Role of the Juxtamembrane Domains of the Transforming Growth Factor-α Precursor and the β-Amyloid Precursor Protein in Regulated Ectodomain Shedding*

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Although regulated ectodomain shedding is a well known process that affects a large group of transmembrane molecules, it is not clear how the shedding system selects its substrates. Here we investigate the structural requirements for the regulated shedding of two substrates of the general shedding system, the transforming growth factor-α precursor, pro-TGF-α, and the β-amyloid precursor protein, β-APP. The ability of different regions of pro-TGF-α or β-APP to confer susceptibility to the shedding system was tested using as a reporter a transmembrane molecule that is not a substrate of this shedding system. For this purpose we chose the TGF-β accessory receptor, betaglycan, since genetic and biochemical evidence showed that betaglycan is not a substrate of the shedding system. We determined that replacement of the 14 extracellular amino acids adjacent to the transmembrane region of betaglycan with the corresponding regions of TGF-α or β-APP rendered betaglycan susceptible to ectodomain shedding. These domain swap constructs were cleaved in response to protein kinase C stimulation, and cleavage was prevented by the metalloprotease inhibitor TAPI, both effects being characteristic of the general shedding system. Domain swap constructs containing the transmembrane and/or the cytoplasmic domains of pro-TGF-α did not undergo regulated ectodomain cleavage. We conclude that despite a lack of sequence similarity, the extracellular regions of pro-TGF-α and β-APP immediately preceding their transmembrane domains are key determinants of ectodomain shedding.

A large, functionally and structurally heterogeneous group of transmembrane proteins can undergo cleavage and release of their extracellular domain into the medium. This proteolytic process is often referred to as “ectodomain shedding” and affects a large set of otherwise unrelated cell surface molecules such as growth factors, growth factor receptors, ectoenzymes, and cell adhesion molecules. In most cases described to date, ectodomain shedding is a regulated process that can be activated by protein kinase C (PKC)1 activation and other mechanisms (1, 2). Although the components of this shedding system have not been identified, recent reports have provided insights into the general characteristics of the shedding machinery. Mutant CHO cell lines selected for lack of regulated shedding of the membrane-anchored growth factor pro-TGF-α are also defective in the cleavage of unrelated molecules such as the cell adhesion molecule L-selectin, the α-receptor for interleukin-6, the β-amyloid precursor protein (β-APP), and a variety of endogenous CHO cell surface proteins (3, 4). Hydroxamic acid-based compounds developed as inhibitors of the shedding of the membrane-anchored growth factor TNF-α by a putative metalloprotease (5–7) can also block shedding of TNF-α receptors, pro-TGF-α, β-APP, L-selectin, interleukin-6 receptor, and Fas ligand with similar potency in all cases (3, 8–10). A few differences have been described in the shedding process of these diverse proteins. Among these, the cytoplasmic domains of pro-TGF-α and the 80-kDa TNF-α receptor are required for the shedding of these two molecules (11, 12), whereas Kit ligand, interleukin-6 receptor, and β-APP, devoid of their cytoplasmic domains, are shed to the same extent as the wild type molecules (13–15). Taken together, these findings suggest the existence of a common shedding machinery acting on most transmembrane molecules whose ectodomain can be released into the medium via regulated proteolytic cleavage. Although small deletions in the membrane proximal segment abolish shedding (15–19), mutational analysis of residues around the cleavage site of β-APP (20), the 60-kDa TNF-α receptor (18), interleukin-6 receptor (15), L-selectin (19), and TNF-α (16) has shown a lack of strict sequence specificity for cleavage. Rather, cleavage occurs at a site located at a fixed distance from the membrane (13, 16, 18–20). These observations raise questions about the role of the cleaved amino acid sequence as a determinant of cleavage specificity.

To investigate these questions, we determined whether any portion of pro-TGF-α or β-APP might confer the ability to be shed to a protein that normally is not subject to the pro-TGF-α and β-APP shedding system. As the test protein we chose the TGF-β accessory receptor, betaglycan. Betaglycan is a membrane-anchored proteoglycan that binds the growth factor TGF-β and facilitates its interaction with signaling receptors (21). Although betaglycan can be shed, we found in the present studies that this is a very slow process that has none of the characteristics of the shedding process described above for pro-hamster ovary; TGF, transforming growth factor; β-APP, β-amyloid precursor protein; TNF, tumor necrosis factor; PMA, phorbol 12-myristate 13-acetate; HA, hemagglutinin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BG, betaglycan; FACS, fluorescence-activated cell sorter; TAPI-2, N-[2-(9-hydroxyaminoacarbonyl)methyl]-4-methylenopyranoxyll-l-3-tertbutyl-l-alanine, 2-aminoethyl amide.

