Metalloprotease-dependent Protransforming Growth Factor-α Ectodomain Shedding in the Absence of Tumor Necrosis Factor-α-converting Enzyme*

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Zinc-dependent metalloproteases can mediate the shedding of the extracellular domain of many unrelated transmembrane proteins from the cell surface. In most instances, this process, also known as ectodomain shedding, is regulated via protein kinase C (PKC). The tumor necrosis factor-α-converting enzyme (TACE) was the first protease involved in regulated protein ectodomain shedding identified. Although TACE belongs to the family of metalloprotease-disintegrins, few members of this family have been shown to participate in regulated ectodomain shedding. In fact, the phenotype of tace−/− cells and that of Chinese hamster ovary cell mutants defective in ectodomain shedding points to the existence of a common PKC-activated ectodomain shedding system, whose proteolytic component is TACE, that acts on a variety of transmembrane proteins. Examples of these proteins include the Alzheimer’s disease-related protein β-amyloid precursor protein (βAPP) and the transmembrane growth factors protransforming growth factor-α (pro-TGF-α) and, as shown in this report, pro-heparin-binding epidermal growth factor-like growth factor (pro-HB-EGF). Here we show that the mercurlurial compound 4-aminophenylmercuric acetate (APMA), frequently used to activate in vitro recombinant matrix metalloproteases, is an activator of the shedding of βAPP, pro-HB-EGF, and pro-TGF-α. Treatment of tace−/− cells or Chinese hamster ovary shedding-defective mutants with APMA activates the cleavage of pro-TGF-α but not that of pro-HB-EGF or βAPP, indicating that APMA activates TACE and also a previously unacknowledged proteolytic activity specific for pro-TGF-α. Characterization of this proteolytic activity indicates that it acts on pro-TGF-α located at the cell surface and that it is a metalloprotease active in cells defective in furin activity. In summary, treatment of shedding-defective cell lines with APMA unveils the existence of a metalloprotease activity alternative to TACE with the ability to specifically shed the ectodomain of pro-TGF-α.

During the last decade protein ectodomain shedding has rapidly evolved from an emerging concept to an important aspect of the cell biology (for a recent review, see Ref. 1). The diversity of the extracellular domains susceptible to being proteolytically released, which include transmembrane growth factors and cytokines, growth factor receptors, cell adhesion molecules, and ectoenzymes, has prompted cell biologists from different backgrounds to study the components and mechanisms involved in ectodomain shedding. In certain cases ectodomain shedding also has a practical interest; for example, the shedding of the ectodomain of the β-amyloid precursor protein (βAPP)1 prevents the formation of the β-amyloid peptide, a component of the lesions found in brains of patients with Alzheimer’s disease (2). Two aspects seem to be common to most, if not all, shedding events: they are controlled by shared regulatory mechanisms, the best characterized of which involves protein kinase C (PKC), and they are mediated by zinc-dependent metalloproteases (for reviews, see Refs. 3 and 4).

