Pro-Tumor Necrosis Factor-α Processing Activity Is Tightly Controlled by a Component That Does Not Affect Notch Processing*

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The extracellular domain of a heterogeneous group of transmembrane proteins can be proteolytically released from the cell surface, a process known as protein ectodomain shedding. Despite the biomedically important role of several substrates of the shedding system, such as the β-amyloid precursor protein (βAPP), little is known about the regulation of protein ectodomain shedding, and the only protease known to be involved is the metalloprotease disintegrin, tumor necrosis factor-α converting enzyme (TACE). Here, we show that previously described pro-transforming growth factor-α shedding-defective cell mutants (M2 cells), known to be defective in ectodomain shedding of several molecules, that include βAPP, fail to shed the ectodomain of pro-TNF-α. The target of the mutation is a component required for TACE activity, since transfection of TACE into M2 cells has no effect on the shedding of pro-TNF-α and somatic cell fusions between M2 cells and TACE null cells recover the ability to shed pro-TNF-α, pro-transforming growth factor-α, and βAPP. Furthermore, we show that TACE is also necessary for the shedding of βAPP since TACE null cells show defective βAPP shedding. Biochemical evidence shows that the component that controls TACE is different from protein kinase C, the only known activator of protein ectodomain shedding, and that this component does not affect biosynthesis or processing of TACE or other metalloprotease disintegrins. The component mutated in M2 cells is likely to control only a subset of metalloprotease disintegrins involved in regulated ectodomain shedding, since Notch processing, a process known to be dependent on the activity of another metalloprotease disintegrin, Kuzbanian, is normal in M2 cells.

The extracellular domain of a functionally and structurally diverse group of transmembrane proteins can be shed from the cell surface via proteolytic cleavage, a process also known as ectodomain shedding. Several features are common to the shedding of the ectodomain of the majority of cell surface proteins analyzed so far. Biochemical evidence show that protein ectodomain shedding is activated via protein kinase C (PKC)1 in most, if not all, cases tested to date (1). Also, hydroxamic acid-based inhibitors, initially developed to block the action of zinc-dependent matrix metalloproteases, have been shown to inhibit the shedding of pro-TNF-α (2–4), the Fas ligand (5), pro-TGF-α (6), the 80-kDa TNF-α receptor (7), IL-6 receptor (IL-6R), the p60 TNF receptor (8), the thyrotropin receptor (9), angiotensin converting enzyme, t-selectin (10), and the amyloid β protein precursor (βAPP) (6). Using a genetic approach, it has been found that CHO cell mutants initially selected for lack of pro-TGF-α shedding are also defective in ectodomain shedding of βAPP, t-selectin, IL-6R, and a number of anonymous CHO cell surface proteins (6, 11). On the other hand, the protease(s) responsible for the shedding of many cell surface molecules seem to have broad sequence specificity since mutational analysis of residues around the cleavage site of pro-TGF-α (12), βAPP (13), IL-6R (14), t-selectin (15), and pro-TNF-α (16) has shown no specific sequence requirements. Some of the transmembrane proteins that undergo ectodomain shedding are of biomedical interest. For example, the amyloid β peptide is an invariant component of the amyloid deposits found in brains of patients with Alzheimer’s disease. The activity that sheds the ectodomain of βAPP (also known as α-secretase) acts within the amyloid peptide precluding the formation of potentially amiloiodogenic, and therefore potentially pathologic, βAPP fragments (17).

Last year, the protease responsible for the shedding of pro-TNF-α was identified and cloned (18, 19). TNF-α converting enzyme (TACE) belongs to the family of metalloprotease disintegrin proteins (also known as ADAM or MDC proteases, reviewed in Refs. 20 and 21). TACE is expressed ubiquitously and several TACE knock-out cell types lose TNF-α processing activity indicating that TACE is responsible for pro-TNF-α shedding in most, if not all, cell types (18).2 Presently, it is not known the number of metalloprotease disintegrins involved in protein ectodomain shedding but it has been recently found that the activity of Kuzbanian, another member of the metalloprotease disintegrin family, is necessary for the cleavage of Notch receptors in the ectodomain, and that this cleavage is important for Notch function in Drosophila. The extracellular domain of Notch is not released to the cell media but remains

1 The abbreviations used are: PKC, protein kinase C; TNF-α, tumor necrosis factor-α; TACE, tumor necrosis factor-α converting enzyme; βAPP, β-amylloid precursor protein; MMP, matrix metalloprotease; DME, Dulbecco’s modified Eagle’s medium; CHO, Chinese hamster ovary; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; PGE, polyacrylamide gel electrophoresis.

