Mechanisms controlling the shedding of transmembrane molecules

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Although the number of cell-surface transmembrane molecules known to release their extracellular domain to the cell media has grown greatly during past years, the protease(s) involved have remained elusive until recently. The analysis of ectodomain shedding of proteins as diverse as membrane-anchored growth factors, such as pro-transforming growth factor \(\alpha\) (proTGF-\(\alpha\)) and pro-tumour necrosis factor \(\alpha\) (proTNF-\(\alpha\)), growth factor receptors, ectoenzymes, cell adhesion molecules and the \(\beta\) amyloid precursor protein (\(\beta\)APP) has revealed several common features. The shedding of most proteins occurs at a fixed distance from the plasma membrane, can be activated via protein kinase C (PKC) and can be prevented by hydroxamic acid-based metalloprotease inhibitors initially developed to block the shedding of proTNF-\(\alpha\) [1]. The structural diversity of proteins that undergo protein ectodomain shedding initially suggested the existence of several proteases involved, each endowed with a restricted specificity. However, Chinese hamster ovary (CHO) cell mutants selected for a lack of proTGF-\(\alpha\) shedding (M1 and M2 cells) showed a general defect in protein ectodomain shedding, indicating the existence of at least a common component necessary for the shedding of multiple transmembrane proteins [2,3].

The first protease shown to be involved in a shedding event acts on proTNF-\(\alpha\) and belongs to the family of metalloprotease disintegrins (also known as the ADAM family), a family of modular transmembrane \(\mathrm{Zn}^{2+}\)-dependent metalloproteases that contain a disintegrin domain. Metalloprotease disintegrins are involved in sperm–egg fusion, muscle fusion in C2C12 myoblasts and the regulation of neural cell fate at different stages of neurogenesis in Drosophila (metalloprotease disintegrins are reviewed in [4,5]). The substrates of most members of the metalloprotease disintegrin family are unknown because most metalloprotease disintegrins have been isolated on the basis of sequence homology. So far, only TNF-\(\alpha\)-converting enzyme (TACE) has been convincingly shown to act on proTNF-\(\alpha\) [6,7]. In addition, the proteolytic activity of Kuzbanian (also known as ADAM 10) has been shown to be necessary for the processing of Notch, the receptor for the Delta ligand [8]; however, it is not clear whether Kuzbanian acts directly on Notch [8a]. Although the finding that the first protease found to be involved in a shedding event (TACE) belongs to the numerous metalloprotease disintegrin family seemed to confirm the notion of the participation of several metalloproteases in protein ectodomain shedding, it has been recently shown that TACE has a central role because it is responsible for the shedding of several unrelated proteins [8b].

The regulation of protein ectodomain shedding remains largely uncharacterized. Although PKC is a modulator of the shedding of most proteins tested so far, the molecular basis of PKC activation is unknown, except for that of \(\beta\)APP. Activation of endogenous PKC seems to augment the formation from the trans-Golgi network of secretory vesicles containing \(\beta\)APP, increasing the availability of \(\beta\)APP at the cell surface [9], where ectodomain shedding takes place [10,11]. This mechanism of modulation of protein ectodomain shedding seems to be a particularity of \(\beta\)APP because PKC frequently activates ectodomain shedding of proteins resident at the cell surface, such as proTGF-\(\alpha\), proTNF-\(\alpha\) or L-selectin. It has been recently shown that calmodulin binds directly to the cytoplasmic domain of L-selectin and has a role in the shedding of its ectodomain, because calmodulin inhibitors such as trifluoperazine (TFP) and calmidazolium induce the shedding of L-selectin. However, it is not known whether PKC mediates the activation of L-selectin shedding via calmodulin.

