

Transforming Growth Factor- α and β -Amyloid Precursor Protein Share a Secretory Mechanism

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Abstract. Cleavage and release of membrane protein ectodomains, a regulated process that affects many cell surface proteins, remains largely uncharacterized. To investigate whether cell surface proteins are cleaved through a shared mechanism or through multiple independent mechanisms, we mutagenized Chinese hamster ovary (CHO) cells and selected clones that were unable to cleave membrane-anchored transforming growth factor α (TGF- α). The defect in TGF- α cleavage in these clones is most apparent upon cell treatment with the protein kinase C (PKC) activator PMA, which stimulates TGF- α cleavage in wild-type cells. The mutant clones do not have defects in TGF- α expression, transport to the cell surface or turnover. Concomitant with the loss of TGF- α cleavage, these clones have lost the ability to cleave many structurally

unrelated membrane proteins in response to PMA. These proteins include β -amyloid precursor protein (β -APP), whose cleavage into a secreted form avoids conversion into the amyloidogenic peptide A β , and a group of cell surface proteins whose release into the medium is stimulated by PMA in wild type CHO cells but not in mutants. The mutations prevent cleavage by PKC-dependent as well as PKC-independent mechanisms, and thus affect an essential component that functions downstream of these various signaling mechanisms. We propose that regulated cleavage and secretion of membrane protein ectodomains is mediated by a common system whose components respond to multiple activators and act on susceptible proteins of diverse structure and function.

A large group of membrane proteins undergo cleavage with release of their ectodomain into the extracellular medium (Ehlers and Riordan, 1991). This group includes membrane-anchored growth factors (Massagué and Pandiella, 1993), growth factor receptors, cell adhesion molecules, ectoenzymes and molecules of unknown function such as the β -amyloid precursor protein (Ehlers and Riordan, 1991; Massagué and Pandiella, 1993). Membrane-anchored growth factors and ectoenzymes provide localized concentrations of these activities, and their cleavage yields soluble forms that can diffuse and act at a distance from the site of production (Ehlers and Riordan, 1991; Massagué and Pandiella, 1993). The membrane-anchored forms of these proteins may be biologically more effective than the soluble forms, as in the case of kit ligand/stem cell growth factor whose soluble form cannot substitute for its membrane-anchored form in critical aspects of mouse development (Flanagan et al., 1991). Cleavage switches the role of growth factor receptors and cell adhesion molecules by converting them into soluble antagonists of the factors they bind (Fer-

nández-Botran, 1991; Gordon, 1991). Cleavage of β -amyloid precursor protein (β -APP)¹ releases a large soluble form (APP_s) that escapes the intracellular degradative process that yields A β (Esch et al., 1990; Sisodia et al., 1990; Golde et al., 1992; Haass and Selkoe, 1993). Deposits of insoluble A β are invariably associated with Alzheimer's disease, suggesting that defects in β -APP release may play a role in the pathogenesis of this disease (Haass and Selkoe, 1993). Thus, cleavage and secretion of membrane protein ectodomains fulfills different biological roles depending on the protein, and alterations in this process may lead to disease.

Cleavage of membrane protein ectodomains is often a regulated process. The extracellular domains of proTGF- α (Pandiella and Massagué, 1991a), kit ligand (Huang et al., 1992), colony-stimulating factor 1 (CSF-1) (Stein and Rettenmier, 1991), CSF-1 receptor (Downing et al., 1989), tumor necrosis factor α (TNF- α) receptors (Porteu and Nathan, 1990; Porteu et al., 1991), IL-6 receptor (Serra-Pages et al., 1994), LAR transmembrane protein tyrosine phosphatase (Müllberg et al., 1992), β -APP protein (Buxbaum et

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1. *Abbreviations used in this paper:* β -APP, β -amyloid precursor protein; CHO, Chinese hamster ovary; CSF, colony-stimulating factor; HA, hemagglutinin epitope; PKC, protein kinase C.

al., 1990), the cell adhesion molecule L-selectin (Kishimoto et al., 1989; Kahn et al., 1994), and angiotensin-converting enzyme (Ramchandram et al., 1994) are released at a slow rate in resting cells but are cleaved minutes after adding certain agonists including protein kinase C activators, calcium ionophores, and serum factors (Pandiella and Massagué, 1991b; Buxbaum et al., 1994).

Despite the biological importance of this process, the cellular components involved in it have eluded identification. Indirect evidence suggests that membrane protein ectodomain cleavage is catalyzed by membrane-associated proteolytic activities that act at the cell surface (Sisodia, 1992; Bosenberg et al., 1993). These enzymes preferentially attack peptide bonds located at a certain distance from the transmembrane region, the amino acid sequence flanking these sites being of secondary importance (Sisodia, 1992). A wide variety of primary sequences can be cleaved by this system, and extensive mutation of the regions proximal to the cleavage site in proTGF- α (Wong et al., 1989; Pandiella et al., 1992), TNF- α (Perez et al., 1990), β -APP (Sahasrabudhe et al., 1992; Sisodia, 1992), and IL-6 receptor (Müllberg et al., 1994) is necessary to prevent their cleavage.

One basic question for which there has been no answer is whether cleavage of this extremely diverse group of membrane proteins occurs through a shared mechanism or multiple independent mechanisms. To investigate this question, and to provide a genetic system for the study of this process, we mutagenized cell cultures and selected cell clones that were unable to cleave membrane TGF- α . We then asked if these clones were also defective in cleavage of other unrelated membrane proteins.

