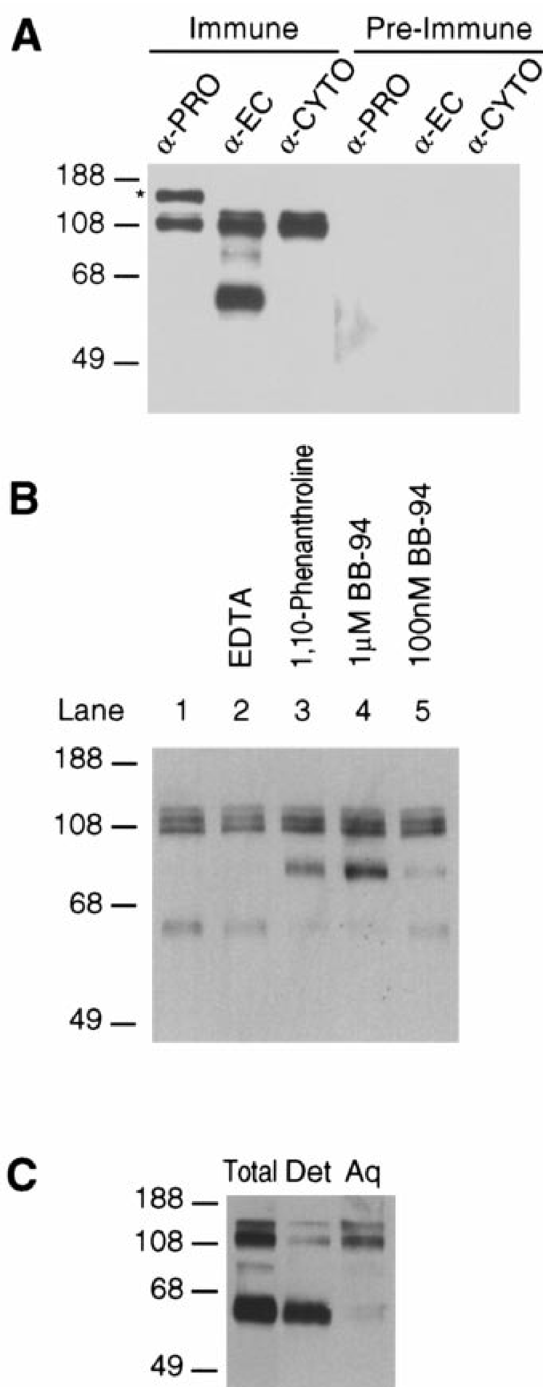

Figure 2 Post-lysis processing of TACE

(**A**) Glycoproteins were isolated from COS-7 cells utilizing lysis buffer B without metalloprotease inhibitors, as described in the Experimental section, and analysed by Western blot under non-reducing conditions. The anti-TACE antibodies used were identical to those shown in Figure 1. Note that the 160 kDa band (*) recognized by the prodomain fused to GST (α-PRO) antibody most likely represents a cross-reacting protein, but not TACE. (see also Figure 1B). (**B**) COS-7 cells were lysed in standard lysis buffer B lacking metalloprotease inhibitors (lane 1) or with the addition of the following metalloprotease inhibitors: 10 mM EDTA (lane 2), 10 mM 1,10-phenanthroline (lane 3), 1 μM BB-94 (lane 4) or 100 nM BB-94 (lane 5). Glycoproteins were precipitated with ConA–Sepharose and analysed by Western blot under non-reducing conditions using the anti-TACE extracellular antibody. (**C**) Detergent partitioning of TACE. COS-7 cells were resuspended in TBS and extracted with Triton X-114. Glycoproteins in the detergent (Det) and aqueous (Aq) phases were separated, precipitated with ConA and analysed by Western blot under non-reducing conditions, using the anti-TACE extracellular antibody. Glycoproteins isolated from COS-7 cells using lysis buffer B and ConA as in (**A**) are shown for comparison (Total). Note that the partitioning of the 110 kDa pro-TACE protein, which has a membrane anchor, into the aqueous phase suggests that the presence of the cytoplasmic tail increases the hydrophilicity of the protein. Molecular-mass markers (kDa) are shown on the left of the panels.