To investigate whether calmodulin has a role in the shedding of other transmembrane molecules, we have analysed the effect of calmodulin inhibitors on the shedding of proTGF-\(\alpha\). As shown in Figure 1(A), TFP promotes, in a
dose-dependent manner, the typical loss of proTGF-α immunoreactivity at the cell surface that follows the proteolytic release of the ectodomain of proTGF-α. Half-maximal activation of proTGF-α shedding is observed with approx. 30 μM TFP, a concentration comparable with that required to inhibit the shedding of L-selectin [12]. Time-course experiments showed that the kinetics of proTGF-α ectodomain shedding induced by TFP (t½ ≈ 15 min) is slightly slower than that induced by the PKC activator PMA (t½ ≈ 4 min) (Figure 1B, and results not shown). Because shedding-defective CHO cell mutants (M2 cells), initially isolated for a lack of proTGF-α shedding, are affected in an unidentified component necessary for the shedding of a variety of molecules including L-selectin, we also tested the effect of TFP in M2 cells. As shown in Figure 1(B), TFP showed little or no effect on proTGF-α shedding in M2 cells, indicating that the component affected in M2 is unrelated to calmodulin.

To characterize more fully the effect of the calmodulin inhibitor TFP on the shedding of proTGF-α, the effect of the hydroxamic acid-based metalloprotease inhibitor TAPI on the shedding promoted by TFP was analysed. TAPI is known to block the shedding of the ectodomain of a variety of proteins, including proTGF-α and L-selectin. The data in Figure 1(C) indicate that, as with the activation produced via PKC, the loss of cell-surface proTGF-α induced by TFP is inhibited by simultaneous treatment with TAPI. Similar results were obtained when the effect of another calmodulin inhibitor, calmidazolium, was analysed under the same conditions (results not shown). The results presented here show that calmodulin inhibitors known to induce the shedding of L-selectin also induce the proteolytic shedding of proTGF-α, indicating that calmodulin is a possible modulator of the general shedding machinery in vivo. However, calmodulin probably does not have a role in the mechanism that activates the shedding of proTGF-α via PKC, because it has been recently found that proTGF-α devoid of a cytoplasmic tail, and therefore incapable of interacting with calmodulin, is shed from the cell surface with the same kinetics as wild-type molecules (J. Ureña, J. Baselga and J. Arribas, unpublished work).

The availability of cell mutants defective in the shedding of several molecules, and the identification of TACE, the protease responsible for the shedding of proTNF-α, have provided useful tools for gaining insights into the mechanisms that regulate the shedding of transmembrane proteins. To characterize the defect of M2

**Figure 1**

Effect of calmodulin inhibitors on the shedding of proTGF-α

(A) CHO cells stably transfected with proTGF-α tagged in the ectodomain with the haemagglutinin (HA) epitope were incubated, as indicated, with different concentrations of TFP in Dulbecco’s modified Eagle’s medium at 37°C for 15 min. Then cells were shifted to 4°C, immunostained with anti-HA, FITC-coupled secondary antibodies and analysed by flow cytometry as described previously [2]. The results are expressed as percentages relative to mean fluorescence of untreated cells and are averages of duplicate determinations. (B) Wild-type or shedding-defective mutant CHO cells (M2 cells) stably transfected with proHA/TGF-α were treated with 40 μM TFP for variable periods as indicated. Then cells were treated and analysed as described for (A). (C) Wild-type CHO cells stably transfected with proHA/TGF-α were treated with 1 μM PMA, 40 μM TFP or 50 μM TAPI, as indicated, in Dulbecco’s modified Eagle’s medium at 37°C for 20 min. Then cells were treated and analysed as described for (A).
cells more fully, we have recently analysed the shedding of proTNF-α in these cells and found that they lack PMA-induced proTNF-α shedding, indicating that, as expected (given the generality of the defect of M2 cells [3]), the component mutated is necessary for the shedding of proTNF-α [13]. This mutant component is likely to be different from TACE because the defect in M2 cells is not rescued by transfection with TACE (Figure 2) [13]. The existence of an unidentified component necessary for the shedding of several molecules, including proTNF-α, and different from TACE, has been further substantiated by using somatic cell fusions between TACE-null cells (TACE−/−) and M2 cells. TACE−/− × M2 hybrids show wild-type shedding of proTNF-α, confirming that the mutation in M2 cells does not affect TACE. Furthermore, although TACE−/− cells show defects in βAPP shedding [13a], TACE−/− × M2 hybrids show normal shedding of proTGF-α and βAPP [13]. Transport and maturation of TACE in wild-type cells are indistinguishable from those in M2 cells [13], arguing that the component mutated is not involved in the regulation of the biosynthesis or subcellular location of TACE. This unidentified component seems to control only a subset of metalloprotease disintegrins because the processing of Notch, which is dependent on the metalloprotease disintegrin Kuzbanian, is normal in M2 cells [13]. Collectively, the results presented here and elsewhere [13] indicate that the activity of TACE is tightly controlled by a novel component of the shedding system that controls the activity of a subset of metalloprotease disintegrins. Because this component is not involved in the regulation of the biosynthesis or subcellular localization of the substrates or shedding machinery, it is tempting to speculate that the novel component would mediate the recognition of the substrate by certain metalloprotease disintegrins, such as TACE.