Materials and Methods

Construction of HA-tagged proTGF α

The pro-HA/TGF- α construct was generated by inserting the HA1 epitope of influenza virus hemagglutinin (Meloche et al., 1992) between aminoacids 42 and 43 of rat proTGF- α (Lee et al., 1985a) using the polymerase chain reaction (PCR). The following oligonucleotides were used: 5'-GGCC-TCTAGAAGAGCGCGGTGGCTGCA-3' containing nucleotides -36 to -19 of the rat proTGF- α cDNA preceded by a XbaI site (underlined), 5'-GCTT-GGATCCTCAGACCACTGTCTCAGAGTGGCAGCA-3' containing nucleotides 455 to 480 of the rat proTGF- α followed by a BamHI site (underlined), 5'-AGCATAATCAGGAACATCATAGTGAGACCACTGCAGC-3', containing nucleotides 109 to 126 of proTGF- α followed by 21 nucleotides encoding the last seven aminoacids of the HA epitope (underlined) and 5'-GTTCTTGATTATGCTAGCCTCTCAACAAGTGCCAGAT-3' containing nucleotides 127 to 144 of proTGF- α preceded by 21 nucleotides encoding the first seven aminoacids of the HA epitope. The product of the final PCR reaction was subcloned into the pVAO expression vector (Pääbo et al., 1986) and confirmed by sequencing.

Cell Transfection and Mutagenesis

CHO cells were cultured in monolayers in DME supplemented with 10% fetal bovine serum (GIBCO BRL, Gaithersburg, MD). The pVAO vector containing the HA tagged proTGF- α was cotransfected with the selectable plasmid pCDneo at a DNA ratio 1:25 into CHO cells using the calcium phosphate precipitate method. Stable transfectants were selected in 500 μ g/ml Geneticin (GIBCO) and subcloned. One clone expressing pro-HA/TGF- α at high levels was mutagenized with 0.35 μ l/ml ethyl methane sulphonate under conditions that killed ~40% of the cells. The cells were allowed to recover for five days. Wild-type and mutant CHO cells were transfected with the pCEP-4 plasmid (Invitrogen, San Diego, CA) containing the cDNA encoding the human β -APP⁶⁹⁵ using the calcium phosphate

precipitate method. Transfectants were selected in 600 μ g/ml hygromycin (Calbiochem Corp., La Jolla, CA) and subcloned.

Fluorescence Analysis

Cells were incubated with 10 μ g/ml of anti-HA monoclonal antibody (12CA5; Babco, Richmond, CA) in PBS containing 5% BSA for 30 min at room temperature, then incubated for 30 min with or without 1 μ M PMA (Sigma Chemical Co., St. Louis, MO) in complete medium at 37°C, and stained for 30 min at 4°C with FITC-conjugated anti-mouse IgG (Becton-Dickinson) in PBS containing 5% BSA. Flow cytometry was done on a FACscan using FACscan Research software (Becton Dickinson Immunocytometry Sys., Mountain View, CA). Cell sorting was done on a FACStar-Plus system (Becton Dickinson). Photomicrographs were obtained using cells immunostained and treated as above.

Metabolic Labeling, Immunoprecipitation, and Biotinylation of Cell Surface Proteins

For immunoprecipitation of TGF- α localized at the cell surface, 10⁶ exponentially growing cells were labeled for 10 or 20 min with 250 μ Ci/ml of [³⁵S]cysteine (DuPont-New England Nuclear, Boston, MA) in cysteine-free medium, at 37°C. The label was chased in complete medium for different times, and cells were then shifted to 4°C and incubated for 30 min with 10 μ g/ml of anti-HA antibody in PBS containing 5% BSA at 4°C. After incubation with anti-HA antibody, cells were shifted to 37°C, incubated in complete medium with or without activators of TGF- α cleavage for various times as indicated, and then lysed in lysis buffer (1% NP-40, 5 mM EDTA, and 5 mg/ml of HA peptide). Insoluble material was removed from cell lysates by centrifugation. The cell lysates and aliquots from the media were incubated with protein A for 40 min at 4°C, and immune complexes washed three times with a solution containing 0.1% Triton X-100 and 0.1% SDS. Samples from cell lysates were resolved in 14% polyacrylamide gels, and samples from media were resolved in 16% polyacrylamide gels. Where indicated, cell lysates were immunoprecipitated with antibodies directed against the cytoplasmic tail of TGF- α (Bosenberg et al., 1992).

For β -APP immunoprecipitation, cells were labeled with 250 μ Ci/ml of [³⁵S]methionine (DuPont-New England Nuclear) for 20 min in methionine-free medium, and chased in complete medium for various times. Cells were then lysed in PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 5 mM EDTA, and insoluble material was removed by centrifugation. Cell lysates were immunoprecipitated with 3 μ l of polyclonal antiserum against the cytoplasmic domain of β -APP (Buxbaum et al., 1990). Media supernatants were immunoprecipitated with 5 μ g/ml of monoclonal antibody against the extracellular domain of β -APP (22C11; Boehringer Mannheim Corp., Indianapolis, IN). Immune complexes were washed three times with a solution containing 0.1% Triton X-100 and 0.1% SDS. Samples were electrophoresed on SDS-PAGE gels of the appropriate density (either 6 or 17% polyacrylamide) to resolve proteins of interest.