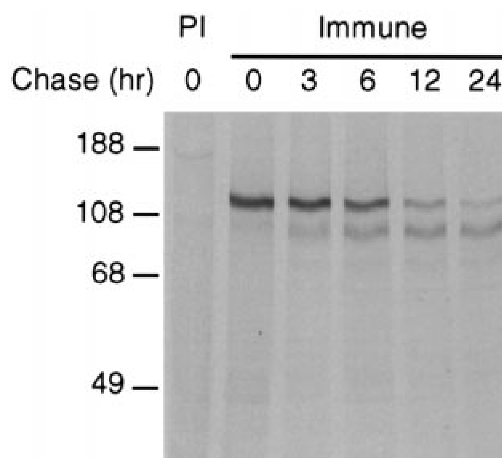

Figure 3 Pulse–chase analysis of TACE maturation in COS-7 cells

COS-7 cells were labelled for 3 h with [^{35}S]methionine/cysteine and then chased in complete media for the indicated time (hr). Cells were lysed in the presence of BB-94 and immunoprecipitations were performed using either Protein A-purified anti-TACE extracellular IgG (Immune) or pre-immune IgG (PI). After washing, the bound material was removed by boiling in sample loading buffer. This sample was diluted 1:50 with lysis buffer B and incubated with ConA–Sepharose to remove non-glycosylated background bands. The beads were washed and bound material was boiled in sample loading buffer, reduced and separated by SDS/PAGE (8% polyacrylamide gel). Molecular-mass markers (in kDa) are shown on the left.

an inhibitor of the protease, with proteolytic removal of the prodomain a prerequisite for protease activity [11,13]. In addition, when a truncated form of TACE lacking the pro and metalloprotease domains was expressed in COS-7 cells, the cytoplasmic domain of the protein remained intact (results not shown). These findings argue strongly for an intramolecular cleavage as opposed to removal of the cytoplasmic tail by another protease in an intermolecular reaction, which should affect all forms of TACE similarly. These results further demonstrate that it is important that 1,10-phenanthroline or a hydroxamate, such as BB-94, be included in the cell lysis buffer for any studies of TACE processing and maturation.

TACE processing in the secretory pathway

As shown in Figure 1, TACE appears to exist in two forms, a full-length form containing the prodomain and a mature one lacking the prodomain. For several other ADAM proteases, it has been shown that mature protein is generated from the proform by proteolytic removal of the prodomain by a furin-type proprotein convertase [11,14,15]. Processing occurs during the transit of the protein through the late Golgi compartments. Consistent with such a model, TACE contains a furin-cleavage site (RXK/RR) at the predicted boundary of the pro and metalloprotease domains. N-terminal sequencing of a recombinant soluble form of TACE has confirmed that this site is used to generate mature, soluble TACE [6]. To evaluate the processing of endogenous TACE, pulse–chase experiments were performed in COS-7 cells (Figure 3). After labelling for 3 h, only the

© 2000 Biochemical Society

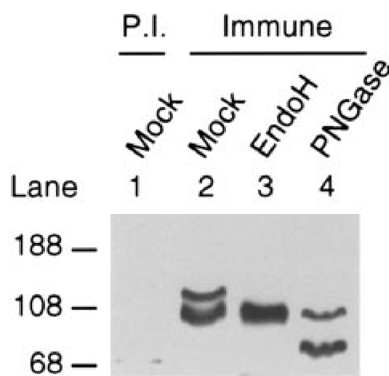

Figure 4 Deglycosylation of TACE

Membranes prepared from THP-1 cells were resuspended in sample loading buffer, reduced and boiled. The samples were adjusted to pH 5.5 with sodium citrate buffer (lanes 1–3) or adjusted to 1% (v/v) Nonidet P-40 and pH 7.5 with sodium phosphate buffer (lane 4). Samples were incubated at 37 °C overnight with the addition of either 500 units of recombinant EndoH (lane 3) or 500 units of PNGase F (lane 4). Samples were analysed by Western blot utilizing the anti-TACE cytoplasmic tail antiserum (lanes 2–4) or pre-immune serum (lane 1). Mock (lanes 1 and 2), samples without EndoH or PNGase F. Molecular-mass markers (in kDa) are shown on the left.

