The extracellular domains of a diverse group of membrane proteins are shed in response to protein kinase C activators such as phorbol 12-myristate 13-acetate (PMA). The lack of sequence similarity in the cleavage sites suggests the involvement of many proteases of diverse specificity in this process. However, a mutant Chinese hamster ovary cell line recently isolated for being defective in PMA-activated shedding of the membrane-anchored growth factor transforming growth factor α precursor (proTGF-α) is concomitantly defective in the shedding of many other unrelated membrane proteins. Here we show that independent mutagenesis and selection experiments yield shedding mutants having the same recessive phenotype and belonging to the same genetic complementation group. Furthermore, two structurally distinct agents, TAPI-2 and 1,10-phenanthroline, which are known to inhibit metalloproteinases, block PMA-activated shedding of proTGF-α, cell adhesion receptor L-selectin, interleukin 6 receptor α subunit, β-amyloid precursor protein, and an entire set of anonymous Chinese hamster ovary cell surface proteins. Certain serine protease inhibitors prevent release of these proteins by interfering with their maturation and transport to the cell surface but do not inhibit ectodomain shedding from the cell surface. The results suggest the existence of a common system for membrane protein ectodomain shedding involving one or several proteolytic activities sensitive to metalloprotease inhibitors, whose ability to act can be disrupted by recessive mutations in a single gene.

The extracellular domain of a large number of transmembrane proteins can be proteolytically released into the medium. This shedding process regulates the fate and physical location of membrane-anchored growth factors (1), growth factor receptors (2), cell adhesion molecules, ectoenzymes (3), and proteins of unknown function such as the β-amyloid precursor protein (βAPP) (4). Many of these proteins are of practical importance.

For example, βAPP is implicated in the pathogenesis of Alzheimer’s disease (5), angiotesin converting enzyme plays an important role in the regulation of blood pressure (6), and tumor necrosis factor α (TNF-α) and the homing receptor L-selectin are implicated in inflammatory responses (7, 8). Ectodomain shedding can convert membrane-anchored growth factors into diffusible factors, membrane receptors into soluble competitors of their own ligand (9) or accessories to ligand binding (2), and cell adhesion receptors into products no longer capable of mediating physical interactions with other cells or the extracellular matrix (8). Membrane protein ectodomain shedding is now recognized as an important aspect of cell regulation and cell-cell interaction.

Despite its broad interest, this shedding mechanism involves molecular components of unknown identity. Shedding appears to occur at or near the cell surface and does not require cytosolic factors that are essential for many forms of membrane traffic (10). Shedding is often stimulated by protein kinase C activators and other agents (11–20). However, the proteins that are shed are not the targets of phosphorylation in this process. The nature of the proteases involved is of great interest because they might constitute ideal targets for therapy in various disease conditions. Given the diversity of amino acid sequences that are cleaved, many different proteases could be involved in this process, each endowed with a specific substrate recognition capacity. This notion has been reinforced by recent reports that the shedding of different ectodomains appears to be inhibited by different protease inhibitors (19–28).

Despite expectations that many different proteolytic activities may be involved in membrane protein ectodomain shedding, recent genetic evidence suggests that these processes may share certain components. We recently isolated a mutant cell line that is defective in the shedding of at least two unrelated molecules, βAPP and proTGF-α, thus providing evidence that the shedding mechanisms of these two molecules share a common component (29). In the present report, we show that independent selection of cell mutants defective in proTGF-α shedding yields cell lines that have identical phenotypes and belong to the same genetic complementation group, indicating a repeated isolation of mutations in the same gene. The defect in these cells prevents shedding of all membrane proteins tested.
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EXPERIMENTAL PROCEDURES

Materials—Phorbol 12-myristate 13-acetate (PMA), ethyl methane sulfonate, disopropylfluorophosphate (DFP), L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butaneone, tosylphenylalanyl chloromethyl ketone (TPCK), and 1,10-phenanthroline were from Sigma. 3,4-Dichloroarsonic acid and phosphoramidon were from Boehringer Mannheim. TAPI-2 was kindly provided by Immunex.

Cell Transfection, Mutagenesis, Selection, and Fusions—CHO cells were cultured in monolayers in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). pcDNA3 vectors containing L-selectin or IL-6 R DNAs were cotransfected either with the selectable plasmid pCEP-4 at a DNA ratio of 1:10 into CHO cells by electroporation. Stable transfectants were selected in 700 mg/ml of hygromycin and subcloned. Clones expressing L-selectin or IL-6 R at high levels were used for subsequent experiments. CHO cell mutants defective in TGF-α cleavage were isolated as described previously (29).