Hydroxamate-derived compounds, which efficiently inhibit zinc-dependent members of the matrix metalloprotease and metalloprotease-disintegrin families, block the PKC-activated shedding of all proteins tested to date, suggesting that proteases that belong to these families are responsible for activated ectodomain shedding (1). The metalloprotease-disintegrin TACE was identified as the first one involved in a particular shedding event: that of the cytokine pro-TNF-α (5, 6). The family of metalloprotease-disintegrins (also known as ADAM (a disintegrin and metalloprotease) or MDC (metalloprotease/disintegrin/cysteine-rich)) comprises 14 members with predicted proteolytic activity (7). Given the diversity of the proteins that can be shed and the number of potentially active metalloprotease-disintegrins, it is frequently assumed that several metalloprotease-disintegrins, each one endowed with a particular specificity, are involved in ectodomain shedding. However, mouse fibroblasts expressing an inactive form of TACE (tace−/− cells) show defects in ectodomain shedding that are not restricted to pro-TNF-α and related molecules (8). Instead, tace−/− cells lack the ability to shed a variety of molecules structurally and functionally unrelated to pro-TNF-α such as the transmembrane growth factor pro-TGF-α, the cell adhesion molecule L-selectin, the pro-TNF-α p75 and p55 re-
In this report, we show that necessary for the regulated shedding of pro-TNF-α, an unidentified gene that is different from PKC or TACE but involved for the Notch receptor (12), or hamster ovary (CHO) mutant cell lines (M2) initially isolated by PKC whose proteolytic component is TACE. Chinese hamster ovary (CHO) mutant cell lines (M2) initially isolated by PKC and on the ligand of the Notch receptor, Delta, in human embryonic kidney cells and Drosophila melanogaster, respectively (19, 20), and MDC9 has been implicated in the shedding of pro-HB-EGF mediated by PKC6 in Vero cells (21). However, in this report, we show that mouse fibroblasts or CHO shedding-defective cell mutants show a lack of constitutive and activated pro-HB-EGF shedding, indicating that TACE is responsible for the shedding of this growth factor in these cell types and opening the possibility that pro-HB-EGF is a substrate of different metalloprotease-disintegrins in different cell lines. Thus, despite expectations, the number of proteins whose PKC-activated shedding is found to be dependent on TACE is continuously growing, while little evidence on alternative proteolytic activities involved in regulated ectodomain shedding has been reported so far.

Since the majority of shedding events activated via PKC seem to be dependent on TACE, we have analyzed the effect of compounds with the potential to induce ectodomain shedding independently of PKC to determine the possible role of other proteolytic activities in ectodomain shedding. Here we present evidence showing that the mercurial compound 4-amino-phenylmercuric acetate (APMA), frequently used to activate recombinant matrix metalloproteases in vitro (22), is an activator of the shedding of several cell surface proteins such as βAPP, pro-HB-EGF, or pro-TGF-α when added to cells. The products of the shedding of βAPP, pro-HB-EGF, and pro-TGF-α induced by APMA are indistinguishable from those produced by TACE, indicating that APMA activates TACE and/or proteolytic activity(ies) with similar characteristics. Treatment of cells with APMA activates TACE and/or proteolytic activity specific for pro-TGF-α. Characterization of this proteolytic activity indicates that it is a metalloprotease inhibited by hydroxamate-derived compounds that does not require processing by furin to be activated by APMA and that it acts on cell surface but not on intracellular pro-TGF-α. In summary, the use of APMA and shedding-defective cell lines as tools has unveiled the existence of a specific metalloproteinase activity alternative to TACE with the ability to shed the ectodomain of pro-TGF-α.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-HA monoclonal antibodies were from Babo (Richmond, CA), the monoclonal anti-βAPP antibodies 22C11 and anti-Alz90 were from Roche Molecular Biochemicals, the polyclonal antibody against the cytoplasmic domain of βAPP was a gift from Dr. Sam Gandy, and the polyclonal antibodies against the cytoplasmic tail of pro-TGF-α have been previously described (23). Goat anti-mouse monoclonal p40/80-NHS-LC conjugated to horseradish peroxidase-conjugated streptavidin, and the SuperSignal chemiluminescence kit were from Pierce. Culture reagents were from Life Technologies Ltd.

cDNAs, Cell Lines, and Transfections—Pro-HB-EGF was amplified from a HeLa cDNA library using appropriate oligonucleotides and subcloned into pCER-4 vector (Invitrogen, San Diego, CA). The HA epitope was inserted at the N terminus of the mature growth factor, between amino acids 77 and 78, using PCR and standard techniques. The pro-HA/TGF-α and the C-terminal pro-HA/TGF-α mutant (V159G) have been described elsewhere (24). Wild type CHO cells expressing pro-HA/TGF-α or pro-HA/TGF-α V158G, the M2 mutant cell line, tace−/− fibroblasts, and the corresponding wild type cell lines have been described previously (16, 24). The furin-defective CHO mutant cell line, RPE-4, kindly provided by Dr. Joseph Sucic, was grown in DMEM F-12 supplemented with 1% nonessential amino acids and 7.5% fetal bovine serum.