For biotinylation of cell surface β -APP, cells were labeled with 500 μ Ci/ml of [³⁵S]methionine and 500 μ Ci/ml of [³⁵S]cysteine for 2 h in methionine- and cysteine-free medium, chased for 45 min in complete medium and washed three times with Krebs Ringer Hepes buffer at 4°C. Cells were then incubated in the same buffer containing 1 mg/ml of sulfo-NHS-LC-Biotin (Pierce Chem. Co., Rockford, IL) for 30 min at 4°C. Excess biotinylation reagent was quenched by washing with 10 mM glycine in Krebs Ringer Hepes buffer. Metabolically labeled biotinylated cells were shifted to 37°C and incubated for 15 min in DME with or without 1 μ M PMA, 1 μ M A23187 or 10% fetal bovine serum. Cell lysates were immunoprecipitated with antibodies against the β -APP cytoplasmic tail as above. Immunoprecipitated β -APP was eluted from washed protein A beads with 100 mM glycine (pH 2.5) and 10 μ M competing β -APP COOH-terminal peptide (corresponding to aminoacids 636–694 of human β -APP⁶⁹⁵). Supernatants were removed, neutralized, and incubated with streptavidin-Sepharose beads (Pierce) for 30 min at 4°C. The streptavidin agarose beads were washed with 0.1% Triton X-100 and 0.1% SDS, and analyzed on 6% polyacrylamide gels.

For cell surface biotinylation of endogenous membrane proteins, cells were labeled with 250 μ Ci/ml of [³⁵S]methionine and 250 μ Ci/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 sulfo-NHS-LC-Biotin as described above. After biotinylation, cells were incubated for 15 min at 37°C in complete medium with or without PMA. Media samples were incubated with streptavidin agarose beads, and the beads washed with

0.1% Triton X-100 and 0.1% SDS, and analyzed on 6–16% gradient polyacrylamide gels.

Results

Isolation of CHO Cell Mutants Defective in TGF- α Cleavage

To generate cell mutants defective in proTGF- α cleavage, we first transfected CHO cells with the construct pro-HA/TGF- α that encodes the rat TGF- α precursor (Lee et al., 1985b) tagged with an influenza virus hemagglutinin epitope (HA) (Meloche et al., 1992). This tag was used to facilitate immunostaining of intact cells expressing proTGF- α on the

surface, and was inserted immediately downstream of the NH₂-terminal TGF- α cleavage site (Fig. 1 A). Immunofluorescence analysis of stably transfected CHO cells stained with anti-HA antibody revealed proTGF- α expression at the cell surface (Fig. 1 D, top left) that was not detected in mock transfectants (not shown). As determined by flow cytometry, addition of the PKC activator PMA markedly reduced the HA immunofluorescence in these transfectants (Fig. 1 B) and induced the release of HA/TGF- α immune complexes into the media (see below). These observations were confirmed by immunofluorescence microscopy (Fig. 1 D, bottom left), and were consistent with the known ability of PMA to induce proTGF- α cleavage (Pandiella and Massagué, 1991a).