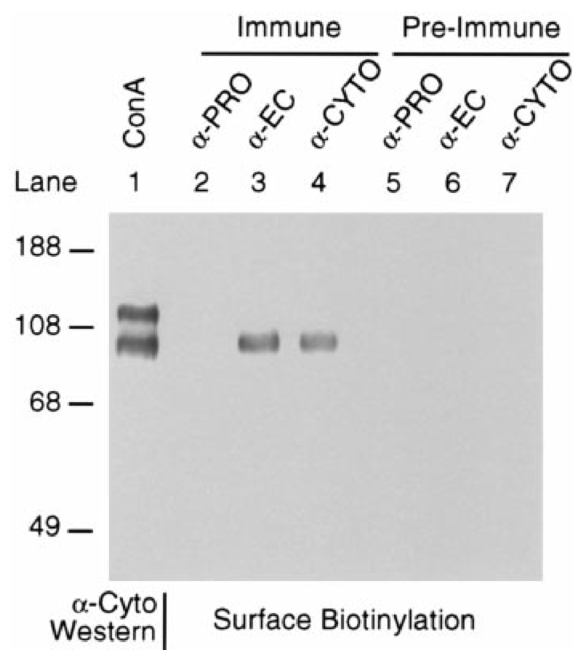

Figure 5 Detection of TACE by cell-surface biotinylation of COS-7 cells

COS-7 cells were labelled, on ice, with the membrane-impermeable NHS-LC-biotin and, after removal of unreacted biotinylation reagent, cells were lysed. Lane 1, glycoproteins from cell lysate were precipitated with ConA–Sepharose and analysed by Western blot using the anti-TACE cytoplasmic tail antibody. Lanes 2–7, TACE from the lysate was immunoprecipitated with anti-TACE prodomain antiserum (α-PRO; lane 2), anti-TACE extracellular domain antiserum (α-EC; lane 3), anti-TACE cytoplasmic tail antiserum (α-CYTO; lane 4) (Immune), or the corresponding pre-immune serum (lanes 5–7 respectively). Immunoprecipitated material was separated by SDS/PAGE, transferred on to nitrocellulose and probed with strepavidin-coupled horseradish peroxidase. All samples were reduced before electrophoresis. Molecular-mass markers (in kDa) are shown on the left.

120 kDa full-length form of TACE was detected. With increasing chase time, the 120 kDa form was converted into a 100 kDa form representing TACE without its prodomain. The rate of prodomain removal of TACE was slow compared with over-expressed MDC9 [11] or MDC15 [14], with approximately half of the full-length protein converted after 12 h.

To define the cellular compartment in which prodomain processing takes place, the ability of two deglycosidases, endoH and PNGase F, to remove N-linked sugars from TACE was assessed (Figure 4). Although N-linked sugars remain sensitive to PNGase F, the majority become resistant to EndoH after they are modified in the medial Golgi. After deglycosylation with PNGase, full-length TACE migrated as a 100 kDa protein; mature TACE migrated as a 70 kDa protein. Full-length TACE was sensitive to EndoH and mature TACE was resistant, indicating that the former is localized in the endoplasmic reticulum and proximal Golgi, whereas the latter had traversed the medial Golgi. This pattern is consistent with removal of the prodomain in the trans-Golgi network by a furin-type proprotein convertase. The ability of AEBSF, a known inhibitor of pro-protein convertases, to inhibit the maturation of TACE in pulse–chase experiments further supports this model (results not shown). Thus mature TACE is generated by the proteolytic removal of the prodomain, and this processing event is tightly linked with the transport of TACE through the medial Golgi.

Cell-surface labelling of TACE

In order to identify which forms of TACE are present on the cell surface, COS-7 cells were surface labelled with a membrane-impermeable biotinylation reagent. TACE was then immuno-precipitated from these samples with one of three antibodies. As shown in Figure 5, a 100 kDa protein corresponding to mature TACE was immunoprecipitated with antibodies raised against either the extracellular or the cytoplasmic domains of TACE, but not with the prodomain antibody. All three antibodies immuno-precipitated TACE from [^{35}S]Cys/Met metabolically labelled cell lysate (results not shown). Identical results were obtained when THP-1 cells were used (results not shown), demonstrating that mature TACE was the predominant form found on the surface of these cells. The inability of the prodomain antiserum to immunoprecipitate mature TACE indicates that the pro-domain is no longer tightly associated with mature TACE on the cell surface. This is in contrast to MDC15 overexpressed in COS-7 cells, where fragments of its prodomain remain associated with the mature protein even after cleavage of the prodomain by a proprotein convertase [14]. Interestingly, recent studies of soluble forms of human TACE expressed in insect cells indicate that the disintegrin and cysteine-rich domains are important for mediating the dissociation of the prodomain from the metallo-protease after it has been proteolytically processed [13].