The various cell lines were permanently co-transfected with the pro-HA/TGF-α or pro-HA/EB-EGF constructs and the selectable plasmid pREP-4 (Invitrogen) at a DNA ratio of 25:1 using the calcium phosphate precipitation method as described previously (14). Transfectants were selected in 600 μg/ml hygromycin and subcloned. The levels of expression of pro-HA/TGF-α or pro-HA/EB-EGF were monitored by flow cytometry using anti-HA antibodies and a FACSscan instrument and software (Becton Dickinson).

Metabolic Labeling and Immunoprecipitation—Approximately 3 × 10⁶ cells expressing pro-HA/TGF-α or a pro-HA/TGF-α C-terminal mutant were metabolically labeled with 500 μCi/ml [35S]cysteine (Biolink 2000, Arlington Heights, IL) for 45 min in cysteine-free medium at 37 °C and chased for variable periods of time in complete medium with or without 1 μM PMA, 0.5 mM APMA, or APMA and BB-94 at 37 or 23 °C as indicated. Conditioned media were harvested in the presence of a protease inhibitor mixture (10 mM 1,10-phenantroline, 1 mM phenylmethylsulfonyl fluoride, 10 μM EDTA, 2 mM pepstatin A, and 10 μM leupeptin). Cells were lysed in lysis buffer (PBS containing 1% Nonidet P-40 and the mixture of protease inhibitors), and insoluble material was removed by centrifugation; cell lysates were immunoprecipitated with polyclonal antibodies against the cytoplasmic domain of pro-TGF-α or anti-HA monoclonal antibodies. Endogenous βAPP was immunoprecipitated with polyclonal antibodies against the cytoplasmic domain of this protein. Immune complexes were collected with protein A-Sepharose, washed three times with washing buffer (PBS containing 0.1% Triton X-100 and 0.1% SDS), analyzed on SDS-polyacrylamide gels of the appropriate reticulation (13% for cell-associated pro-TGF-α and 15% for soluble TGF-α), and quantified using a BAS-1800 instrument and software (Fujifilm).

For immunoprecipitation of pro-TGF-α located at the cell surface, CHO or tace−/− cells expressing pro-HA/TGF-α were metabolically labeled as above, chased for 15 min in DMEM, and shifted to 4 °C. Cells were then incubated with PBS containing 5% bovine serum albumin and 5 μg/ml anti-HA monoclonal antibodies, washed extensively with DMEM, shifted to 37 °C, and further chased for 20 min in the presence or absence of 0.5 mM APMA. Conditioned media were harvested, and cell lysates were prepared as indicated above. Immune complexes were recovered with protein A-Sepharose, washed, and analyzed on SDS-polyacrylamide gels.

Western Blotting—Approximately 3 × 10⁶ parental HeLa, CHO, M2, or tace−/− cells or the same cells permanently transfected with pro-HA/EB-EGF were washed with DMEM and treated with 1 ml of DMEM with or without 1 μM PMA or 0.5 mM APMA for variable periods of time. Conditioned media were harvested in the presence of proteinase inhibitors, and cells were lysed as described above. Aliquots from conditioned media and from cell lysates were subjected to Western blotting analysis using the monoclonal anti-βAPP antibodies 22C11 or Alz90 as recom-b
mended by the manufacturers or 5 μg/ml anti-HA. Signals were detected with SuperSignal West Dura Extended Duration Substrate (Pierce). Films were digitized and quantified using MacBAS® software (Fujifilm).

Internalization Assay—Exponentially growing tace—/— cells permanently expressing pro-HA/TGF-α were treated with different concentrations of APMA in DMEM for 20 min (A and B) or treated with 0.5 mM APMA for variable periods of time as indicated (C and D). Cells were then lysed, and aliquots from the conditioned media and cell lysates were subjected to Western blot analysis with antibody 22C11, directed against the extracellular domain of βAPP, as described under “Experimental Procedures.” X-ray films were quantified using the MacBAS® software.