For cell fusions, 2 × 10⁶ cells of a hygromycin-resistant clone and 2 × 10⁶ cells of a histidine-resistant clone were plated in 60-mm dishes. 16 h later, the cultures were briefly covered with 3 ml of 45% polyethylene glycol (PEG, 1300–1600, American Type Culture Collection) in MEM, 10 mM Hepes with a final pH of 7.3. The PEG solution was immediately aspirated, leaving only the minimum amount needed to cover the cells. The cultures were incubated for 10 min at 37°C. Cells were washed three times with MEM and twice with MEM containing nonessential amino acids and 10% fetal bovine serum using warm medium. After 10 h of incubation in the latter medium, the cultures were trypsinized and plated into 150-mm dishes. Hybrid cell clones were selected in histidine-free MEM containing nonessential amino acids, 10% dialyzed fetal bovine serum, 0.5 mM histidinol, and 800 μg/ml of hygromycin for 2 weeks.

Flow Cytometry Analysis—Cells were washed with Dulbecco’s modified Eagle medium for 1 h at 37°C and then treated with or without protease inhibitors for 5 min and treated with or without PMA and/or protease inhibitors for an additional 20 min. Cells were then incubated for 45 min at 4°C with 10 μg/ml of anti-HA monoclonal antibody (12CA5, Babco) or 25 μg/ml of anti-L-selectin DREG-200 monoclonal antibody (19), in phosphate-buffered saline (PBS) containing 0.1% SDS, and analyzed by FACS those cells that retain cellsurface anti-HA staining after treatment with the phorbol ester PMA, a known inducer of TGF-α cleavage. For cell fusions, 2×10⁶ cells of a hygromycin-resistant clone and 2×10⁶ cells of a histidine-resistant clone were plated in 60-mm dishes. 16 h later, the cultures were briefly covered with 3 ml of 45% polyethylene glycol (PEG, 1300–1600, American Type Culture Collection) in MEM, 10 mM Hepes with a final pH of 7.3. The PEG solution was immediately aspirated, leaving only the minimum amount needed to cover the cells. The cultures were incubated for 10 min at 37°C. Cells were washed three times with PEG and MEM and twice with PEG containing nonessential amino acids and 10% fetal bovine serum using warm medium. After 10 h of incubation in the latter medium, the cultures were trypsinized and plated into 150-mm dishes. Hybrid cell clones were selected in histidine-free MEM containing nonessential amino acids, 10% dialyzed fetal bovine serum, 0.5 mM histidinol, and 800 μg/ml of hygromycin for 2 weeks.

Flow Cytometry Analysis—Cells were washed with Dulbecco’s modified Eagle medium for 1 h at 37°C and then treated with or without protease inhibitors for 5 min and treated with or without PMA and/or protease inhibitors for an additional 20 min. Cells were then incubated for 45 min at 4°C with 10 μg/ml of anti-HA monoclonal antibody (12CA5, Babco) or 25 μg/ml of anti-L-selectin DREG-200 monoclonal antibody (19), in phosphate-buffered saline (PBS) containing 5% bovine serum albumin and 10 mM ethylenediaminetetraacetic acid. Cells were then washed with PBS and lysed in lysis buffer containing 5 μg/ml of HA peptide in order to block any unbound anti-HA antibody. Insoluble material was removed by centrifugation. Immune complexes were collected by incubation of cell lysates and medium samples with protein A-Sepharose for 45 min at 4°C, washed three times with PBS containing 0.1% Triton X-100 and 0.1% SDS, and analyzed by SDS-PAGE.

For biotinylation of cell surface proteins, cells were labeled with 250 μCi/ml of [35S]methionine and 250 μCi/ml of [35S]cysteine for 2 h in methionine- and cysteine-free medium, chased for 30 min in complete medium, shifted to 4°C, and incubated with 1 mg/ml sulfo-NHS-LC-biotin (Pierce) for 1 h at 4°C. Unreacted biotinylation agent was quenched by washing the cells with PBS containing 50 mM Tris. Cells were then incubated for 30 min at 37°C in complete medium, 5 min with or without 200 μM PMA and/or 2 μg/ml of anti-L-selectin antibody (19) or 200 μM PMA and/or 2 μg/ml of anti-I-A (Becton Dickinson) antibody for an additional 20 min with or without the addition of the indicated protease inhibitors and for an additional 15 min with or without addition of 1 μM PMA. Cells were then washed three times with cold PBS and lysed in lysis buffer. Medium samples and cell lysates were incubated with streptavidin-agarose beads, and the beads were washed with 0.1% Triton X-100 and 0.1% SDS and analyzed on 12–18% gradient polyacrylamide gels.