Immunofluorescence of TACE in COS-7 cells

To define the steady-state localization of TACE in COS-7 cells, immunofluorescence was performed on cells using antibodies raised against the extracellular domains of TACE. The majority of TACE was localized in a perinuclear compartment, with some diffuse localization consistent with surface and endoplasmic reticulum staining (Figure 6A). No significant staining was seen with the pre-immune antibody (Figure 6B). In order to confirm that the immune staining was specific to TACE, COS-7 cells were transiently transfected with a truncated form of TACE lacking the pro and metalloprotease domains (TACE ΔMP). Immuno-fluorescence staining of cells expressing low levels of this construct showed staining patterns similar to those seen in untransfected

© 2000 Biochemical Society

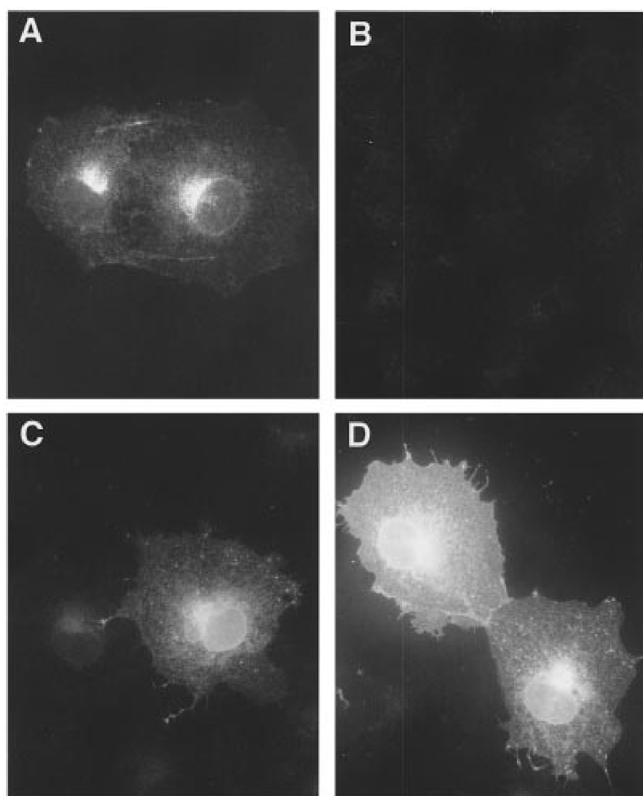

Figure 6 Immunofluorescence staining of TACE in COS-7 cells

Wild-type COS-7 cells (**A** and **B**), or COS-7 cells transiently expressing human TACE ΔMP (**C** and **D**), were fixed and permeabilized. The cells were incubated with either anti-TACE extracellular IgG (**A**, **C** and **D**) or the corresponding pre-immune IgG (**B**), followed by Texas Red-coupled goat anti-rabbit secondary antibodies. Endogenous TACE was seen in a predominantly perinuclear localization, with some diffuse staining consistent with surface and endoplasmic reticulum localization (**A**). When overexpressed at low levels, TACE ΔMP had a staining pattern similar to that seen with endogenous TACE, indicating that the antibody staining is likely specific for TACE (**C**). Furthermore, at the higher expression levels seen in some cells it was clear that some TACE ΔMP was present on the cell surface and in the endoplasmic reticulum, in addition to being present within a perinuclear compartment (**D**). Note that the exposure time for panels (**C**) and (**D**) was shorter than that of panels (**A**) and (**B**). This explains the lower intensity of endogenous TACE staining in nontransfected cells in (**C**) compared with (**A**). Only low levels of fluorescence were detected when cells were stained with pre-immune IgG (**B**). All images were taken using a Zeiss Axiophot microscope.

cells, indicating that the staining is likely specific (Figure 6C). Cells expressing higher levels of TACE ΔMP showed additional surface staining (Figure 6D). Thus although at least some endogenous TACE was detected at the cell surface, the majority of TACE appeared to be localized at an intracellular perinuclear compartment.