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at the cell surface, bound to the transmembrane cytoplasmic domains through disulfide bonds (23, 24). Interestingly, the structure and function of Kuzbanian is conserved across evolution (25), indicating that mammalian Notch is a substrate of ADAM10, the mammalian ortholog of Kuzbanian.

Little is known about the regulation of the activity of metalloprotease disintegrins. Several authors have suggested that the mechanism of activation of certain members of the metalloprotease disintegrin family is similar to that of some members of the matrix metalloprotease family (MMPs) (18, 19, 25–27). MMPs are synthesized aszymogens containing a prodomain with an odd number of cysteine residues, one of which blocks the active site zinc atom through a coordinate bond. MMPs become active after proteolytic removal of the propeptide. This mechanism of control of the metalloprotease activity is known as the “cysteine switch” (reviewed in Ref. 28). The boundary between the prodomain and the metalloprotease domain of some MMPs contains a typical furin-like cleavage site composed of four basic residues. It has been shown that the MMPs stromelysin-3 and MT1-MMP are indeed proteolytically processed by furin in the Golgi compartment (29, 30). Several members of the metalloprotease disintegrin family, such as TACE, MDC9, and Kuzbanian, contain in the prodomain a conserved sequence which is comparable to the cysteine switch sequences of MMPs and four basic amino acids between the prodomain and the metalloprotease domain, indicating that the mechanism of activation of these members of the metalloprotease disintegrin family includes a cysteine switch and proteolytic removal of the prodomain by furin-like convertases.

Here, we show that a previously described shedding defective mutant cell line, M2, is unable to process pro-TNF-α. The target of the mutation is a gene different from TACE or PKC, since transfection of TACE does not restore TNF-α processing activity, M2 cells show no defects in PKC function and somatic cell fusions between M2 cells and TACE null cells recover the wild type phenotype. Furthermore, although the regulated shedding of βAPP is defective in TACE null cells and in M2 cells, hybrids between TACE null cells and M2 mutants show normal levels of βAPP regulated shedding. The biosynthesis and processing of TACE in mutant cells is indistinguishable from those in wild type cells. The component necessary for the activity of TACE does not seem to control all the members of the metalloprotease disintegrin family. The mechanism of activation of these members of the metalloprotease disintegrin family, included in the shedding of relevant proteins such as pro-TNF-α or βAPP.

**EXPERIMENTAL PROCEDURES**

cDNAs and Antibodies—Plasmids containing human cDNAs encoding pro-TNF-α or mouse TACE and monoclonal antibody M222 against the ectodomain of TACE were kindly provided by Immunix. Monoclonal antibodies against TNF-α were provided by Immunex or purchased from Boehringer Mannheim. cDNA and antibodies against mouse MDC9 were kindly provided by Dr. Carl Blobel. Antibodies against the cytoplasmic tail of βAPP were provided by Dr. Samuel Gandy. Mouse Notch tagged at the COOH terminus with the Myc epitope was kindly provided by Dr. Raphael Kopan. Monoclonal antibodies against different PKC isoforms were purchased from Transduction Laboratories.

**Construction of TACE Tagged with the Myc Epitope**—The Myc epitope was introduced at the very carboxyl terminus of mouse TACE using the unique HindIII site of mouse TACE and two complementary oligonucleotides encoding the sequence DSKETGCEQKLISEDLE-

(Stop), where the underlined part represents the Myc epitope.

**Cells, Transfections, Indirect Immunofluorescence, and Fusions**—TACE-deficient ear fibroblasts were isolated from TACE-deficient mouse as described. TACE-deficient fibroblasts were transfected (using LipofectAMINE) with human pro-TNF-α cDNA in the pCDNA3zeo expression vector from Invitrogen (2 × 10⁶ cells with 15 µg of cDNA, 200 µl of LipofectAMINE). The transfectants were selected in growth media (1% fetal bovine serum in DMEM/F-12) containing 500 µg/ml Zeocin for 2 weeks. 24 individual colonies were isolated and screened for human pro-TNF-α expression on the cells using an enzyme-linked immunoabsorbent assay. Clones expressing high levels of pro-TNF-α were selected for further studies and maintained in growth media containing 200 µg/ml Zeocine. Wild type and M2 mutant cells defective in pro-TGF-α ectodomain shedding have been described elsewhere (6, 31).