RESULTS

Effect of APMA on the Shedding of Several Transmembrane Proteins—To determine whether APMA, an activator of matrix metalloproteases (22) and metalloprotease-disintegrins (25), is also an activator of the shedding of transmembrane proteins, we initially analyzed its effect on the shedding of one of the substrates of TACE, βAPP. Western blot analysis of CHO cell lysates with the antibody 22C11, directed against the N terminus of βAPP, revealed the typical array of immature and fully modified, mature forms of βAPP (Fig. 1) that have been extensively characterized (26). As shown in Fig. 1, A and B, APMA induced a dose-dependent decrease in the amount of mature βAPP and, concomitantly, a release of soluble βAPP, also in a dose-dependent manner. The effect of APMA is rapid (t½ ~ 12 min) (Fig. 1, C and D) and similar in different cell lines, including HeLa (Fig. 1, B and D). To extend these results, we also analyzed the shedding of the EGF receptor ligands pro-TGF-α and pro-HB-EGF. APMA was also found to activate the shedding of these molecules from CHO cells with a dose dependence and time course indistinguishable from those found for βAPP (data not shown). Therefore, these results show that APMA is an activator of the shedding of diverse molecules such as βAPP, pro-TGF-α, or -HB-EGF.

Characterization of the Products of the Shedding Induced by APMA—To characterize the effect of APMA, we compared the electrophoretic migration of the products of the shedding of βAPP, pro-TGF-α, or pro-HB-EGF induced by APMA with that of the products produced by the PKC activator PMA, which activates the shedding of these molecules mediated by TACE (8, 13) or MDC9 (21). Several types of proteolytic activities, collectively known as secretases, act on βAPP. TACE possesses α-secretase activity since it cleaves βAPP, upon PKC activation, within the β-amylloid peptide, releasing the ectodomain of βAPP (Fig. 2A, schematic). As shown in Fig. 2A, the migration of the ~15-kDa stub that remains associated with cells after activation with APMA is indistinguishable from that generated by α-secretase under basal conditions or after PMA treatment. In addition, using monoclonal antibodies directed against the N terminus or the C terminus of the ectodomain of βAPP, equivalent soluble fragments are detected in the conditioned media of cells treated with PMA or APMA (Fig. 2A). In agreement with previous results, permanently transfected CHO cells express several pro-HB-EGF species of 20–30 kDa that differ in their glycosylation state and, therefore, in their electrophoretic migration (27). The HA epitope was introduced at the N terminus of the mature growth factor (Fig. 2B, schematic) to facilitate the detection of cell-associated pro-HB-EGF as well as soluble HB-EGF. The different forms of pro-TGF-α also differ in their glycosylation state and have been characterized previously (14). The electrophoretic migration of the ectodomains of pro-TGF-α or pro-HB-EGF or the transmembrane/cytoplasmic domain of pro-TGF-α generated after APMA treatment are indistinguishable from those generated after PMA treatment (Figs. 2, B and C). These results indicate that APMA activates one (or more) proteolytic activity(ies) that cleaves βAPP, pro-TGF-α, and pro-HB-EGF at a site very proximal, if not identical, to that cleaved by the shedding activities activated by PKC. Therefore, APMA could be an activator of the metalloprotease-disintegrins previously found to act on the molecules analyzed, or alternatively, APMA could activate novel endogenous proteolytic activities with similar, if not identical, specificity(ies).