Stimulating cells with phorbol esters is known to increase the TACE-mediated ectodomain shedding of several proteins [5,7,8]. To determine whether this may be due to a relocalization of TACE, cells were pretreated with 20 ng/ml PMA for 30 min before fixing. The global distribution of TACE was not detectably altered in these cells (results not shown). However, we cannot rule out that a small subpopulation of TACE does redistribute and mediate increased shedding. Interestingly, TNFα has been shown to reside largely in an intracellular compartment in macrophages treated with an hydroxamate inhibitor [29]. These results raise the intriguing possibility that TACE may function predominantly intracellularly. Several studies have examined the shedding of the TACE substrates TNFα and TGFα [16,18–20]. TNFα cleavage appears to occur in a post-endoplasmic reticulum compartment and is tightly linked to transport to the surface, whereas TGFα located on the cell surface is rapidly cleaved and released into the media. However, these studies have not ruled out the possibility that a significant amount of shedding may actually occur in an intracellular compartment. In light of the localization of TACE, it will be interesting to compare what proportion of ectodomain shedding occurs at the surface versus intracellular compartments.

In summary, the present study describes the maturation and subcellular localization of TACE, a member of the ADAM family of metalloproteases responsible for the ectodomain shedding of several proteins. TACE exists predominantly in two forms, as the full-length protein and as a mature form lacking the prodomain, with the latter representing approximately two-thirds of the total population. This study has disclosed that in the absence of metalloprotease inhibitors, such as BB-94, the cytoplasmic tail of mature TACE is removed after cell lysis. The need for prior prodomain removal and disruption of the membrane by detergent in order for cytoplasmic-tail cleavage to occur is consistent with a model whereby the protease domain of TACE mediates this conversion in an intramolecular reaction. Although this is a post-lysis event, the processing must be taken into account when considering strategies for isolating active TACE and provides a simple assay for protease activity. Prodomain removal occurs after the protein exits the medial Golgi but before its arrival at the cell surface, and is likely to be mediated by a furin-type proprotein convertase. Although some mature form of TACE is detected on the cell surface, immunofluorescence indicates that the majority of TACE localizes to a perinuclear compartment. The predominant localization of TACE in an intracellular compartment raises the question of whether TACE functions intracellularly or if the proteolytic activity of TACE is limited to the cellular surface by a yet unknown mechanism.

We thank Dr Greg L. Pahel and Dr H. Luke Carter (Glaxo Wellcome Inc., Research Triangle Park, NC, U.S.A.) for expression and purification of the recombinant TACE 651 and Jui-lan Su (Glaxo Wellcome Inc. Research Triangle Park, NC, U.S.A.) for the production of the monoclonal anti-TACE antibody 3-7.49. We thank Dr L. Howard and Dr L. Lum for a critical reading of the manuscript, and other members of the laboratory of C.P.B. for valuable discussions and advice. This work was supported, in part, by a grant from Glaxo Wellcome (to C.P.B.), a National Institutes of Health Training Grant 5T32GM07739-17 and the Louis and Rachel Rudin Family Foundation (to J.S.), and by the Memorial Sloan-Kettering Cancer Center Support Grant NCI-P30-CA-08748 and the DeWitt Wallace Fund for Memorial Sloan-Kettering Cancer Center.

REFERENCES

1 Schlöndorff, J. and Blobel, C. (1999) J. Cell Sci. **112**, 3603–3617
2 Blobel, C. P. (1999) in Advances in Developmental Biochemistry, vol. 5 (Wassarman, P. M., ed.), pp. 165–198, JAI Press Inc., Stamford
3 Black, R. A. and White, J. M. (1998) Curr. Opin. Cell Biol. **10**, 654–659
4 Primakoff, P. and Myles, D. G. (2000) Trends Genet. **16**, 83–87
5 Black, R., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S. et al. (1997) Nature (London) **385**, 729–733
6 Moss, M. L., Jin, S.-L. C., Milla, M. E., Burkhart, W., Cartner, H. L., Chen, W.-J., Clay, W. C., Didsbury, J. R., Hassler, D., Hoffman, C. R. et al. (1997) Nature (London) **385**, 733–736
7 Peschon, J. J., Slack, J. L., Reddy, P., Stocking, K. L., Sunnarborg, S. W., Lee, D. C., Russel, W. E., Castner, B. J., Johnson, R. S., Fitzner, J. N. et al. (1998) Science **282**, 1281–1284
8 Buxbaum, J. D., Liu, K. N., Luo, Y., Slack, J. L., Stocking, K. L., Peschon, J. J., Johnson, R. S., Castner, B. J., Cerretti, D. P. and Black, R. A. (1998) J. Biol. Chem. **273**, 27765–27767
9 Sadhukhan, R., Santhamma, K. R., Reddy, P., Peschon, J. J., Black, R. A. and Sen, I. (1999) J. Biol. Chem. **274**, 10511–10516