RESULTS

Recessive Loss of Ectodomain Shedding Activity in Independently Isolated CHO Mutant Cells—Regulated shedding of membrane protein ectodomains was studied using two independently isolated mutant CHO cell clones. One of these clones, M1, was previously isolated from CHO cells expressing a transfected pro-TGF-α tagged with the HA epitope (HA-proTGF-α) (29). M1 was isolated by treating these transfectants with the single base mutagen ethyl methane sulfonate and then sorting by FACS those cells that retain cell surface anti-HA staining after treatment with the phorbol ester PMA, a known inducer of TGF-α cleavage. A control nonmutagenized cell population run in parallel did not yield shedding defective cells. Interestingly, the M1 line and other cell lines clonally propagated from the mutant pool are also defective in jAPP shedding activity. Activation of shedding by protein kinase C-independent mechanisms is also defective in these cells. However, these cells are normal with respect to membrane protein biosynthesis and transport (29). Although M1 and other cell lines established from the original mutant pool have the same phenotype, the entire mutant pool could be derived from a single mutant clone enriched during the consecutive rounds of sorting. In order to determine the frequency of isolation of a shedding defective phenotype by mutation of the same gene, we isolated an independent cell line, M2, by repeating the mutagenesis and sorting protocol with a fresh batch of CHO cells. Like M1 cells, M2 cells were unable to shed cell surface proTGF-α (Fig. 1A) or jAPP (data not shown) in response to PMA. Furthermore, hybrids generated by fusion of M1 or M2 with parental CHO cells had wild type shedding activity (Fig. 1A). M1 × M2 cell hybrids lacked shedding activity, showing that they belong to the same genetic complementation group (Fig. 1A). Thus, the M1 and M2 cell lines have the same recessive phenotype and belong to the same complementation group. These results argue that the defect in membrane protein ectodomain cleavage in these independent cell lines is caused by recessive mutations in the same gene.

General Loss of Ectodomain Shedding Activity—Previous results showed that the defect in the M1 mutant cell line inhibited PMA-dependent shedding of various CHO cell surface proteins of unknown identity (29). To better establish the shedding defect, the M1 and M2 cell lines and parental CHO cells were transfected with expression vectors encoding L-selectin or IL-6 Rα, two proteins that undergo PMA-induced ectodomain cleavage and are structurally and functionally unrelated to each other and to proTGF-α or βAPP (19, 28). The cell-bound and released forms of these proteins and of TGF-α as a control were
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**Fig. 1.** Shedding of L-selectin, IL-6 Rα, and TGF-α in wild type and mutant CHO cells and cell hybrids. A, wild type CHO cells (WT), their mutant derivatives M1 and M2, and hybrids between the indicated cell lines, all expressing HA-tagged proTGF-α, were treated with or without PMA for 20 min. The levels of cell surface immunostaining with anti-HA antibody were analyzed by flow cytometry. The results are expressed as percentages relative to the mean fluorescence of cells not treated with PMA and are the averages ± S.D. of triplicate determinations. B, wild type or mutant (M1) CHO cells transfected with L-selectin, IL-6 Rα, or proTGF-α were metabolically labeled with [35S]cysteine and [35S]methionine and then chased in complete medium for 45 min with or without PMA. Cell lysates were immunoprecipitated with antibodies against the L-selectin ectodomain or against the IL-6 Rα or proTGF-α ectodomains, respectively. Medium samples were immunoprecipitated with antibodies against the ectodomains of L-selectin, IL-6 Rα, or proTGF-α, respectively. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

Immunoprecipitated from metabolically labeled cells treated with or without PMA. As described previously (29), PMA induced a loss of the two cell surface proTGF-α forms (17 and 20–22 kDa) with a concomitant increase in soluble TGF-α in parental CHO cells but had no effect in the mutants (Fig. 1B, bottom).