For transient expression, the various constructs were transfected using the DEAE dextran method as described (32). To verify the simultaneous expression of co-transfected molecules in this system pro-TNF-α and Myc/TACE, both subcloned into pcDNA3, were transiently co-transfected in M2 cells. Transiently transfected cells were analyzed by indirect immunofluorescence using monoclonal antibodies against the ectodomain of pro-TGF-α, monoclonal anti-Myc antibodies, fluorescein isothiocyanate-conjugated anti-mouse, and tetramethyl-rhodamine isothiocyanate-conjugated anti-rabbit antibodies (Vector Laboratories, Burlingame, MA). More than 70% cells expressing pro-TGF-α were found to express detectable levels of Myc/TACE as well (data not shown).

For cell fusions, 2 × 10⁶ M2 mutant cells of a histidinol resistant clone and 2 × 10⁶ TACE null cells stably transfected with pro-TNF-α were plated into 60-mm dishes. 16 h later, the cultures were briefly covered with 3 ml of 45% polyethylene glycol (M, 1300–1600, American Type Culture Collection) in DME, 25 ml Hepes, with a final pH 7.3. The polyethylene glycol/DMEM solution was immediately aspirated, leaving only a minimum amount needed to cover the cells, and the cultures were incubated for 10 min at 37 °C. Cells were then washed three times with DMEM and twice with DME containing non-essential amino acids and 10% fetal bovine serum using warm media. After 10 h incubation in the later medium the cultures were trypsinized and plated into 150-mm dishes. Hybrid cell clones were selected in histidine-free DMEM, 10% diazoyl fetal bovine serum, 0.5 mM histidinol, and 200 µg/ml Zeocin for 5 weeks.

**Metabolic Labeling and Immunoprecipitation**—Approximately 4 × 10⁶ exponentially growing wild type or mutant CHO cells transiently transfected with human pro-TNF-α, mouse TACE, Myc/TACE, mouse Myc/Notch, or mouse MDC9 subcloned into the pcDNA3 vector (Invitrogen) were labeled for 30 min with 500 µCi/ml Trans-³⁵S-label (NEN Life Science Products Inc.) in methionine and cysteine-free medium at 37 °C. The label was chased in complete medium for the indicated times in the presence or absence of 1 µM PMA. Cells were then washed with cold PBS and lysed in PBS containing 1% Nonidet P-40 and 1 mM EDTA (lysing buffer). Aliquots from the media and cell lysates were immunoprecipitated with the appropriate antibodies as indicated and immunoprecipitates were analyzed by SDS-PAGE.

For analysis of phosphoproteins, wild type, or M2 cells were labeled metabolically with [³²P]Phosphate, treated with or without 1 µM PMA for 10 min and lysed. Aliquots from whole cell lysates were analyzed by two-dimensional gel electrophoresis using standard methods.

**Cell Fractionation**—10⁶ exponentially growing wild type or M2 cells were treated with or without 1 µM PMA for 5 min, washed, scraped in cold PBS containing 10% glycerol, and sonicated (three cycles, 30 s power on, 1 min power off) on ice. Soluble fraction was obtained after centrifugation at 100,000 × g for 1 h. The pellet obtained after centrifugation at 100,000 × g was extracted in lysis buffer and centrifuged at 130,000 × g in a Microfuge. The supernatant, containing solubilized membrane proteins, was considered the membrane fraction.

**Flow Cytometry**—Cells stably transfected with pro-TGF-α taggged in the ectodomain with the HA epitope (31) were treated with or without PMA for variable periods of time at 37 °C. Cells were then shifted to 4 °C, incubated for 45 min with 10 µg/ml anti-HA monoclonal antibodies in PBS containing 5% bovine serum albumin and stained for 30 min at 4 °C with fluorescein isothiocyanate-conjugated anti-mouse IgG (Beckton Dickinson) in PBS containing 5% bovine serum albumin. Flow cytometry was done on a FACSCan instrument and software (Beckton Dickinson).