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1 The abbreviations used are: PKC, protein kinase C; CHO, Chinese hamster ovary; TGF, transforming growth factor; β-APP, β-amyloid precursor protein; TNF, tumor necrosis factor; PMA, phorbol 12-myristate 13-acetate; HA, hemagglutinin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BG, betaglycan; FACS, fluorescence-activated cell sorter; TAPI-2, N-[2-(9-hydroxyaminoacarbonyl)methyl]-4-methylenopyranoxyll-l-3-tertbutyl-l-alanine, 2-aminoethyl amide.
TGF-α, β-APP, and other proteins. Taking advantage of this fact, we generated chimeras between betaglycan and domains of TGF-α and β-APP and determined which domains can confer susceptibility to the shedding system. Here we report that short juxtamembrane sequences of TGF-α and β-APP endow betaglycan with the ability to be cleaved by the regulated shedding system.

EXPERIMENTAL PROCEDURES

Materials—Phorbol-12-myristate 13-acetate (PMA) and the calcium ionophore A23187 were from Sigma. TAPI-2 was kindly provided by Immunex.

Cells and Transfections—Wild type and CHO cell lines defective in pro-HA/TGF-α shedding have been described elsewhere (4). Wild type and mutant CHO cells were stably transfected with the pCEF-4 plasmid (Invitrogen) containing the cDNA encoding the different molecules used in this work using the calcium phosphate precipitate method. Transfectants were selected in 600 μg/ml hygromycin (Calbiochem) and subcloned. For transient transfection of L17 cells (22), the various cDNA constructs were subcloned into pCMV5 vector and transfected using the DEAE dextran method as described (22).

Flow Cytometry Analysis—Cells expressing various constructs were washed with Dulbecco’s modified Eagle’s medium for 1 h at 37 °C and then treated with or without 50 μM TAPI-2 for 5 min and/or without 1 μM PMA, 1 μM calcium ionophore A23187, or 10% fetal bovine serum and/or TAPI as indicated for additional periods of time. Cells were then incubated for 45 min at 4 °C with 10 μg/ml anti-HA monoclonal antibody (12CA5, Babco) or 10 μg/ml of anti-myc monoclonal antibody 9E10 (23) in phosphate-buffered saline (PBS) containing 5% bovine serum albumin and stained for 30 min at 4 °C with fluorescein isothiocyanate-conjugated anti-mouse IgG (Becton Dickinson) in PBS containing 5% bovine serum albumin. Flow cytometry was done on a FACscan instrument and software (Becton Dickinson).

Metabolic Labeling and Immunoprecipitation—Approximately 2.107 exponentially growing CHO cells expressing various molecules were labeled for different periods of time with 250 or 1,000 μCi/ml [35S]cysteine and 250 or 1,000 μCi/ml Tran35S-label (NEN Life Science Products) in methionine- and cysteine-free medium at 37 °C. The label was chased in complete medium for various periods of time in the presence or absence of 50 μM TAPI-2 and/or 1 μM PMA, 1 μM calcium ionophore A23187, or 10% fetal bovine serum as indicated. Cells were then washed with cold PBS and lysed in PBS containing 1% Nonidet P-40 and 5 mM EDTA (lysis buffer). Aliquots from the media and from cell lysates were immunoprecipitated with anti-HA (Babco) or anti-Myc monoclonal antibodies. Immune complexes were collected by incubation of cell lysates and media samples with protein A-Sepharose (for anti-HA antibody) or protein G-Sepharose (for anti-Myc antibody) 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 quantification of secreted material, specific bands corresponding to betaglycan or pro-TGF-α were excised from the gel, counted in a Beckman scintillation counter, and the amount of secreted material expressed as percentage relative to anti-HA or anti-myc immunoprecipitable counts at the end of the chase from cell lysates.