© 2000 Biochemical Society

10 Van Wart, H. E. and Birkedal-Hansen, H. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5578–5582
11 Roghani, M., Becherer, J. D., Moss, M. L., Atherton, R. E., Erdjument-Bromage, H., Arribas, J., Blackburn, R. K., Weskamp, G., Tempst, P. and Blobel, C. P. (1999) J. Biol. Chem. **274**, 3531–3540
12 Loechel, F., Overgaard, M. T., Oxvig, C., Albrechtsen, R. and Wewer, U. M. (1999) J. Biol. Chem. **274**, 13427–13433
13 Milla, M. E., Leesnitzer, M. A., Moss, M. L., Clay, W. C., Carter, H. L., Miller, A. B., Su, J. L., Lambert, M. H., Willard, D. H., Sheeley, D. M. et al. (1999) J. Biol. Chem. **274**, 30563–30570
14 Lum, L., Reid, M. S. and Blobel, C. P. (1998) J. Biol. Chem. **273**, 26236–26247
15 Loechel, F., Gilpin, B. J., Engvall, E., Albrechtsen, R. and Wewer, U. M. (1998) J. Biol. Chem. **273**, 16993–16997
16 Merlos-Suarez, A., Fernandez-Larrea, J., Reddy, P., Baselga, J. and Arribas, J. (1998) J. Biol. Chem. **273**, 24955–24962
17 Dallas, D. J., Genever, P. G., Patton, A. J., Millichip, M. I., McKie, N. and Skerry, T. M. (1999) Bone **25**, 9–15
18 Solomon, K. A., Covington, M. B., DeCicco, C. P. and Newton, R. C. (1997) J. Immunol. **159**, 4524–4531
19 Arribas, J. and Massague, J. (1995) J. Cell Biol. **128**, 433–441
20 Arribas, J., Coodly, L., Vollmer, P., Kishimoto, T. K., Rose-John, S. and Massague, J. (1996) J. Biol. Chem. **271**, 11376–11382
21 Nemunaitis, J., Poole, C., Primrose, J., Rosemurgy, A., Malfetano, J., Brown, P., Berrington, A., Cornish, A., Lynch, K., Rasmussen, H. et al. (1998) Clin. Cancer Res. **4**, 1101–1109
22 Krätzschmar, J., Lum, L. and Blobel, C. P. (1996) J. Biol. Chem. **271**, 4593–4596
23 Harlow, E. and Lane, D. (1988) Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY
24 Lum, L., Wong, B. R., Josien, R., Becherer, J. D., Erdjument-Bromage, H., Schlondorff, J., Tempst, P., Choi, Y. and Blobel, C. P. (1999) J. Biol. Chem. **274**, 13613–13618
25 Nelson, K. K., Schlöndorff, J. and Blobel, C. P. (1999) Biochem. J. **343**, 673–680
26 van't Hof, W. and Resh, M. D. (1997) J. Cell Biol. **136**, 1023–1035
27 Blobel, C. P., Myles, D. G., Primakoff, P. and White, J. W. (1990) J. Cell Biol. **111**, 69–78
28 Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1995) Current Protocols in Molecular Biology, vol. 3, John Wiley and Sons Inc., New York
29 McGeehan, G. M., Becherer, J. D., Bast, Jr., R. C., Boyer, C. M., Champion, B., Connolly, K. M., Conway, J. G., Furdon, P., Karp, S., Kidao, S. et al. (1994) Nature (London) **370**, 558–561

Received 25 October 1999/13 December 1999; accepted 19 January 2000

© 2000 Biochemical Society