**Reverse Transcriptase-Polymerase Chain Reaction**—Total mRNA from wild type or M2 cells was obtained using conventional methods, reverse transcribed using oligo(dT) and amplified using oligonucleotides specific for TACE (5'-GAGATGTGGACGACTTGGGTTGGTCGA-TGCAAC-3') or Kuzbanian (5'-CCATCAGCTTGGGTTGACATAC-3' and 5'-CCATCTGTTAATCTCTGGTCG-3'). The identity of the amplified fragments was confirmed by analysis with restriction enzymes.
RESULTS

**TNF-α Shedding in Wild type and M2 Shedding Defective CHO Cell Mutants**—The CHO mutant cell line M2, initially selected for lack of regulated pro-TGF-α ectodomain shedding, is defective in a functional component necessary for the normal shedding of the ectodomain of βAPP, L-selectin, IL-6R, and a variety of endogenous CHO cell surface molecules (6). To test if the component mutated in M2 cells is also necessary for the shedding of pro-TNF-α, wild type and mutant CHO cells were transiently transfected with human pro-TNF-α. In agreement with previous results, immunoprecipitation of metabolically labeled pro-TNF-α transfectants with antibodies directed against the ectodomain of pro-TNF-α yielded the expected product of 26 kDa that is exposed at the cell surface (for example, see Ref. 3 and Fig. 1). The ectodomain of cell surface pro-TNF-α is shed generating soluble 17-kDa TNF-α by a mechanism that can be activated via PKC (Ref. 8 and Fig. 1). In contrast to wild type CHO cells, M2 cells showed a lack of basal or activated pro-TNF-α shedding activity as judged by pulse-chase experiments (Fig. 1, A and C), indicating that the gene affected in the M2 mutant cell line is also necessary for the shedding of pro-TNF-α.

Effect of TACE Expression on the Shedding of Pro-TNF-α in Wild Type and M2 Cells—Mouse fibroblasts genetically deficient in the zinc-binding domain of TACE release 80–90% less TNF-α than a comparable population of wild type cells (see Fig. 2A). Transfection of TACE null fibroblasts with TACE restores TNF-α processing activity. To determine whether pro-TNF-α shedding activity can be restored in M2 mutant cells by overexpression of TACE, wild type and M2 cells were co-transfected with pro-TNF-α and murine TACE and the shedding of pro-TNF-α was assayed as above. Previous reports showed that TACE is predominantly expressed as a cell surface processed protein of approximately 85 kDa. Slight variations in the electrophoretic migration of cell surface TACE occur in different human cell lines (18). As shown in Fig. 1D, Western blot analysis of wild type and M2 TACE transfectants with monoclonal antibodies directed against the ectodomain of murine TACE yielded a main product of 110 kDa (Fig. 1, panel D). Transient expression of TACE had little or no effect on pro-TNF-α ectodomain shedding in wild type CHO cells (Fig. 1, A-C), indicating that the CHO cell endogenous TACE activity does not limit the rate of pro-TNF-α shedding. In the shedding defective mutant cell line M2, expression of TACE did not induce a detectable level of TNF-α shedding activity (Fig. 1, B and C), showing that TACE is not functional in the mutant background of M2 cells and indicating that the mutation in M2 affects a component different from, but necessary for, the function of TACE.

**Somatic Cell Fusions between M2 Mutant Cells and TACE Null Cells**—To directly establish whether the deficiency of the shedding defective mutant cell line affects a component different from TACE, M2 cells were fused to TACE null fibroblasts (TACE-/−) stably transfected with human pro-TNF-α. As expected, pro-TNF-α shedding activity is greatly reduced in

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**Fig. 1. Pro-TNF-α ectodomain shedding in wild type, M2 cells, and TACE transfectants.** Wild type or M2 cells transiently transfected with pro-TNF-α (A) or co-transfected with pro-TNF-α and TACE (B) were metabolically labeled with Trans35S-label for 30 min and chased in the absence or presence of PMA. Cell lysates and media supernatants were immunoprecipitated with antibodies against the ectodomain of pro-TNF-α and the immunoprecipitates analyzed by SDS-PAGE. C, dried gels were quantitated with a PhosphorImager. The results are average ± S.D. of triplicate determinations. D, wild type or M2 cells transiently co-transfected with pro-TNF-α and TACE or control empty vectors were lysed and cell lysates analyzed by Western blotting with monoclonal antibodies against the ectodomain of TACE.
TACE−/− fibroblasts and M2 cells when compared with control fibroblasts and CHO cells, respectively (Figs. 1A and 2A, and data not shown). However, somatic cell fusions between TACE−/− fibroblasts and M2 cells show appreciable levels of pro-TNF-α shedding (Fig. 2A), confirming that the defect in M2 mutant cells affects a component different from TACE.