Construction and Expression of myc-tagged and Chimeric Betaglycan cDNAs—The pro-HA/TGF-α construct was described in (4). The myc/betaglycan construct (myc/BG) was generated by inserting the myc epitope (EQKLISEEDL) six codons downstream of the putative signal sequence as described previously (21).

Results

Betaglycan Ectodomain Secretion Is a Slow Process Unresponsive to PKC Activation—The ectodomain of betaglycan can be released to the medium by proteolytic cleavage at a site proximal to the transmembrane domain (21, 24). However, this mechanism has been poorly characterized. To analyze the secretion of betaglycan, a vector encoding rat betaglycan tagged with a c-Myc epitope following the signal sequence was stably transfected into CHO cells. Immunoprecipitation of metabolically labeled myc/BG transfecteds with anti-Myc antibody yielded two main products similar to those observed previously with wild type betaglycan. Based on previous characterization of the membrane-anchored forms (25), these products are identified as the heterogeneous 180–250-kDa proteoglycan core and the 110-kDa betaglycan core protein devoid of glycosaminoglycan chains (Fig. 1A).

Confirmation of previous results, a low level of myc/BG ectodomain was specifically immunoprecipitated from the conditioned media of these transfecants (see Fig. 1B).

Ectodomain shedding can be induced by activators of PKC, calcium ionophores, or serum factors, each acting, in part, through independent mechanisms (2). To find out whether the secretion of betaglycan can be activated by these agents, CHO cells expressing myc/BG were treated with the PKC activators PMA, 250 μCi/ml calcium ionophore A23187, or fetal bovine serum and, the levels of betaglycan in the cells and in media were analyzed. None of these agents was able to induce a decrease in the levels of cell surface myc/BG, as determined by immunoprecipitation of metabolically labeled cell lysates (Fig. 1A) and media (Fig. 1B) or by FACS analysis of cell surface c-Myc
immunofluorescence (Fig. 2A). In contrast, these activators induced a marked decrease in the levels of cell surface pro-
TGF-α (Figs. 1 and 2C), as reported previously (4). The propor-
tion of betaglycan and pro-TGF-α released into the media by 
metabolically labeled transfectants was determined after a 7-h 
chase or 1-h chase, respectively. These different time periods 
were chosen because the half-life of betaglycan in CHO cells 
(approximately 1.5 h). The amount of labeled betaglycan recovered 
from media after the chase was only 3.5–5% of the betaglycan 
secreted by wild type CHO cells (Fig. 2C). In contrast, 
TGF-α immunoprecipitation showed a failure of M1 cells to 
shed pro-TGF-α (Fig. 2C), as shown previously (4). Addition-
ally, we tested the effect of the hydroxamic acid-based inhibitor 
TAPI-2 on betaglycan shedding. TAPI-2 inhibits the shedding of 
TNF-α and most transmembrane molecules tested so far (4, 
6–10, 26). However, TAPI-2 had no effect on the secretion of 
betaglycan (Fig. 2B). Collectively, these results indicate that 
betaglycan ectodomain shedding in CHO cells is an inefficient 
process that involves a mechanism distinct from the shedding 
system that acts on pro-TGF-α and other transmembrane 
molecules.

Juxtamembrane Sequences as Determinants of Shed-
ing—To identify regions of pro-TGF-α and β-APP which may 
confer susceptibility to shedding, we generated a panel of 
betaglycan chimeras containing various pro-TGF-α or β-APP 
sequences (Fig. 3). The cytoplasmic domain, the transmembrane 
domain, or both domains of betaglycan were replaced by the 
complementary domains of pro-TGF-α. Additionally, we gener-
ated a betaglycan construct with a replacement of 14 jux-
tamembrane amino acids with the corresponding sequence 
from pro-TGF-α (Fig. 3). To survey the shedding of these 
chimeric molecules efficiently, we used a highly transflectable 
clone (L17) of the Mv1Lu mink lung epithelial cell line in 
transient transfection assays (22). Transiently transfected, 
metabolically labeled L17 cells were chased for different peri-
ods of time with or without PMA addition, and shedding was 
monitored by immunoprecipitation of metabolically labeled 
products. The different chimeric betaglycan molecules showed 
the same biosynthesis profiles as wild type betaglycan (Fig. 4 
data not shown). In the case of the constructs BG-To-BG, 
BG-BG-To, and BG-To-To, approximately 1% of total myc an-
tibody-immunoprecipitable counts at the end of the pulse were

