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To cut or not to cut: New rules for proteolytic shedding of membrane proteins

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Sheddases are specialized proteases that control the abundance and function of membrane proteins by cleaving their substrate's extracellular domain (ectodomain), a process known as shedding. Hundreds of shedding substrates have been identified, but little is known about the mechanisms that govern ectodomain shedding. Iwagishi *et al.* now report that negatively charged amino acids in the membrane-proximal juxtamembrane domain of substrates make them resistant to shedding by the metalloprotease ADAM17. These findings will help researchers better understand the regulation of shedding and may aid in the development of drugs targeting sheddases.

Ectodomain shedding is a proteolytic process, during which the ectodomain of a membrane protein is released from cells (1). The contributing protease, referred to as sheddase, cleaves the substrate typically within its juxtamembrane domain, which is the part of the ectodomain closest to the transmembrane domain (Fig. 1). Ectodomain shedding acts as an irreversible molecular switch and controls the abundance and the activity of integral membrane proteins (*e.g.* through the degradation of cell adhesion proteins and cell surface receptors or through release of membrane-bound cytokines and growth factors) (1). Shedding occurs for hundreds of membrane proteins, which mostly have a single transmembrane domain or are membrane-tethered through a glycosylphosphatidylinositol anchor. Given this large number of substrates, ectodomain shedding controls numerous processes in development and physiology, and regulation of this process is essential. In the absence of tight regulatory mechanisms, shedding contributes to pathology, such as increased inflammation and Alzheimer's disease (1). Thus, understanding the mechanisms governing ectodomain shedding is of vital importance.

Unlike caspases and many other proteases that only cleave specific amino acid motifs, most sheddases have a relaxed substrate specificity. As a consequence, point mutations around a shedding substrate's cleavage sites rarely fully prevent shedding (1). Additionally, sheddases typically cleave at a relatively fixed distance from the membrane surface, such that deletions or insertions around the cleavage site may simply shift the cleavage site to an alternative site nearby but do not fully inhibit shedding. Efforts to assign substrates to sheddases must therefore be well-controlled to avoid the possibility that cleavage at the new site is not simply mediated by another sheddase. For shedding to occur, it is also important that the juxtamembrane domain is accessible and not buried within a globular domain. Moreover, some substrates, such as the Alzheimer's disease–linked amyloid precursor protein, require a helical conformation in their juxtamembrane domain to be shed. Furthermore, post-translational substrate modifications in the vicinity of the cleavage site may affect shedding, as observed with the *O*-glycosylation of the cell adhesion molecule 1 (CADM1) (2). However, the effect of modifications on shedding is substrate-dependent (3). Thus, further study is needed to uncover strategies for how transmembrane proteins avoid sheddase activity.

Shedding substrates are cut by more than 30 sheddases known to date, including the a disintegrin and metalloproteases (ADAMs) ADAM10 and ADAM17 (1). For both sheddases, numerous substrates have been identified, but their substrate spectrum is likely to be much larger than what is currently known. Similarly, for individual substrates, the contributing sheddase(s) as well as their exact cleavage sites remain to be identified and are difficult to predict, raising the question of what features flag substrates to be targeted for or prevented from shedding.

In their recent work, Iwagishi *et al.* (4) report a new element governing the shedding activity of ADAM17 on its substrates. The study analyzed the ADAM17-mediated shedding of the activated leukocyte cell adhesion molecule (ALCAM), which is crucial for the entrance of B cells into the brain in neuroinflammatory diseases such as multiple sclerosis (5). The authors identified two ALCAM isoforms that differ in the juxtamembrane domain amino acid sequence. The isoform containing stretches of negatively charged amino acids barely underwent ADAM17-mediated shedding in the RAW264.7 macrophage cell line, whereas the isoform without multiple negatively charged amino acids was efficiently shed. Detailed mutational analysis revealed that inserting stretches of negatively charged amino acids into the juxtamembrane domain of the well-shed ALCAM prevented its shedding by ADAM17. Similar results are reported for a second ADAM17 substrate, the receptor tyrosine kinase ErbB4. These findings are in line with previous observations for the interleukin-6 receptor (IL-6R) (6), another ADAM17 substrate, where an SNP resulted in an aspartate-to-alanine exchange and increased IL-6R shedding.