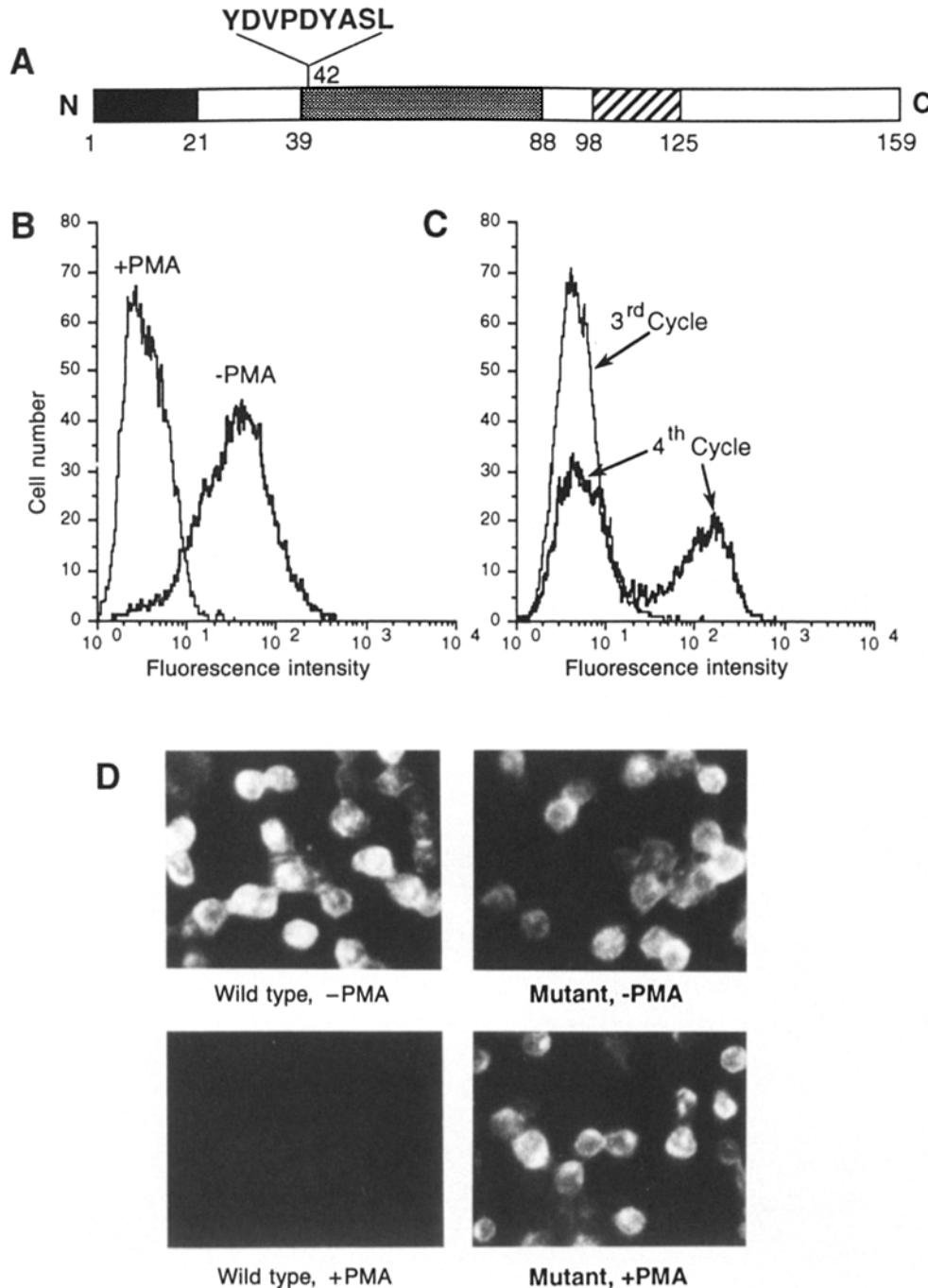


Figure 1. Isolation of CHO cell mutants defective in TGF- α cleavage. (A) Schematic of the pro-HA/TGF- α construct showing the signal sequence (black box), the TGF- α domain (shaded box), the transmembrane domain (hatched box), the HA epitope sequence and its site of insertion. Numbers denote amino acid positions. (B) Flow cytometry analysis of CHO cells stably transfected with pro-HA/TGF- α , immunostained with anti-HA and incubated with or without PMA. (C) Flow cytometry of the mutagenized transfectant cell population after the third and fourth cycles of selection for cells that remained HA⁺ after treatment with PMA. (D) Photomicrographs of pro-HA/TGF- α CHO transfectants (Wild type) and one of the TGF- α cleavage-deficient clones (Mutant) immunostained with HA antibody after treatment with or without PMA.

To select for cells defective in PMA-induced proTGF- α cleavage, one of the pro-HA/TGF- α transfectant clones was mutagenized with ethyl methane sulphonate and the cells allowed to recover for five days. The mutagenized population was immunostained with HA antibody, treated with PMA, and the 1% brightest cells in this population were selected in a cell sorter. The sorted population was expanded and the selection cycle repeated four times. After the first three rounds of cell sorting, no cells could be detected that remained bright upon PMA addition (Fig. 1 D). However, after the fourth round of selection, 38% of the cell population retained high HA immunofluorescence upon treatment with PMA, as determined by flow cytometry of an aliquot from this population (Fig. 1 C). Of 10 clones randomly isolated from this population, four showed complete absence of PMA-induced proTGF- α cleavage, as determined by immunofluorescence (Fig. 1 D) and flow cytometry (not shown). Each of these clones maintained this phenotype for at least three months in culture (\sim 160 cell doublings) after which three of the clones progressively reverted to the wild-type phenotype presumably owing to a growth advantage in the spontaneous revertants. The mutant phenotype of one of the clones has remained unchanged after 12 mo of culture.

TGF- α Biosynthesis, Transport, and Turnover in Wild-type and Mutant CHO Cells

To determine whether the phenotype of these mutants was due to a specific defect in proTGF- α cleavage or to some general defect in proTGF- α biosynthesis or transport to the cell surface, we compared the rates of proTGF- α synthesis, transport to the cell surface and turnover in parental and mutant CHO cells. TGF- α is synthesized as a 18-kD precursor that is heterogeneously glycosylated yielding forms of 20–22 kD. These forms are then transported to the cell surface where rapid cleavage of the NH₂-terminal pro-region leaves a 17-kD membrane-anchored form (Pandiella and Massagué, 1991a; Bosenberg et al., 1992). In the absence of activators, TGF- α slowly turns over without significant release of mature growth factor to the media (Pandiella and Massagué, 1991a; Bosenberg et al., 1992). TGF- α products of molecular size and order of appearance consistent with this process appeared at similar rates in parental and mutant

CHO cells. Metabolic pulse-chase labeling followed by precipitation of cell lysates with antibodies directed against the proTGF- α cytoplasmic region (Fig. 2, *whole cell*) showed the appearance of a 18-kD immunoreactive species that was chased into 20–22 kD forms and, soon thereafter, into a 17-kD form.

To determine the rate of appearance of pro-HA/TGF- α at the cell surface, the same set of metabolically labeled cells was incubated with anti-HA antibody, washed and lysed. Immune complexes recovered from these lysates with protein A-agarose showed that the newly synthesized 20–22 kD form appeared on the cell surface at the same rate in both the wild-type and mutant cells, and was rapidly converted into the 17-kD form in both cell lines (Fig. 2, *cell surface*). Loss of cell surface proTGF- α was clear after 90 min of chase in both wild-type and mutant cells (Fig. 2). This loss is attributable to general membrane protein turnover. In the particular experiment shown here, the wild-type cells exhibited a somewhat faster rate of TGF- α loss, likely due to a basal level of proTGF- α cleaving activity. This possibility is supported by the accumulation, after 60 min, of a 15-kD immunoreactive product (see Fig. 3, *whole wild type cell*) that corresponds to proTGF- α transmembrane/cytosolic tail fragment generated by cleavage of the ectodomain (Pandiella and Massagué, 1991a; Bosenberg et al., 1992). Similar results were obtained with the other three mutant CHO clones (data not shown). These results indicated that the rates of TGF- α synthesis, maturation and transport to the cell surface in these mutants are indistinguishable from those in non-mutagenized CHO cells.