Shedding of HB-EGF in tace—/—Cells—One of the strongest evidences indicating that TACE has a role in the shedding of βAPP and pro-TGF-α is the lack of shedding of these molecules in tace—/— cells (8, 13, 16). In addition, the shedding of both molecules is affected in a mutant cell line (M2), initially isolated for lack of pro-TGF-α shedding, that is defective in many unrelated shedding events (14–16). To characterize the shedding of HB-EGF to the same extent as that of pro-TGF-α or βAPP, we analyzed the shedding of HB-EGF in M2 and...
and immunoprecipitated with antibodies against the cytoplasmic tail of A
epitope, and the transmembrane (TM) domain of the cell lysates were subjected to Western blotting analysis with the indicated antibodies. Immune complexes were collected with protein A-Sepharose and analyzed by SDS-PAGE. Aliquots of conditioned media from cells treated in the same manner were subjected to Western blotting analysis with the signal peptide (SP), the β-amyloid peptide (black box), the α-secretase cleavage site (arrow), the transmembrane domain (TM) and the location of the epitopes recognized by the diverse anti-βAPP antibodies used. Exponentially growing parental CHO cells were metabolically labeled and chased for 20 min in complete medium with or without 1 μM PMA or 0.5 mM APMA as indicated. Then cells were lysed, and cell lysates were immunoprecipitated with polyclonal anti-Tail antibodies. Immune complexes were collected with protein A-Sepharose and analyzed by SDS-PAGE. Aliquots of conditioned media from cells treated in the same manner were subjected to Western blotting analysis with the signal peptide (SP), the mature, soluble growth factor (HB-EGF), the HA epitope, and the transmembrane (TM) and cytoplasmic (Cyt.) domains. CHO cells expressing pro-HA/HB-EGF were treated as in A and lysed, and cell lysates were subjected to Western blotting with anti-HA monoclonal antibodies. C, schematic of pro-HA/TGF-α showing the signal peptide (SP), the mature, soluble growth factor (TGF-α), the HA epitope, the transmembrane (TM) and cytoplasmic (Cyt.) domains, and the location of the epitope recognized by anti-Tail antibodies. Metabolically labeled CHO cells expressing pro-HA/TGF-α were treated as in A and immunoprecipitated with antibodies against the cytoplasmic tail of pro-TGF-α or anti-HA antibodies and protein A-Sepharose. Immunoprecipitates were analyzed by SDS-PAGE.

Fig. 2. Characterization of the products of the shedding induced by APMA. A, schematic representation of βAPP showing the signal peptide (SP), the β-amyloid peptide (black box), the α-secretase cleavage site (arrow), the transmembrane domain (TM) and the location of the epitopes recognized by the diverse anti-βAPP antibodies used. Exponentially growing parental CHO cells were metabolically labeled and chased for 20 min in complete medium with or without 1 μM PMA or 0.5 mM APMA as indicated. Then cells were lysed, and cell lysates were immunoprecipitated with polyclonal anti-Tail antibodies. Immune complexes were collected with protein A-Sepharose and analyzed by SDS-PAGE. Aliquots of conditioned media from cells treated in the same manner were subjected to Western blotting analysis with the indicated antibodies. B, schematic of pro-HA/HB-EGF showing the signal peptide (SP), the mature, soluble growth factor (HB-EGF), the HA epitope, and the transmembrane (TM) and cytoplasmic (Cyt.) domains. CHO cells expressing pro-HA/HB-EGF were treated as in A and lysed, and cell lysates were subjected to Western blotting with anti-HA monoclonal antibodies. C, schematic of pro-HA/TGF-α showing the signal peptide (SP), the mature, soluble growth factor (TGF-α), the HA epitope, the transmembrane (TM) and cytoplasmic (Cyt.) domains, and the location of the epitope recognized by anti-Tail antibodies. Metabolically labeled CHO cells expressing pro-HA/TGF-α were treated as in A and immunoprecipitated with antibodies against the cytoplasmic tail of pro-TGF-α or anti-HA antibodies and protein A-Sepharose. Immunoprecipitates were analyzed by SDS-PAGE.

tace−/− cells. As expected, treatment of wild type cells with PMA activates the shedding of HB-EGF as judged by the decrease in the amount of cell surface-associated pro-HB-EGF and a concomitant release of soluble mature HB-EGF (Fig. 3A, and see also Fig. 2B). The lack of effect of PMA on M2 cell mutants or tace−/− cells strongly suggests that TACE and the gene defective in M2 cells are necessary for the shedding of pro-HB-EGF, at least in these cell lines (Figs. 3A, 5, and B), and, since it has been previously found that MDC9 can shed pro-HB-EGF from Vero cells (21), opens the possibility that pro-HB-EGF is a substrate of different metalloprotease-dissintegrins in different cell lines.