Immunoprecipitation of metabolically labeled L-selectin transfectants with antibodies against the L-selectin intracellular domain yielded products of 50 and 74 kDa (Fig. 1B). Based on previous characterization, these products correspond to the biosynthetic precursor of L-selectin and the fully glycosylated cell surface form, respectively (19) (see also Fig. 3B). Upon cell treatment with PMA, the cell surface form is converted into a soluble form (Fig. 1B), leaving a cell-associated 6-kDa transmembrane/ectoplasmic fragment (19). In contrast to parental CHO cells, M1 and M2 cells showed a lack of basal or activated L-selectin shedding activity (Fig. 1B and data not shown). Likewise, IL-6 Rα, which is expressed in transfected CHO cells as an 80-kDa cell surface protein and shed as a 57-kDa soluble ectodomain in response to PMA (28), was not shed in the mutants (Fig. 1B, middle panel). The results obtained with M1 and M2 transfectants were identical, and only one of the mutants (M2) is shown here for simplicity. These results indicate that the gene affected in these mutants is essential not only for proTGF-α and βAPP shedding but also for the shedding of L-selectin and IL-6 Rα.

Effect of Serine Protease Inhibitors on Ectodomain Shedding Activity—Previous studies showed that TGF-α release can be blocked by the addition of certain serine protease inhibitors (27), whereas shedding of IL-6 Rα (28) or L-selectin (Ref. 19 and references therein) is not sensitive to such inhibitors. These results could be interpreted as evidence for the involvement of different proteolytic activities. However, these results were obtained using different methods and cell lines. For example, 3,4-DCI inhibited the release of TGF-α from CHO cells as determined by immunoprecipitation of metabolically labeled proteins (27) but did not inhibit the shedding of L-selectin as determined by FACS analysis of immunostained human granulocytes (21). Therefore, we decided to test the effect of 3,4-DCI on the shedding of L-selectin, IL-6 Rα, TGF-α, and βAPP under the same experimental conditions. As in previous studies on proTGF-α cleavage (27), metabolically labeled cells were treated with 3,4-DCI for 30 min prior to PMA addition. Under these conditions, 3,4-DCI inhibited the release of L-selectin and IL-6 Rα as well as TGF-α (Fig. 2). Two other serine protease inhibitors, TPCK and DFP, previously shown to prevent TGF-α release under these conditions (27) prevented the PMA-induced release of L-selectin and IL-6 Rα as well as endogenous βAPP (data not shown).

The current availability of a HA-tagged proTGF-α construct that can be recognized on the cell surface by anti-HA antibody allowed us to assess the effect of these protease inhibitors on the shedding of cell surface proTGF-α using FACS analysis. In marked contrast with the results obtained by immunoprecipitation of metabolically labeled products, FACS analysis of CHO transfectants surface-stained with anti-HA antibody showed that 3,4-DCI did not inhibit the PMA-induced shedding of cell surface proTGF-α (Fig. 3A). As described previously (21), 3,4-DCI did not prevent the PMA-induced loss of cell surface L-selectin as determined by FACS analysis. The same results were obtained when TPCK or DFP were analyzed in this type of assay (data not shown).

To confirm the inability of 3,4-DCI to prevent ectodomain cleavage at the cell surface, a protocol was designed to specifically follow the fate of proteins that are present on the cell surface at the time of PMA addition. Cells were metabolically labeled and then chased long enough to allow labeled membrane proteins to reach the cell surface. Cells were then incubated with antibodies against proteins of interest and treated with PMA and/or 3,4-DCI. The immune complexes formed on the cell surface were recovered by precipitation from cell lysates and medium samples and analyzed by SDS-PAGE. When tested in this manner, 3,4-DCI did not inhibit PMA-induced

**Fig. 2.** Effect of 3,4-DCI on the release of L-selectin, IL-6 Rα, and proTGF-α ectodomains. CHO cells transfected with L-selectin, IL-6 Rα, or HA proTGF-α were metabolically labeled and chased for 45 min in the presence or the absence of 100 μM 3,4-DCI. Where indicated, PMA was added during the last 30 min of the chase period. Aliquots from medium samples were immunoprecipitated with antibodies against the corresponding protein ectodomains.