PMA-inducible Cleavage in Wild-type and Mutant CHO Cells

We next examined the cleavage of proTGF- α in wild-type and mutant CHO cells in response to PMA. Since proTGF- α cleavage occurs at the cell surface (Bosenberg et al., 1992, 1993), we monitored the cleavage process by immunoprecipitating cell surface proTGF- α as well as total proTGF- α . Metabolically labeled cells were chased 20 min to allow transport of newly synthesized proTGF- α to the cell surface. The chase was then continued for up to 60 min with or without PMA addition. In the absence of PMA, cell surface

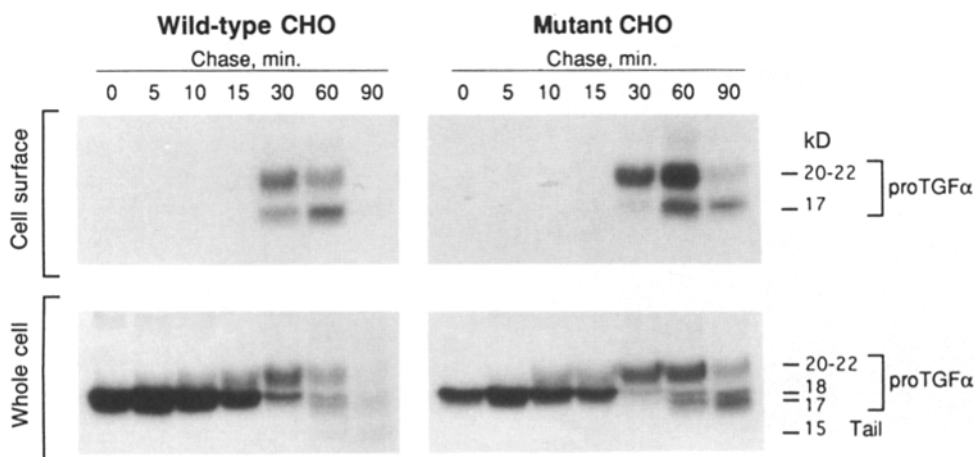


Figure 2. Biosynthesis and turnover of TGF- α in wild-type and mutant CHO cells. Exponentially growing cells were labeled with [³⁵]cysteine for 10 min and chased in complete medium for the indicated times. For determinations of cell surface proTGF- α , cells were incubated with HA antibody, lysed and immune complexes precipitated with protein A-agarose as described under Materials and Methods. For determination of proTGF- α in the whole cell, cell lysates were precipitated with antiserum against the cytoplasmic tail of proTGF- α .

proTGF- α turned over in both cell lines with little or no release of soluble TGF- α into the medium (Fig. 3; note that the indicated times started to count at the time of PMA addition 20 min after initiation of the chase, hence the apparent differences in turnover rates with Fig. 2).

In wild-type CHO cells proTGF- α was rapidly cleaved in response to PMA with release of soluble factor into the medium and accumulation of 15-kD tail in the cell (Fig. 3, *bottom left*), as previously described (Pandiella and Massagué, 1991a; Bosenberg et al., 1992). In the mutants, however, PMA addition had no effect on proTGF- α cleavage. ProTGF- α turnover in these cells occurred without appearance of soluble factor in the medium or 15-kD tail in the cells, either in the presence or absence of PMA (Fig. 3). These results confirmed the presence of a defect in proTGF- α cleavage. Similar results were obtained in the other three mutant lines (data not shown).

β -APP Biosynthesis and Secretion in Wild-type and Mutant CHO Cells

β -APP expression and processing via the amyloidogenic and non-amyloidogenic pathways are present in most tissues and cell types (Tanzi et al., 1989; Weidemann et al., 1989). Metabolically labeled wild type CHO cells expressed a β -APP immunoreactive species of 115 kD that was chased

into a form of 145 kD, as shown by immunoprecipitation with antibodies against the cytoplasmic domain of β -APP (Fig. 4). These forms correspond in size to immature and fully glycosylated β -APP, respectively (Buxbaum et al., 1990). In the absence of PMA treatment, the rates of synthesis and turnover of β -APP in wild type and mutant CHO cells were similar (Fig. 4). Furthermore, the level of cell surface β -APP was also similar in both cell lines, as judged by immunoprecipitation of surface-biotinylated β -APP (see Fig. 6).

Processing of β -APP through the non-amyloidogenic pathway, yielding the secreted form APP_s and a residual tail fragment of 10 kD, can be stimulated with PMA (Buxbaum et al., 1990). This effect was observed in wild-type CHO cells but not in the mutants defective in proTGF- α cleavage (Fig. 4). Only a low level of constitutive β -APP release, that was not affected by PMA, could be detected in these cells (Fig. 4). In the three clones that spontaneously reverted to wild-type phenotype with respect to TGF- α cleavage, β -APP cleavage was also restored (data not shown), providing further evidence for a genetic link between the TGF- α and β -APP secretory mechanisms.

Since various β -APP isoforms exist, and the identity of those endogenously expressed in CHO cells has not been established, we transfected wild-type CHO cells and two of the mutant clones with an expression vector encoding the human β -APP⁶⁹⁵ isoform which is known to be amyloidogenic (Sisodia et al., 1990). Immunoprecipitates of CHO cells stably transfected with β -APP⁶⁹⁵ showed additional β -APP immunoreactive products of the size predicted for mature and immature β -APP⁶⁹⁵ forms, respectively (Fig. 5, *arrows*). The rates of maturation and turnover of transfected β -APP⁶⁹⁵ were indistinguishable from those of the endogenous β -APP in both wild-type and mutant CHO cells (data not shown). Addition of PMA induced β -APP⁶⁹⁵ cleavage in wild-type but not mutant CHO cells, as judged by disappearance of the mature β -APP⁶⁹⁵ form. These results confirmed a defect in cleavage of authentic β -APP in the mutants. Three randomly selected subclones from each of two mutant clones transfected with β -APP⁶⁹⁵ were analyzed, yielding the same results in all cases (data not shown).