APMA-induced Shedding in Different Cell Lines—The results presented elsewhere and in this report indicate that the shedding of βAPP, pro-TGF-α, and pro-HB-EGF relies on the presence of active TACE. An obvious explanation of the results presented this far is that APMA is an activator of TACE. To test this hypothesis, we analyzed the shedding of endogenous βAPP, transfected pro-HB-EGF, or transfected pro-TGF-α in the shedding-defective mutant cell line M2 and in tace−/− cells. As previously described (8, 14), the PMA-inducible shedding of βAPP is defective in M2 and tace−/− cells (see also Fig. 4). APMA did not induce the shedding of βAPP in M2 cells and produced a modest effect on tace−/− cells (Fig. 4), strongly suggesting that the effect of APMA in wild type cells is mediated by TACE. Similar results were observed when the shedding of pro-HB-EGF was analyzed in M2 or tace−/− cells treated with APMA (data not shown). Therefore, these results indicate that APMA induces the shedding of βAPP and pro-HB-EGF in wild type cells by activating TACE.

In contrast to the case of βAPP or pro-HB-EGF, APMA did induce the shedding of pro-TGF-α in M2 and tace−/− cells to an extent comparable to that induced by the PKC activator PMA in wild type cells (Fig. 5). This result suggests the existence of an alternative proteolytic activity, not activated via PKC, endowed with the ability of cleaving pro-TGF-α in cells where TACE is nonfunctional (tace−/− and M2 mutant cells). This proteolytic activity is inhibited by 25 μM BB-94 in the three cell types analyzed (data not shown), indicating that it is a zinc-dependent metalloprotease.

It has been proposed that the prodomain of several zinc-dependent metalloproteases likely involved in ectodomain shedding, such as TACE, ADAM10, or MDC9, is removed by a furin-like proprotein convertase (19, 28, 29). To determine whether processing by furin is required for the activation of ectodomain shedding induced by APMA, we analyzed the shedding of pro-TGF-α from CHO cells devoid of furin activity.

Thus, we permanently transfected pro-HA/TGF-α into RPE.40 cells, a mutant CHO cell line that shows a lack of furin activity (30). As shown in Fig. 5, A and B, APMA activates the shedding of pro-HA/TGF-α from permanently transfected wild type CHO or RPE.40 cells to a similar extent, indicating that processing by furin is not required for the metalloprotease activities activated by APMA.

APMA Induces the Shedding of Pro-TGF-α at the Cell Surface—Pro-TGF-α C-terminal mutants that fail to interact with the adaptor protein TACIP18 in vitro are retained in an early compartment of the secretary pathway that partially co-localizes with endoplasmic reticulum markers in vivo (24, 31, 32). Pro-TGF-α located in this compartment is resistant to PMA-induced ectodomain shedding, which occurs at or near the cell surface (23, 24).

To delimit the subcellular location where the shedding activated by APMA takes place, we analyzed its effect on the shedding of a pro-TGF-α C-terminal mutant. In agreement with previous results, the levels of cell surface pro-TGF-α are higher in cells transfected with the wild type molecule than in cells transfected with a C-terminal pro-TGF-α mutant (Fig. 6A) despite the higher level of expression of the latter (Fig. 6B). As shown in Fig. 6B, APMA did not induce the shedding of pro-TGF-α V159G indicating that this pro-TGF-α mutant is located in a compartment where it is not accessible to the APMA-activated proteolytic activity.