The regulation of the subcellular location of components of the shedding machinery or its substrates has been suggested as a likely mechanism for controlling the shedding of transmembrane molecules. However, only for βAPP has this mechanism been proved [9]. Several years ago the shedding of proTGF-α was found to be dependent on the integrity of the most C-terminal, cytoplasmic, residue: valine. Recently we and others have shown that proTGF-α C-terminal mutants are localized to the endoplasmic reticulum ([14], and J. Ureña, J. Baselga and J. Arribas, unpublished work). The modest percentage of proTGF-α C-terminal mutants that reach the cell surface are shed with identical kinetics to that of wild-type molecules (J. Ureña, J. Baselga and J. Arribas, unpublished work). Therefore, retention in the endoplasmic reticulum provides an explanation for the defective shedding of proTGF-α C-terminal mutants. Currently we are characterizing the mechanisms that localize proTGF-α C-terminal mutants to the endoplasmic reticulum. The identification of proteins that recognize the C-terminus of proTGF-α will be useful for evaluating the impact of the regulation of the proTGF-α subcellular location in the control of the shedding of its ectodomain.

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Proteinase-activated receptors: a growing family of heptahelical receptors for thrombin, trypsin and tryptase

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Introduction

Although proteases usually function as degradative enzymes, certain enzymes have biological activity that is mediated through a new and growing family of G-protein-coupled receptors (GPCRs) (reviewed in [1]). The best example is thrombin, which has activity distinct from its role in the coagulation cascade. In 1991, Coughlin and colleagues isolated a cDNA encoding a GPCR that mediates many of thrombin’s cellular effects [2]. Initially named the thrombin receptor, it was subsequently renamed proteinase-activated receptor 1 (PAR-1) after the discovery of related receptors. Thrombin activated platelets from PAR-1 knockout mice, but had no effect on fibroblasts from these animals, proving that additional thrombin receptors exist [3]. Indeed, PAR-1 has no role in the activation of mouse platelets by thrombin. A cDNA encoding PAR-3 was subsequently amplified by reverse transcriptase-mediated PCR from rat platelet RNA with degenerate primers based on conserved regions of other GPCRs [4]. Although low concentrations of thrombin had no effect on platelets from PAR-3 knockout mice, high concentrations caused a delay and diminished activation, suggesting the existence of yet another thrombin receptor. A gene bank search for PAR-related sequences identified an expressed sequence tag similar to other PARs; full-length cDNA was subsequently obtained [5,6]. This cDNA encoded a unique thrombin receptor, designated PAR-4. Thus thrombin interacts with three related receptors: PAR-1, PAR-3 and PAR-4. Pancreatic trypsin and tryptase, a major secretory granule protease of human mast cells, also possess biological activity that might be receptor-mediated. In 1994 Sundelin and colleagues screened a genomic