Resistance to Diverse Cleavage-activating Signals

PMA activates TGF- α cleavage via PKC whereas calcium ionophores and serum factors activate this process in part by PKC-independent mechanisms (Pandiella and Massagué, 1991b). Likewise, β -APP release is activated by calcium ionophores via a PKC-independent mechanism (Buxbaum et al., 1994), and is activated also by serum factors (Fig. 6). In order to determine the effect of these agents on proTGF- α and β -APP cleavage, we monitored cell surface pro-HA/TGF- α levels by labeling cells with HA antibody (as in Fig. 2) and cell surface β -APP levels by cell surface biotinylation followed by two-step precipitation with antibodies against β -APP cytoplasmic tail and streptavidin-agarose beads. Like PMA, the calcium ionophore A23187 and fetal bovine serum promoted release of TGF- α and β -APP in wild-type CHO cells but not in the mutant clones (Fig. 6, and data not shown). These results argued that the signals conveyed by these various mechanisms converge on a component that lies downstream of different signaling pathways, and this component is essential for cleavage of different membrane proteins and is defective in the mutants.

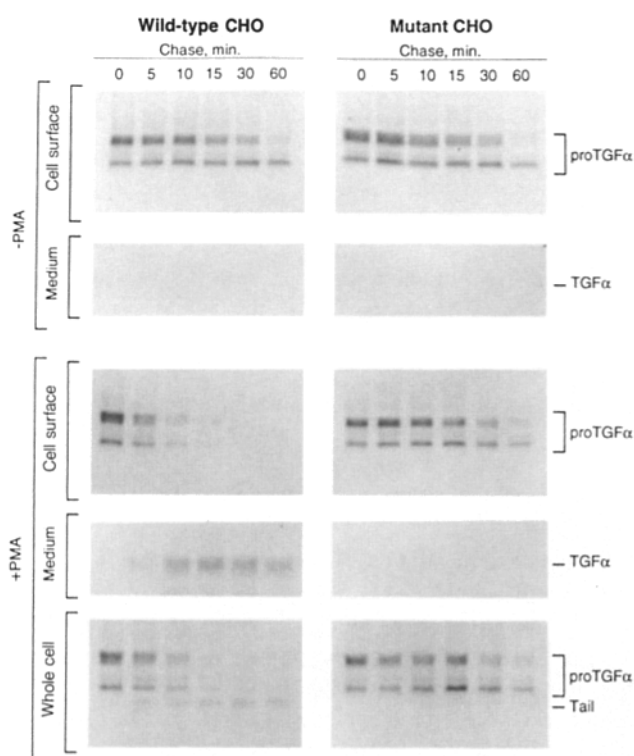


Figure 3. ProTGF- α cleavage in wild-type and mutant CHO cells. Exponentially growing cells were pulsed with [³⁵S]cysteine for 20 min, chased in complete medium for 20 min and then incubated with HA antibody. Cells were then further chased for the indicated times in the presence or absence of 1 μ M PMA. Immune complexes present in cell lysates and media were precipitated with protein A-agarose and analyzed by SDS-PAGE. Separately, lysates from PMA-treated cells were precipitated with antiserum against the cytoplasmic tail of proTGF- α .