To further characterize the subcellular location where APMA-activated shedding occurs, we analyzed the effect of APMA on pro-TGF-α located at the cell surface. To follow the fate of cell surface pro-TGF-α, metabolically labeled cells expressing pro-TGF-α were chased to allow labeled membrane proteins to reach the plasma membrane. Cells were then incubated at 4 °C with anti-HA antibodies to label only cell surface pro-TGF-α and treated with APMA. As shown in Fig. 6C, APMA induced the shedding of cell surface pro-TGF-α as judged by the decrease in cell surface pro-TGF-α and concom-

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Shedding of pro-HA/HB-EGF in tace−/− and mutant M2 cells. A, parental CHO cells, the shedding-defective mutant cell line (M2), or tace−/− cells permanently transfected with pro-HA/HB-EGF were treated with or without 1 μM PMA for variable periods of time. Then cells were shifted to 4 °C, and the levels of cell surface pro-HA/HB-EGF were analyzed by flow cytometry. The results are expressed as percentages relative to untreated cells and are the average ± S.D. of triplicate experiments. B, M2 or tace−/− cells permanently transfected with pro-HA/HB-EGF were treated with or without 1 μM PMA for 30 min. Then cells were lysed, and aliquots of the cell lysates and conditioned media were subjected to Western blotting analysis with anti-HA antibodies.

Effect of PMA and APMA on the shedding of βAPP in different shedding-defective cell lines. Parental CHO cells, M2 shedding-defective cell mutants, or tace−/− cells were treated with or without 1 μM PMA or 0.5 mM APMA, as indicated, for 20 min. Then cells were lysed, and aliquots from cell lysates (A) or conditioned media (B) were subjected to Western blotting with 22C11, a monoclonal antibody against the extracellular domain of βAPP. X-ray films were digitized and quantified; results are expressed as percentages relative to the amount of immunoreactive βAPP in lysates of untreated cells and are the average ± S.D. of triplicate experiments. NT, not treated.

It is broadly accepted that protein ectodomain shedding is a widespread mechanism of regulating the activity and physical location of a variety of cell surface molecules. The proteolytic activities involved remain largely unidentified despite the expectations generated after the identification, 5 years ago, of the metalloprotease-disintegrin TACE as the “sheddase” responsible for the shedding of pro-TNF-α (5, 6). Since there are at least 14 metalloprotease-disintegrins with a potentially active catalytic site (7) and several of them are simultaneously expressed in a particular cell type (34), it has been frequently assumed that each one has a restricted specificity. Nevertheless, the phenotype of tace−/− cells do not seem to support this hypothesis because these cells show a gross defect in activated protein ectodomain shedding (8). tace−/− cells are incapable of shedding the ectodomains of numerous molecules functionally and structurally unrelated, including pro-TGF-α (8), pro-HB-EGF (this report), or βAPP (13, 16). Conversely, few direct evidences (such as lack of a particular shedding event in knockout cell lines) implicate other metalloprotease-disintegrins in ectodo-
In this report, we present experiments showing the existence of a metalloprotease activity, alternative to TACE, that shows a certain degree of specificity since it acts on pro-TGF-α but not on other putative substrates of TACE, such as APP or pro-HB-EGF. While this manuscript was in preparation, Schlöndorff et al. (18) reported the existence of at least two different proteases different from TACE capable of shedding the ectodomain of the TNF-related protein TRANCE in CHO cells. The differential sensitivity of these two TRANCE shedding activities to different stimuli and metalloprotease inhibitors suggests that each one could be acting to cleave TRANCE under different physiological conditions (18). This could also be the case of the APMA-induced metalloprotease in mouse fibroblasts, although further characterization of this proteolytic activity is required to establish its physiological relevance.