To test if the general shedding system is reconstituted in the cell fusions generated, we analyzed the shedding of molecules expressed by parental and hybrid cells whose shedding is defective in M2 cells. The shedding of pro-TGF-α was tested in wild type, M2, and fusions by flow cytometry. As described previously, PMA treatment produced the typical loss of fluorescence at the cell surface of wild type but not mutant CHO cells expressing pro-TGF-α (Fig. 2B) (31). The shedding of pro-TGF-α from M2 X TACE−/− cells is comparable to that of wild type control cells and CHO X TACE−/− fibroblasts, corroborating that the shedding system is functional in M2 X TACE−/− fibroblasts cell hybrids.

βAPP Shedding in TACE Null Cells and Hybrids between TACE Null Cells and M2 Mutant Cells—Different βAPP isoforms are ubiquitously expressed, thus we analyzed the shedding of endogenous βAPP in TACE−/− cells and cell hybrids by immunoprecipitation of metabolically labeled products with specific antibodies. As previously shown, βAPP is expressed as intracellular immature ~115-kDa forms and as cell surface fully modified ~145-kDa forms (33). Upon PKC activation, the 145-kDa mature forms are shed from the cell surface releasing soluble APP into the cell media and leaving a 15-kDa transmembrane/ectoplasmic tail bound to the cells (34). Pulse-chase experiments showed products consistent with this process in control mouse fibroblasts (Fig. 3A, TACE+/-+) and CHO cells (Fig. 3C and Ref. 31).

Previous results showed that the shedding of βAPP is sensitive to TAPI, a metalloprotease inhibitor known to inhibit TACE (6). To analyze the role of TACE on the shedding of βAPP, TACE+/-+ and TACE−/− cells were treated with or without PMA and endogenous βAPP was immunoprecipitated. In agreement with recent results that involve TACE in the shedding of βAPP, TACE−/− cells show decreased secretion of soluble βAPP that is most apparent in PMA-treated cells, indicating that TACE is necessary for the activated shedding of βAPP (Fig. 3, A and B). As in the case of pro-TNF-α and pro-TGF-α, the shedding of βAPP in cell fusions between M2 and TACE null cell lines is comparable to that in wild type cells, arguing that TACE is necessary for the shedding of βAPP and that the factor mutated in M2 cells is necessary for the activity of TACE.

M2 Cells Do Not Show Global Defects in PKC Function—The only known common activator of protein ectodomain shedding is the PKC. PKC, or a protein necessary for PKC function, could be the target of the mutation that disrupted the shedding machinery in the M2 cell line. Previous indirect evidence indicated that the mutation that affects M2 cells does not affect PKC since two activators, each acting, in part, through PKC independent mechanisms, failed to promote shedding of pro-TGF-α or βAPP (31). To directly determine if PKC activity is normal in the mutant cell line M2, we analyzed in two-dimensional gels the phosphoproteins of wild type and M2 cells treated with or without PMA. The level of phosphate incorporation to the main substrate of PKC after PMA treatment, presumably the MARCKS protein (22), in wild type cells was found indistinguishable to that of M2 cells (Fig. 4A, arrow), indicating that PKC activity is not the target of the mutation that disrupts shedding. Next, we investigated the possibility that the mutation affected a protein necessary for the correct localization of PKC. To assess the ability of PKC to induce translocation of the PKC α and γ isoforms, the main PKC modulable PKC isoforms present in CHO cells (data not shown), wild type and M2 cells were treated with or without PMA, lysed, and the soluble and membranous fractions separated by ultracentrifugation. As shown in Fig. 4B, PMA treatment effectively depleted both PKC isoforms from the soluble fraction of wild type and mutant cells. Concomitantly, the amount of PKC in the membrane fraction increased similarly in both cell types, indicating that the translocation of PKC α and γ is normal in M2 cells. Furthermore, the level of expression and subcellular distribution of the rest of PKC isoforms expressed in CHO cells is similar in wild type and M2 cells (Fig. 4C). These results directly show that PKC function is normal in M2 cells and indicate that the target of the mutation that disrupts the shedding machinery in M2 cells affects a gene different from PKC.