**Fig. 3A.** Immunoprecipitation of metabolically labeled products, FACS analysis of CHO transfectants surface-stained with anti-HA antibody showed that 3,4-DCI did not inhibit the PMA-induced shedding of cell surface proTGF-α (Fig. 3A). As described previously (21), 3,4-DCI did not prevent the PMA-induced loss of cell surface L-selectin as determined by FACS analysis. The same results were obtained when TPCK or DFP were analyzed in this type of assay (data not shown).
loss of either proTGF-α or L-selectin from the cell surface (Fig. 3B, left panels) and did not inhibit the release of soluble L-selectin and TGF-α into the medium (Fig. 3B, right panels).

These results suggested that 3,4-DCI and related protease inhibitors prevented the release of newly synthesized membrane proteins but not the shedding of these proteins once they have reached the cell surface. To determine whether these protease inhibitors interfered with transport of membrane proteins to the cell surface, a proTGF-α pulse-chase metabolic labeling experiment was done in the presence or the absence of the inhibitors. ProTGF-α is synthesized as an 18-kDa precursor that matures into forms of 20–22 kDa that reach the cell surface and are converted to a 17-kDa product by removal of the N-terminal pro-region (30) (Fig. 4, 60 min lanes). Under basal conditions, this 17-kDa proTGF-α form slowly turns over without significant release of TGF-α into the medium (30). The addition of 3,4-DCI at the start of the chase period markedly delayed the maturation of the 18-kDa precursor into the 20–22-kDa form, as seen by immunoprecipitation from cell lysates (Fig. 4, right panels). Furthermore, 3,4-DCI delayed the appearance of these forms on the cell surface, as determined by precipitation of cell surface anti-HA immune complexes (31). These results argue that 3,4-DCI, TPCK, and DFP prevent ectodomain release by inhibiting membrane protein maturation and transport rather than by specifically blocking cell surface shedding activity.

Effect of Metalloprotease Inhibitors on Ectodomain Shedding Activity—Recent reports have described the inhibition of TNF-α shedding by compounds with the characteristics of metalloprotease inhibitors (23–25). One of these compounds, TAPI, and an analog with the naphthyl-alanine side chain replaced by a tertbutyl group (TAPI-2) are also effective at preventing the cleavage of TNF-α receptor (26). In contrast to the serine protease inhibitors mentioned above, the metalloprotease inhibitor TAPI-2 did not prevent TGF-α maturation or transport to the cell surface (Fig. 4), although it partially inhibited basal cleavage of the 20–22-kDa surface form into the 17-kDa form (compare Fig. 4, left panels, last two lanes). Furthermore, TAPI-2 did not prevent the maturation or transport of βAPP, IL-6 Rα, or L-selectin (data not shown). Therefore, we tested the effect of TAPI-2 on the shedding of TGF-α, βAPP, L-selectin, and IL-6 Rα.

CHO cells expressing transfected proTGF-α, L-selectin, or IL-6 Rα were metabolically labeled, chased, and then treated with or without PMA or TAPI-2. It should be noted that TAPI-2 was added to the cells 30 min after the radioactive pulse; this period of time is enough to allow transport of proTGF-α (Fig. 4), L-selectin (Fig. 3), and β-APP (31) to the cell surface. Therefore the effects of TAPI cannot be due to interference with maturation or transport of these molecules. In order to analyze the membrane forms of these proteins or endogenous βAPP, cell lysates were immunoprecipitated with antibodies against the extracellular domain of IL-6 Rα or against the cytoplasmic domains of L-selectin, βAPP, or proTGF-α. In order to analyze the soluble forms, medium samples were immunoprecipitated with the same antibodies against IL-6 Rα or antibodies against soluble L-selectin or TGF-α. Cleavage of TGF-α and βAPP was also followed by the appearance of their residual 15-kDa transmembrane/cytoplasmic fragments in the immunoprecipitates.
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Fig. 5. Effect of TAPI-2 on the shedding of L-selectin, IL-6 Rα, βAPP, and TGF-α. CHO cells expressing these proteins were metabolically labeled and chased in complete medium with or without TAPI-2 for 25 min with PMA addition for the final 20 min, as indicated. Cell lysates (top panel in each set) were immunoprecipitated with antibodies against the IL-6 Rα extracellular domain or against the cytoplasmic domains of L-selectin, proTGF-α or βAPP, respectively. The cytoplasmic tail of βAPP (βAPP, bottom panel) is visualized in a longer exposure of the corresponding portion of the gel. Medium samples (bottom panels in other sets) were immunoprecipitated with antibodies against the ectodomain of L-selectin, IL-6 Rα, or TGF-α, respectively.