Early experiments showed that protein ectodomain shedding is a process regulated by several independent pathways (35), the best characterized of which acts via PKC (4). In fact, the well characterized PKC activators phorbol esters are the most frequently used way of activating protein ectodomain shedding in cultured cells. The lack of PKC-mediated shedding of multiple cell surface molecules in tace−/− cells strongly suggests that TACE is a major metalloprotease involved in PKC-activated shedding. To assess the possible participation of PKC-independent proteolytic activities in protein ectodomain shedding, we have looked for compounds to activate ectodomain shedding via PKC-independent mechanisms. The mercurial compound APMA has been extensively used to activate in vitro recombinant MMPs. These metalloproteases are synthesized as zymogens in which an unpaired cysteine residue of the prodomain binds to the active site zinc atom. In vitro APMA reacts with the free thiol group formed after conformational perturbation of the zymogen stabilizing an active form of the enzyme that leads to autoproteolytic processing of the propeptide (22). Here we have shown that treatment of cells with APMA induces the shedding of βAPP, pro-TGF-α, and pro-HB-EGF presumably by a PKC-independent mechanism. Because the products of the shedding reaction mediated by TACE and those of the shedding induced by APMA are indistinguishable, we analyzed whether TACE can be activated by APMA by comparing the effect of this compound on wild type and tace−/− cells. As a result of this analysis, we found that while the activation of the shedding of βAPP and pro-HB-EGF in wild type cells could be mainly mediated by TACE, the shedding of pro-TGF-α can also be mediated by an alternative, endogenous specific metalloprotease. Although in this work we do not address the mechanism by which APMA activates TACE, the known ability of APMA to activate the removal of prodomains from MMPs and metalloprotease-disintegrins opens the possibility that prodomains could play a role in the regulation of metalloprotease-disintegrins in cells. The prodomain of TACE is constitutively cleaved, presumably by furin-like proprotein convertases, in a variety of cells (5). It has been shown that the prodomain of another metalloprotease-disintegrin, MDC15, remains bound to the rest of the molecule after cleavage, opening the possibility that prodomains could have a role in the regulation of the activity of metalloprotease-disintegrins even after
they have been cleaved (36). Thus, it is conceivable that the prodomain of TACE could modulate its activity; in this scenario APMA could help to release the prodomain of TACE and other metalloprotease-disintegrins. The effect of APMA seems to be independent of the possible processing of prodomains of TACE and the other(s) APMA-activated metalloprotease(s) by furin since treatment of the furin-defective mutant cell line RPE.40 with APMA activates the shedding of pro-HA/TGF-α as efficiently as in wild type cells. Therefore, the evidence at hand indicates that APMA effectively activates furin-processed as well as furin-unprocessed metalloprotease disintegrins.

BB-94, a compound known to inhibit MMPs and metalloprotease-disintegrins in vitro and most shedding events in cells, also blocks the shedding of pro-TGF-α induced by APMA in tace−/− or M2 cells, indicating that the alternative metalloprotease that sheds pro-TGF-α could be another metalloprotease-disintegrin or a MMP. It has been recently shown that MMP-7 can shed pro-TNF-α in a model of herniated disc resection (37) indicating that at least a TACE substrate can be cleaved by MMP-7. However MMP-7 is not a likely candidate for the metalloprotease pro-TGF-α shedding activity that we report here since it is an epithelial-specific MMP not expressed in fibroblasts.

Protein ectodomain shedding takes place at or near the cell surface (1). Since treatment with APMA is a nonphysiological way of activating metalloproteases, it could be argued that it produces a physically “uncontrolled” activation of metalloproteases that can artifically cleave substrates in inappropriate cellular compartments. However, three independent evidences indicate that the metalloprotease(s) activated by APMA act only on proteins that are located in suitable subcellular sites. The immature forms of βAPP are not cleaved after APMA treatment, arguing that intracellular βAPP is not accessible to the APMA-induced metalloprotease. Also, C-terminal pro-TGF-α mutants that are retained in intracellular compartments are resistant to the effect of APMA, while cell surface pro-TGF-α is susceptible. Furthermore, APMA-induced shedding of pro-TGF-α can also be detected when internalization has been inhibited, excluding the possibility that this proteolytic activity occurs in the early endosomes. Therefore, the subcellular compartments where APMA-activated shedding takes place are similar to those where constitutive or PKC-activated shedding occurs.

In summary, using APMA, tace−/−, and M2 cells we have shown the existence of a metalloprotease alternative to TACE with the ability to specifically shed pro-TGF-α and not other TACE substrates. Future identification of this protease will help to determine its physiological relevance.

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