**FIG. 2.** Cell surface levels and secretion of betaglycan and pro-TGF-α in wild type and mutant CHO cells with 
activators of the shedding system and effect of TAPI-2. Panel A, 
CHO cells stably transfected with HA/pro-TGF-α or myc/BG were 
treated with or without shedding-promoting reagents as in Fig. 1, 
immunostained with anti-Myc (thick line) or anti-HA (thin line) anti-
bodies, and subjected to flow cytometry analysis. Panel B, wild type 
(WT) or M1 CHO cells expressing myc/BG were metabolically labeled 
for 3 h and chased for 7 h with or without PMA and with or without 
TAPI-2. Then media samples were immunoprecipitated with anti-myc 
antibodies. Panel C, wild type or M1 CHO cells expressing pro-HA/ 
TGF-α were metabolically labeled for 30 min and chased for 45 min with 
or without PMA and with or without TAPI-2. Then media samples 
were immunoprecipitated with anti-HA antibodies. Immunoprecipitates 
were analyzed by SDS-PAGE.
released to the medium at the end of the chase, and the same result was obtained with the wild type betaglycan construct transfected under the same conditions (data not shown). The amount of released betaglycan ectodomain did not change in response to PMA. In contrast, the betaglycan construct containing the juxtamembrane domain of pro-TGF-α released significant amounts of betaglycan ectodomain into the media. Quantitation of the immunoprecipitated material indicated that 40% of the metabolically labeled betaglycan was released. Furthermore, the rate and extent of the release were increased in response to PMA (Fig. 4). These results indicate that the juxtamembrane domain of TGF-α is sufficient to confer susceptibility to the general shedding system.

To confirm these results in a better controlled system, we stably transfected the construct BG-Tα juxt into CHO cells. Additionally, we generated a related construct containing the juxtamembrane 14 amino acids of β-APP (construct BG-β-APP juxt) and stably transfected this construct into CHO cells. As shown in Fig. 5A, PMA treatment of CHO cells expressing myc/BG-Tα juxt or myc/BG-β-APP juxt induced the release of the ectodomain of the chimeric molecules as detected by immunoprecipitation via the c-myc epitope. The shedding of both chimeras was abolished completely by treatment with 50 μM TAPI-2 (data not shown).

We next analyzed the kinetics of ectodomain shedding of these two chimeric molecules and compared it with that of TGF-α. In agreement with previous results, the levels of cell surface pro-TGF-α decreased dramatically soon after treatment with PMA as determined by FACS analysis (Fig. 5B). The levels of cell surface pro-TGF-α were decreased 4-fold after a 5-min of treatment with PMA and became undetectable after 10 min of PMA addition. The ectodomain of the chimeric molecules BG-Tα juxt and BG-β-APP juxt were shed from CHO cells with approximately the same kinetics as pro-TGF-α. Furthermore, upon addition of medium lacking PMA, the original levels of cell surface pro-TGF-α and BG-Tα juxt were recovered with similar kinetics (t_{1/2} ~ 2.5 h) (Fig. 5C). These results demonstrate that the juxtamembrane domains of pro-TGF-α and β-APP are similarly effective at conferring susceptibility to cleavage by the regulated shedding system.

DISCUSSION

Genetic and biochemical evidence points to the existence of a general regulated ectodomain shedding system acting on a wide variety of cell surface proteins. Examination of surface-labeled membrane proteins in CHO cells indicated that the transmembrane proteins whose ectodomain is proteolytically released into the medium constitute a diverse group and account for 2–5% of the total cell surface protein in CHO cells (3). The vast majority of these proteins are shed in response to PKC activation, and their release is prevented by a mutation in a common component (3). To date only two proteins, colony-stimulating factor-1 (9) and betaglycan (this report), have been reported to be shed by an independent mechanism. In the present report, we show that, as in the case of colony-stimulating factor-1, the shedding of betaglycan is not stimulated by well known activators of the general shedding system and is not inhibited by the general shedding inhibitor TAPI. Furthermore, in the case of betaglycan, the rate and extent of release into the medium are very limited and not affected by mutations that disrupt the general shedding system.