with anti-cytoplasmic domain antibodies. The results of these experiments show that TAPI-2 inhibited the PMA-induced shedding of all these proteins (Fig. 5). Half-maximal inhibition of shedding activity was observed with 10 μM TAPI-2 (Fig. 6), a concentration that is comparable with that required for half-maximal inhibition of TNF-α cleavage (25) or TNF-α receptor cleavage (26). Among other known metalloprotease inhibitors tested, 1,10-phenanthroline inhibited the shedding of TGF-α, βAPP, L-selectin, and IL-6 Rα, whereas EDTA, EGTA, or phosphoramidon were without effect at the concentrations tested (Table I). The effect of 1,10-phenanthroline on the shedding of TGF-α was prevented by the addition of 5 mM ZnCl₂ (data not shown), suggesting that the inhibitory effect of 1,10-phenanthroline is due to its metal-chelating activity.

Additional experiments were designed to determine the effect of TAPI-2 on endogenous CHO proteins that are shed in response to PMA. For these experiments, metabolically labeled proteins were chased to allow their transport to the cell surface. The cell surface was then biotinylated by treatment with sulfo-NHS-LC-biotin and washed. Cells were incubated for a short period with or without PMA and/or TAPI-2. Biotinylated proteins were retrieved from the medium using immobilized streptavidin. The results show that numerous labeled proteins with molecular masses ranging from 30 to >200 kDa were released into the medium of PMA-treated cells but not that of control cells (Fig. 7). On the basis of cpm bound to streptavidin-agarose beads, the amount of biotinylated cell surface proteins released to the medium corresponds to 2–4% of biotinylated proteins present in the corresponding cell lysates. The SDS-PAGE profiles of metabolically labeled, biotinylated proteins recovered from the medium and cell lysates did not match, arguing that this technique specifically detects the minority of cell surface proteins that undergo PMA-induced ectodomain cleavage (data not shown). The addition of TAPI-2-inhibited PMA-induced release of all these proteins (Fig. 7), arguing that all membrane protein ectodomain shedding in these cells is catalyzed by metalloprotease activity. Collectively, these results favor the hypothesis that one or several proteases with characteristics of a metalloprotease are responsible for the shedding of and entire set of transmembrane proteins, and their action can be disrupted by mutations in a single gene.

**DISCUSSION**

Shedding of cell surface protein ectodomains is a general mechanism that affects the activity of many membrane molecules with different functions (1, 3). The four membrane proteins (proTGF-α, βAPP, L-selectin, and IL-6 receptor α) chosen for the present studies are well known examples of this phenomenon. A sense of how many different proteins are affected by this process is provided by present (Fig. 7) and previous (29) experiments showing that treatment with the protein kinase C activator PMA leads to the rapid shedding of approximately 2% of the surface protein in CHO cells. The shed proteins are of widely different molecular weights, and their electrophoretic profiles are distinct from that of the general cell surface protein population. These observations indicate that a significant subset of cell surface proteins including proTGF-α, βAPP, L-selectin, IL-6 Rα, and many others are shed into the medium, and often, if not always, this process can be activated through protein kinase C-dependent and -independent mechanisms.

Despite their common fate, no clear similarity can be found between the various membrane proteins that undergo ectodomain shedding. In particular, no sequence similarity can be found in the cleavage site of these molecules to suggest that a common protease mediates the shedding of all these molecules. Based on these considerations and due to previously noted differences in sensitivity to protease inhibitors (21, 27), it had been proposed that different proteases are involved in the shedding of different membrane proteins (20, 23–25, 32). However, the phenotype of our two independent mutant cell lines indicate that one component of the shedding machinery that lies downstream of protein kinase C is required for shedding of all proteins tested (this report and Ref. 29). Moreover, the present results show that in our mutant selection experiments we repeatedly isolated independent mutant cell lines that have the same recessive shedding phenotype and belong to the same complementation group. These observations strongly suggest that shedding of membrane protein ectodomains is mediated by a common system with an essential component encoded by a nonredundant gene in CHO cells. The substrates of this shedding system include TGF-α, βAPP, IL-6 Rα, L-selectin, and essentially all other cell surface proteins that are shed in response to PMA.