Biosynthesis and Processing of Metalloprotease Disintegrins in Wild Type and M2 Cells—It has been proposed that the control of the activity of several metalloprotease disintegrins is

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similar to that of certain MMPs and includes a cysteine switch and proteolytic removal of the prodomain by furin-like proprotein convertases (18, 19, 25–27). In principle, the mutation in the shedding defective mutant cell line could affect a gene necessary for the processing of the prodomain of TACE. To compare the biosynthesis and processing of TACE in wild type and M2 cells, we performed pulse-chase experiments. To facilitate immunodetection we used mouse TACE tagged at the carboxyl terminus with the Myc epitope. In transiently transfected CHO cells, Myc/TACE is synthesized as a 100-kDa form that is rapidly modified, probably through glycosylation, to a form of 110 kDa. The 110-kDa form is chased to a form of 90 kDa, presumably after cleavage of the prodomain. The amount of the 90-kDa form reaches a maximum after 2 h chase, when it represents 10–20% of the total counts specifically immunoprecipitable after the pulse (Fig. 5 and data not shown). The rate and extent of appearance of the 90-kDa form in mutant cells was indistinguishable from those in wild type cells (Fig. 5), indicating that the defect in M2 cells does not affect components necessary for the biosynthesis or processing of TACE.

The partial processing of TACE could be physiological or, alternatively, could be due to the saturation of a component necessary for the biosynthesis or processing of TACE. To test if the processing of another member of the metalloprotease disintegrin family is also partial in the same conditions, wild type and mutant CHO cells transiently transfected with MDC9 were pulse-chased, and MDC9 was immunoprecipitated with specific antibodies. MDC9 is synthesized as a precursor of ~115 kDa that is completely processed after 2 h to a form of ~85 kDa (Fig. 5). These results are in agreement with previous results indicating that the 84-kDa form of MDC9 is predominant in a variety of tissues where little or no 115-kDa form is detected (25). Collectively, the results presented show that although the processing of TACE is partial in CHO cells, M2 mutant cells do not show defects in the machinery necessary for the biosynthesis and processing of different members of the metalloprotease disintegrin family.

**Notch Processing in Wild Type and M2 Cells**—The results presented so far reveal the existence of a novel component, disrupted in M2 cells, that controls the activity of TACE and perhaps other metalloprotease disintegrins. It has been recently shown that the activity of Kuzbanian, another member of the metalloprotease disintegrin family, is necessary for Notch processing. Thus, we investigated if the component defective in M2 cells is a general regulator of metalloprotease disintegrins first by analyzing if M2 cells express Kuzbanian, and then examining the processing of Notch in M2 cells. To determine if the mammalian homolog of Kuzbanian (also known as ADAM10) is expressed in wild type and M2 cells, we performed reverse transcriptase-polymerase chain reaction analysis using mRNA obtained from these cells and oligonucleotides specific for Kuzbanian. As shown in Fig. 6A wild type as well as M2 cells express detectable amounts of mRNA encoding Kuzbanian.

In cultured cells, Notch is synthesized as a 300-kDa precursor that is constitutively cleaved yielding a 110-kDa fragment.

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that contains the transmembrane and cytoplasmic domains and a 180-kDa fragment spanning the extracellular domain. Both fragments remain bound through disulfide bonds and are exposed to the cell surface (24). As expected, metabolic pulse-chase labeling of CHO cells transiently transfected with Notch tagged in the carboxyl terminus with the Myc epitope followed by precipitation of cell lysates with anti-Myc antibodies revealed the appearance of a 300-kDa immunoreactive species that was chased into 110-kDa forms. The rate and extent of appearance of Notch fragments was similar in wild type and M2 cells (Fig. 6B), showing that the component that controls the activity of TACE, defective in M2 cells, is not required for the processing of Notch. Furthermore, the constitutive cleavage of Notch was not augmented by PMA in wild type or M2 cells (Fig. 6C), arguing that Notch processing is not regulated by PKC. Collectively, the results presented show the existence of a novel component of the shedding system that specifically controls the activity of the PKC regulated metalloprotease disintegrin TACE and, perhaps, other members of the metalloprotease disintegrin family involved in protein ectodomain shedding.