This new rule for ADAM17 substrates—the absence of stretches of negative charges—has important consequences for

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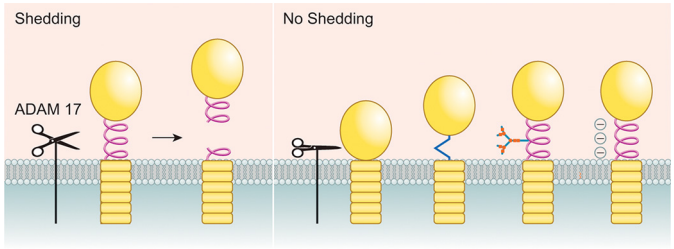

Figure 1. Rules for proteolytic shedding of membrane proteins. *Left*, ADAM 17 cleaves a transmembrane protein in the juxtamembrane (JM) domain, releasing the extracellular part. *Right*, alterations of the potential substrate may interfere with shedding. From *left* to *right*, JM domain is not accessible; JM domain does not have a helical conformation, which is required for some substances to be shed; JM is post-translationally modified (*e.g.* by glycosylation); negatively charged amino acids in the JM domain interfere with shedding, as shown by Iwagishi (4).

functional rescue experiments *in vitro* and *in vivo*. For many shedding substrates, it remains unclear whether their physiological function is mediated by the full-length form, the shed ectodomain, or both. To address this question, it is often necessary to test whether a nonsheddable variant of a substrate is able to rescue the functional deficits seen in substrate-deficient cells or mice. However, given the aforementioned relaxed substrate specificity of many sheddases, the generation of nonsheddable substrate mutants has often been difficult—*e.g.* for the ADAM17-mediated shedding of the SARS-CoV-2 receptor ACE2 (7)—but may now be possible through the introduction of negatively charged amino acid stretches into the substrate's juxtamembrane domain. This approach could also be useful for substrates of ADAM10, a close homolog of ADAM17.

The introduction of negative charges into the juxtamembrane may happen under physiological conditions upon alternative splicing of juxtamembrane domain-encoding exons. Iwagishi *et al.* (4) show this to be the case for ALCAM. The alternatively spliced exon encodes for 14 amino acids, six of which are aspartate and glutamate in the nonshed isoform, whereas there is only a single glutamate in the efficiently shed isoform. Similar observations were made for ErbB4. Likewise, alternative splicing of CADM1 generates a nonsheddable variant that is *O*-glycosylated around the cleavage site or a sheddable variant without that modification. These examples suggest that alternative splicing of exons encoding juxtamembrane domains may be a more generally applicable mechanism to control the shedding and, thus, the (patho-)physiological function of membrane proteins. In fact, the different splice forms of ErbB4 have different signaling functions (8), and one, but not the other, CADM1 isoform is linked to bladder cancer (9).

In summary, the molecular mechanisms for ectodomain shedding are partly understood and comprise both positive and negative features of the substrate. The recent work from Iwagishi *et al.* (4) adds one more negative feature that substrates must avoid to be cleaved by ADAM17, namely stretches of negatively charged amino acids. Physiological relevance is demonstrated with the finding that alternative splicing can introduce either a shedding-susceptible or a negatively charged, shedding-resistant juxtamembrane domain. Future studies need to address the exact mechanism by which the negatively charged amino acids block ADAM17-mediated shedding. Biophysical experiments and *in vitro* protease assays may reveal whether the negative charges simply reduce the affinity of the substrate to the protease or even to protease-associated proteins. ADAM17 forms a complex with a nonproteolytic subunit, iRhom1 or iRhom2. Both proteins are integral membrane proteins that bind to the transmembrane and probably the juxtamembrane domains of ADAM17 and may act as adaptors that help to recruit substrates to ADAM17 (10). Thus, it also appears possible that the negative charges in the substrate's juxtamembrane domain interfere with binding to iRhoms and thus, indirectly, reduce shedding. Another challenge for the future is to understand which positive and negative features of the substrate similarly apply to other major sheddases, such as BACE1 and BACE2. More investigations of the shedding of individual membrane proteins and systematic proteome-wide shedding analyses that are now feasible will give us a more detailed picture of the molecular machinery governing ectodomain shedding and will be helpful for future functional studies, unraveling disease mechanisms and drug development for the many shedding-related human diseases.

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Abbreviations—The abbreviations used are: ADAM, a disintegrin and metalloprotease; ALCAM, activated leukocyte cell adhesion molecule; JM, juxtamembrane.

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