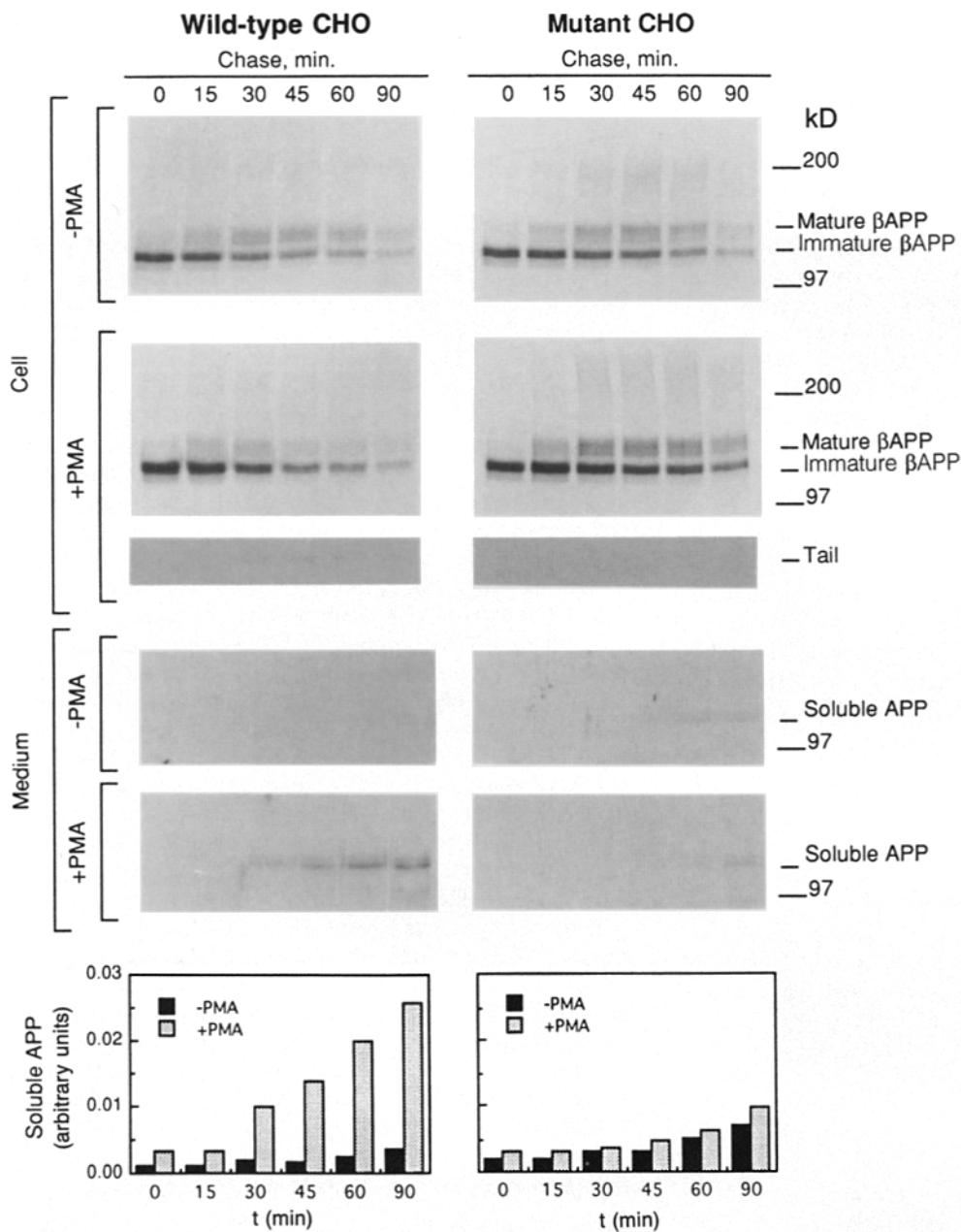


Figure 4. β -APP cleavage in wild-type and mutant CHO cells. Metabolically labeled wild type and mutant CHO cells were chased for the indicated times in complete medium with or without PMA. Cell lysates and media supernatants were immunoprecipitated with antibodies against the cytoplasmic tail or the extracellular domain of β -APP, respectively, and the immunoprecipitates analyzed by SDS-PAGE. Bottom panels show the densitometric quantification of the labeled bands corresponding to soluble β -APP in the media samples.

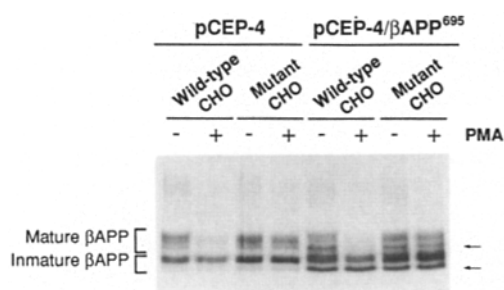


Figure 5. Transfected β -APP⁶⁹⁵ is resistant to cleavage in mutant CHO cells. Wild-type and mutant CHO cells transfected with the human β -APP⁶⁹⁵ were metabolically labeled and chased with complete medium for 45 min with or without PMA. Cell lysates were precipitated with antibodies against β -APP cytoplasmic tail, and the immunoprecipitates analyzed by SDS-PAGE.

Release of Endogenous CHO Membrane Proteins by a Shared Secretory Mechanism

The evidence that two structurally and functionally different proteins, proTGF- α and β -APP, share a secretory system in CHO cells raised the possibility that this mechanism mediates the activated release of many other cell surface proteins. To test this possibility, wild-type and mutant CHO cells were metabolically labeled, then cell surface biotinylated, incubated for a brief period with or without PMA, and the material released into the medium was retrieved with streptavidin-agarose beads. In the absence of PMA, there was little release of cell surface proteins from either wild-type or mutant CHO cells (Fig. 7). In response to PMA, wild-type CHO cells released cell surface proteins ranging from 30 to over 200 kD, as determined by electrophoresis of the streptavidin-bound radiolabeled material (Fig. 7). On the ba-

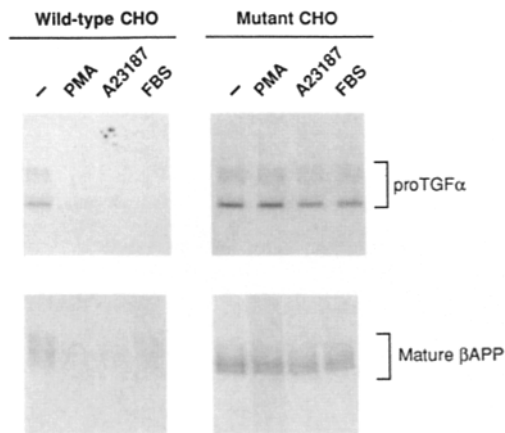


Figure 6. Different mechanisms fail to activate proTGF- α and β -APP cleavage in mutant CHO cells. For analysis of cell surface proTGF- α , cells were metabolically labeled for 20 min, chased for 20 min in complete medium, incubated with HA antibody and then incubated with 1 μ M PMA, 1 μ M A23187, 10% fetal bovine serum or no additions for 20 additional min. Cell lysates were precipitated with protein A-agarose and the precipitates analyzed by SDS-PAGE. For analysis of cell surface β -APP, wild-type and mutant CHO cells transfected with human β -APP⁶⁹⁵ were metabolically labeled and chased for 45 min, incubated with sulfo-NHS-LC-Biotin, and then incubated for 15 min in complete medium with the indicated agonists. Cell lysates were subjected to a two-step precipitation with antibodies against β -APP cytoplasmic tail and streptavidin-agarose beads, as described in Materials and Methods. The final precipitates were analyzed by SDS-PAGE.

sis of recovered cpm, the amount of protein that bound to the streptavidin beads was only \sim 1% of the total protein secreted into the media, and the radiolabeled protein profiles of both samples on SDS-PAGE gels were different (data not shown). This suggested that PMA specifically activated the release of

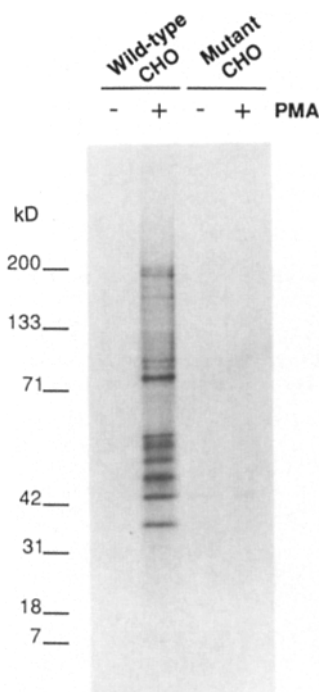


Figure 7. PMA-induced release of endogenous CHO cell surface proteins. Metabolically labeled cells were incubated with sulfo-NHS-LC-Biotin to tag cell surface proteins, and then incubated with or without 1 μ M PMA. Biotinylated proteins were recovered from the media with streptavidin-agarose beads. The precipitates were analyzed on 6–16% gradient polyacrylamide gels.

cell surface proteins but not general exocytosis in CHO cells. Importantly, PMA did not activate the release of any of these proteins in the mutants (Fig. 7). These results indicate that the component affected by the mutations plays an essential role in a general mechanism for activated release of membrane protein ectodomains.