DISCUSSION

Despite the biomedical interest of some of the proteins that undergo ectodomain shedding, best exemplified by βAPP, the proteases involved have remained elusive until recently. The first protease responsible for a shedding event to be identified, TACE, acts on pro-TNF-α and belongs to the metalloprotease disintegrin family (also known as ADAM or MDC family) (18, 19). In agreement with recent results, here, we show that TACE is not only necessary for the shedding of pro-TNF-α, but also for the shedding of βAPP since TACE null cells secrete significantly less soluble βAPP than control cells. Previous results from our laboratory showed that the shedding of βAPP is sensitive to TAPI (6), a well characterized inhibitor of TACE. Therefore, the evidence at hand strongly supports that TACE is responsible for, at least, part of the shedding activity that acts on βAPP (also known as α-secretase activity) in mouse fibro-
blasts and CHO cells.

Since the target of the mutation that affects M2, a cell line initially isolated for lack of pro-TGF-α shedding, is also necessary for the shedding of pro-TNF-α (this report), we investigated the possible effect of TACE expression in M2 cells. Expression of TACE showed little or no effect on the shedding of pro-TNF-α in M2 mutant cells, therefore we concluded that the factor defective in M2 cells is probably different from TACE. Somatic cell fusions between TACE null cells and M2 cell mutants further confirmed that the target of the mutation in the M2 cell line is not TACE but a factor necessary for its activity. On the other hand, direct biochemical evidence shows that the factor mutated in M2 cells is also different from PKC, the only known modulator of most shedding events.

Previous experiments showed that the factor defective in M2 cells is also necessary for the shedding of pro-TGF-α, IL-6R, l-selectin, and a variety of anonymous cell surface molecules (6, 31). The proteases responsible for the shedding of these molecules are not known, but they are probably similar to TACE, since their activity can be inhibited by TAPI. Alternatively, TACE could be a general “sheddase” controlled by the factor affected in M2 cells. In any case, the proteolytic activity(ies) acting on most, if not all, transmembrane proteins susceptible are quite unspecific since mutational analysis of residues around the cleavage site of pro-TGF-α (12), βAPP (13), IL-6R (14), l-selectin (15), and pro-TNF-α (16) has shown a lack of sequence specificity for shedding. Furthermore, short juxtamembrane sequences of different molecules, such as pro-TGF-α or βAPP are sufficient to confer susceptibility to the shedding system (11). Conceivably, the factor mutated in M2 cells could be involved in restricting the proteolytic activity(ies) participating in protein ectodomain shedding and impeding unwanted proteolysis of proteins whose ectodomain should remain intact. Alternatively, it could be a potent activator of TACE or could be involved in the recognition of the substrates of TACE and other metalloprotease disintegrins.

By analogy to the activation of some MMPs, it has been hypothesized that metalloprotease disintegrins containing four basic amino acids between the prodomain and the metalloprotease domain, such as TACE or MDC9, are activated by removal of the prodomain in the Golgi compartment by furin-like proprotein convertases. Analyzing the processing of transiently transfected TACE in CHO cells, we found that, although the conditions used do not lead to saturation of the machinery that
processes different metalloproteases, the processing of TACE is partial. The biological significance of the molecules that are not processed is unknown, however, the results presented clearly show that the amount of processed TACE in wild type cells is comparable to that of M2 cells, indicating that the factor mutated in M2 cells is not required for the activation of metalloprotease disintegrins by removal of the propeptide.

Soon after TACE was identified, the transmembrane protein Notch was found to be a substrate of another member of the metalloprotease disintegrin family, Kuzbanian (23). To test if the factor mutated in M2 cells does also control the processing of Notch, we transfected Notch into wild type and M2 cells and analyzed its processing. The rate and extent of Notch processing is indistinguishable in wild type and mutant cell lines and the factor mutated in M2 cells does also control the processing of Notch, we transfected Notch into wild type and M2 cells and analyzed its processing. The rate and extent of Notch processing is indistinguishable in wild type and mutant cell lines and is not activated by PKC, indicating that the regulation of Notch processing is different from that of other substrates of metalloprotease disintegrins and further supporting the notion that the factor mutated in M2 cells is not a general one involved in activation or trafficking of metalloprotease disintegrins. The results presented are compatible with a hypothesis in which the factor mutated in M2 cells controls the activity of a subset of metalloprotease disintegrins, that would include TACE, involved in protein ectodomain shedding.

In summary, genetic and biochemical analysis of shedding defective mutants and TACE null cells reveals the involvement of TACE in the shedding of bAPP and the existence of a novel component of the shedding system that tightly controls the activity of TACE and perhaps other metalloprotease disintegrins involved in protein-ectodomain shedding.

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