Among the original lines of evidence suggesting that shedding of different membrane protein ectodomains may be mediated by different proteases is the observation that certain serine protease inhibitors (3,4-DCI, TPCF, and DFP) can inhibit the release of newly synthesized proTGF-α (27) but are ineffective against shedding of L-selectin (21). The inhibitory effect on TGF-α release was observed by adding these compounds to metabolically labeled cells and measuring the conversion of newly synthesized proTGF-α into TGF-α and the 15-kDa cell-associated fragment. However, shedding of membrane protein ectodomains takes place at or near the cell surface (10, 33). In the present studies, these protease inhibitors were ineffective against PMA-induced cleavage of cell surface proTGF-α, L-selectin, IL-6 Rα, and βAPP. This result was obtained irrespective of whether shedding was assayed by immunoprecipitating metabolically labeled cell surface proteins and soluble ectodo-
mains or by performing flow cytometry of cell surface proTGF-α or L-selectin immunostaining. When added to cells immediately after a metabolic pulse, 3,4-DCI, TPCK, and DFP did prevent the release of various ectodomains. However, it is now clear that this effect is due to inhibition of membrane protein transport to the cell surface by these agents. Whether this is mediated by their antiprotease activity remains to be determined. We conclude that 3,4-DCI, TPCK, and DFP are ineffective against the cell surface shedding of proTGF-α, L-selectin, IL-6 Rα, and βAPP ectodomains. Therefore, the previously noted differences in sensitivity to these protease inhibitors no longer constitute evidence that different proteases catalyze the shedding of these proteins.

Recently, several compounds with the characteristics of metalloprotease inhibitors have been shown to block the shedding of TNF-α (23–25) and the 80-kDa TNF-α receptor ectodomain (26). These compounds are derivatives of hydroxamic acid that are known to inhibit metalloproteases by binding to their active site (34). In the present studies, we tested the effects of one such compound, TAPI-2 (26), on the diverse group of membrane proteins whose shedding is activated by PMA in CHO cells. TAPI-2 does not interfere with the biosynthesis of proTGF-α (present work) or TNF-α receptor (26). The results show that TAPI-2 inhibits basal as well as PMA-activated shedding of TGF-α, βAPP, L-selectin, and IL-6 Rα and is equally potent in each case. Furthermore, TAPI-2 also inhibits the shedding of all endogenous CHO membrane proteins that undergo this process in response to PMA. The general metalloprotease inhibitor 1,10-phenanthroline, which acts by chelating heavy metals, is also an effective inhibitor of PMA-activated ectodomain cleavage, whereas other ion chelators including EDTA and EGTA are ineffective. It has been previously shown that the shedding of CD43, CD44, and CD16 is inhibited by 1,10-phenanthroline but not by EDTA or EGTA (21). This result might be explained by a higher affinity of 1,10-phenanthroline...
for the zinc ion found in the active site of most metalloproteases. In our experiments, the effect of 1,10-phenanthroline could be prevented by the addition of Zn$^{2+}$, indicating that the inhibitory effect of 1,10-phenanthroline is due to its metal-chelating activity. Phosphoramidon, which inhibits certain metalloproteases by binding to their active site, is without effect on the shedding of all the membrane proteins tested here. The evidence argues that TAPI-2 and 1,10-phenanthroline specifically inhibit a regulated shedding activity likely to be catalyzed by one or several metalloproteases that release the ectodomains of a diverse group of unrelated cell surface proteins.

The present results provide strong evidence for the existence of a single general mechanism for membrane protein ectodomain shedding. The evidence at hand is consistent with two alternative models. In one model, a family of metalloproteases with different substrate specificity would catalyze the shedding of a diverse group of membrane proteins in the same cell; all these proteases would depend on a shared regulatory component that can be disrupted by mutations in a single gene. Because extracellular proteases are often activated by proteolysis, one possibility is that this gene encodes a protease that activates all the others. In the second model, the target of the mutations would be the shedding protease itself. This model would imply the existence of a protease with unusually broad substrate specificity, perhaps one that would recognize a certain secondary structure (or a lack thereof) as the cleavage site. The mutant cell lines described here may help identify these components of the shedding machinery.

Acknowledgments—We thank R. Black (Immunex) for TAPI-2 and advice, T. Delohery for cell sorting and FACS analysis, and the members of the Massague lab for helpful discussion.

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J. Biol. Chem. 1996, 271:11376-11382.
doi: 10.1074/jbc.271.19.11376

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