Discussion

Many membrane proteins involved in various aspects of cell communication and regulation undergo cleavage with release of their ectodomains into the media. However, very little is known about the nature of the secretory mechanism involved in this process. One feature common to many of these cleavage processes is their inducibility by activators of PKC or calcium influx into the cytosol. Aside from this, no similarities can be found between the various proteins that undergo this process or, most remarkably, between the primary sequences at the cleavage sites (Wong et al., 1989; Perez et al., 1990; Pandiella et al., 1992; Sahasrabudhe et al., 1992; Sisodia, 1992; Müllberg et al., 1994). These properties raise the possibility that membrane protein ectodomain cleavage involves a large number of specific proteolytic systems. However, the genetic approach taken here to investigate this question provides support for the alternative possibility, that cleavage of diverse membrane protein ectodomains is mediated by a common mechanism whose regulatory and/or catalytic components might be relatively few and of broad specificity.

We selected CHO cell mutants that retained cell surface proTGF- α immunostaining in the presence of PMA. The mutants isolated by this procedure appear free of defects in membrane protein biosynthesis and dynamics, as judged from the unaltered rates of proTGF- α maturation, transport to the cell surface and turnover. Nevertheless, these cells show a complete resistance to PMA-induced proTGF- α cleavage. In the presence of PMA, proTGF- α remains at the cell surface and turns over without being converted into soluble factor, as it does in non stimulated wild-type CHO cells. The evidence indicates that a specific defect in proTGF- α cleavage is present in these cells.

To investigate the generality of the affected process we focused on β -APP cleavage. This choice was made because β -APP is unrelated to TGF- α in expression pattern, cleavage site and probable function, and also because defects in β -APP processing may be involved in the pathogenesis of Alzheimer's disease. Consistent with the widespread expression of β -APP, CHO cells express immunoreactive β -APP polypeptides of 115 and 145 kD that presumably correspond to the immature and fully glycosylated forms, respectively (Weidemann et al., 1989; Wang et al., 1991). In agreement with previous reports, the release of APPs into the cell media was stimulated upon cell treatment with PMA (Buxbaum et al., 1990). However, in the cleavage-defective CHO mutants, PMA does not accelerate APPs secretion, indicating that the component affected in these mutants is also necessary for regulated secretion of β -APP. These mutants are also defective in the processing of a transfected human β -APP isoform (β -APP⁶⁹⁵). As with TGF- α , the rates of synthesis, maturation and turnover of endogenous or transfected β -APP are very similar in wild type and mutant CHO cells. Moreover, revertants that have regained the ability to cleave

TGF- α have concomitantly regained the ability to cleave β -APP, further supporting the conclusion that the defective component is one involved in regulated cleavage of both proteins.

An examination of endogenous cell surface proteins that undergo PMA-induced release provides further evidence for a general process that mediates the release of many different membrane proteins. In response to PMA, CHO cells release cell surface proteins that range in size from 30 to over 200 kD. Although the identity of these CHO proteins is not known, they are likely to represent a structurally and functionally diverse group. PMA-induced release of these proteins is defective in the mutants, indicating that these proteins share a common secretory process with proTGF- α and β -APP.

ProTGF- α and β -APP ectodomain release can be activated by various independent mechanisms. Calcium ionophores act by a mechanism independent of PMA-responsive PKC isoforms whereas serum factors act by a calcium-independent mechanism (Pandiella and Massagué, 1991b). All these agents failed to stimulate cleavage of β -APP or proTGF- α in our mutants, indicating that the defective component functions downstream of the signals conveyed by these various activators.

In conclusion, we have provided genetic evidence for a general mechanism that catalyzes regulated cleavage and secretion of membrane protein ectodomains. The identity of the component(s) disrupted by these mutations remains to be determined. They could include a regulator of protease activity, a regulator of protease access to its substrates, or a shared protease. The presence of a regulatory element in this system can be inferred from the fact that the inducers of cleavage are activators of protein phosphorylation and yet, neither proTGF- α nor β -APP are the relevant substrates since mutation of their potential phosphorylation sites does not affect cleavage (Bosenberg et al., 1992; Da Cruz e Silva et al., 1993; Hung and Selkoe, 1994). The cytoplasmic domain of proTGF- α is essential for cleavage (Bosenberg et al., 1992) but the cytoplasmic domain of β -APP is not (Sisodia, 1992; DeStrooper et al., 1993; Haass et al., 1993; Hung and Selkoe, 1994), arguing that the target of the present mutations is not one that recognizes this domain. ProTGF- α cleavage occurs at the cell surface (Bosenberg et al., 1993), can be activated in permeabilized cells devoid of cytosol (Bosenberg et al., 1993), and does not require membrane traffic (Bosenberg et al., 1993), suggesting that regulated cleavage of these proteins requires relatively few membrane-associated components. Isolation of genes that complement the mutations described here should reveal the identity of these molecules.

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