Since betaglycan is in principle accessible to the general shedding system, we have used it as a reporter to identify TGF-α and β-APP sequences that would confer susceptibility to the general shedding system. Using a panel of betaglycan/TGF-α chimeras, we demonstrate that the juxtamembrane 14 amino acids of pro-TGF-α, which contain the natural pro-TGF-α cleavage site, are sufficient to confer susceptibility to the general shedding system. Furthermore, since the juxtamembrane domain of β-APP is as effective as that of pro-
Substrates of the Shedding Machinery

FIG. 5. Shedding of betaglycan molecules containing the juxtamembrane domains of pro-TGF-α and β-APP. Panel A, CHO cells permanently transfected with myc/BG-Tα juxt or myc/BG-β-APP juxt were metabolically labeled for 30 min, chased for 1 h in the presence or absence of PMA, and lysed. Cell lysates and media samples were immunoprecipitated with anti-Myc antibodies and immunoprecipitates were analyzed by SDS-PAGE. Panel B, CHO cells permanently transfected with various constructs were treated with or without PMA for the indicated periods of time. The level of cell surface immunostaining with anti-HA or anti-Myc antibodies and subjected to flow cytometry. The results are expressed as percentages relative to mean fluorescence of cells not treated with PMA and are the average ± S.D. of triplicate determinations. Panel C, CHO cells expressing pro-HA/TGF-α or pro-β-APP juxt were treated with PMA for 30 min, washed with Dulbecco’s modified Eagle’s medium, and further incubated in Dulbecco’s modified Eagle’s medium at 37 °C. At the indicated times cells were shifted to 4 °C and stained with anti-HA or anti-Myc antibodies and subjected to analysis by flow cytometry. The results are expressed as percentages relative to mean fluorescence of cells not treated with PMA and are the average ± S.D. of triplicate determinations.

TGF-α at supporting betaglycan shedding, we conclude that the short juxtamembrane regions of pro-TGF-α and β-APP are determinants of ectodomain shedding. In contrast to this role of the juxtamembrane region, the transmembrane region and the cytoplasmic region of pro-TGF-α, tested jointly or separately, failed to support betaglycan ectodomain cleavage.

It has been noted previously that the preferred cleavage site for ectodomain shedding is located at a certain distance from the membrane. Combined with a lack of evidence that the primary sequence of the cleaved region is important for cleavage these findings have led to the notion that shedding occurs by the action of an enzyme or set of enzymes which are sterically restricted to substrates adjacent to the membrane but are otherwise broad in their sequence specificity (15–19). The present observations clearly demonstrate that some feature of the 14 amino acid juxtamembrane sequences of pro-TGF-α and β-APP, not present in the corresponding region of betaglycan, is necessary for cleavage. No sequence similarities can be found between these juxtamembrane regions of pro-TGF-α and β-APP except for the presence of a cluster of hydrophobic amino acids at or following the cleavage sites (see Fig. 4). However, mutations in this hydrophobic cluster have limited effect on the shedding of pro-TGF-α (27), suggesting that this feature is not sufficient for recognition by the shedding system. It is therefore possible that the key determinant is in the as yet unknown secondary structure of this region. Alternatively, it is conceivable that this region in pro-TGF-α, β-APP, and other shed proteins might be disordered, and a lack of secondary structure renders them susceptible to the shedding system.

Previous reports have emphasized the importance of the β-APP membrane proximal segment on the basal cleavage of this molecule (20, 28), and recently it has been proposed that PKC activators enhance secretion of β-APP by enhancing budding of transport vesicles from the trans-Golgi network (29). Our observation that the juxtamembrane domain of β-APP introduced in betaglycan is sufficient to allow induced shedding of the chimeric molecule suggests that the proteolytic component of the β-APP shedding enzyme, often referred to as α-secretase, is activated via PKC. Stimulation of β-APP transport to the cell surface and activation of the α-secretase-mediated shedding are therefore two distinct and complementary mechanisms for activation of β-APP release by PKC.

In summary, our evidence suggests that a general shedding system regulated via PKC acts on diverse transmembrane proteins and selects their targets by recognition of a certain secondary structure (or a lack thereof) located in the juxtamembrane domain. Although the diversity of sequences cleaved by this system suggests the involvement of multiple proteases, it would appear from the present observations and previous results with hydroxamic acid inhibitors that these enzymes may be structurally and functionally related to each other.

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