Transforming the cell surface through proteolysis

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Protein shedding, or the proteolytic cleavage of a protein from the surface of a cell, is emerging as an important mechanism in the regulation of cellular activity but it is poorly understood. Growing evidence suggesting that protein shedding and protein function are closely linked may lead to new strategies for the treatment of a wide range of diseases.

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Introduction

Cells have the remarkable ability to direct the functions and activities of their thousands of proteins through several mechanisms. One of these is phosphorylation — a reversible modification that affects function by changing protein–protein interaction propensities. An alternative and more permanent way to direct cellular processes is by proteolysis. Intracellularly, proteolysis can regulate the presence of specific proteins, influencing fundamental processes such as the cell cycle or apoptosis, and proteolysis regulates important processes outside the cell, such as cell migration and wound repair. Proteolysis can also function as a control mechanism on the cell surface. For example, the G-protein-coupled thrombin receptor can be activated by limited proteolysis [1]: thrombin cuts the amino terminus of its receptor, causing a conformational change that activates the receptor. Thus, regulated proteolysis has an important role on the cell surface.

This review focuses on the importance of proteolysis in the release of the extracellular portion of a protein by cleavage at the cell surface, referred to here as protein shedding. The term protein shedding has been used to describe the loss of molecules from the cell surface either through enzymatic cleavage by proteases or lipases, or by exocytosis in membrane-derived vesicles. Here, however, protein shedding will be used exclusively to describe the proteolytic cleavage of proteins from the surface of the cell.

A number of different cell types shed proteins from their cell surface and the proteins released are also diverse, including cytokines and cytokine receptors, growth factors and growth factor receptors, cell-adhesion molecules, Fc receptors, and G-protein-coupled receptors (Table 1). These proteins have a wide range of functions, and there is a growing interest in exploring the relationship between protein shedding and protein function.

Recent reviews have provided excellent discussions about the characteristics of the enzymes involved in proteolytic release, the cleavage of leukocyte membrane molecules, and the generation and function of soluble cytokine receptors and growth factors [2–6]. Here, we focus on the events that liberate the ectodomain of a transmembrane protein (Figure 1 and Table 1), which we have classified into four main categories. The first group is comprised of the shedding processes induced by cellular activators, the second group consists of shedding processes that appear to be constitutive, the third comprises proteolytic cleavage events mediated by antibodies, and we also discuss emerging evidence suggesting that some receptors are proteolytically

Table 1

Proteins that are shed from the cell surface.

Shedding method	Proteins released				
	Cell adhesion molecules	Cytokines/ cytokine receptors	Growth factors/ growth factor receptors	G-protein coupled receptors	Miscellaneous
Activation	CD43*†, CD44*§, CD62L*†, CD23*	TNFα*, TNFR-I*†‡§, TNFR-II*‡,IL-1RII*, IL-4R*, IL-6R*#	proTGFα*§, CSF-1R*, HGFR*, c-kit receptor*§, TrkA neurotrophin receptor*	TSHR*	CD14*#, CD16-I* CD16-II*,CD30*, Fas*, LAR*, ACE*, βAPP*, class I MHC*, syndecan*
Antibody¶	CD43 (anti-CD43), CD44 (anti-CD44, anti-CD95), CD62L (anti-CD62L, anti-Leu-13, anti CD45, anti-CD16, anti-CD95), CD23 (anti-CD20)				CD14 (anti-CD14)
Ligand¥	CD62L (neoglycopolymer), CD44? (hyaluronate), CD43?	IL-3R (IL-3), TNFR-I (TNFα), TNFR-II (TNFα)	TrkA neurotrophin receptor (NGF)	TSHR (TSH), V2 vasopressin receptor (photoreactive vasopressin agonist)	CD14? (LPS)

The symbols designate those cell-surface molecules that are shed in response to the corresponding activator. This list is not all inclusive; each molecule has not necessarily been tested with every activating agent: *PMA, †fMLP, ‡LPS, §Ca^{2+} ionophores, #toxins. ¶Shed molecule (antibody or antibodies that induce the shedding). ¥Shed molecule (ligand that induces its shedding). ACE, angiotensin converting enzyme; βAPP, β-amyloid precursor protein; TNF, tumor necrosis factor; TSH, thyrotropin; IL, interleukin; TGF, transforming growth factor; MHC, major histocompatibility complex; CSF, colony stimulating factor; HGF, hepatocyte growth factor; NGF, nerve growth factor; PMA, phorbol 12-myristate 13-acetate; fMLP, formylmethionylleucylphenylalanine: LPS, lipopolysaccharide.

cleaved in response to ligand binding. Ligand-induced proteolysis is a potential mechanism for regulating receptor–ligand interactions, and extends a relationship between the function and regulation of receptors.

Activation-induced shedding

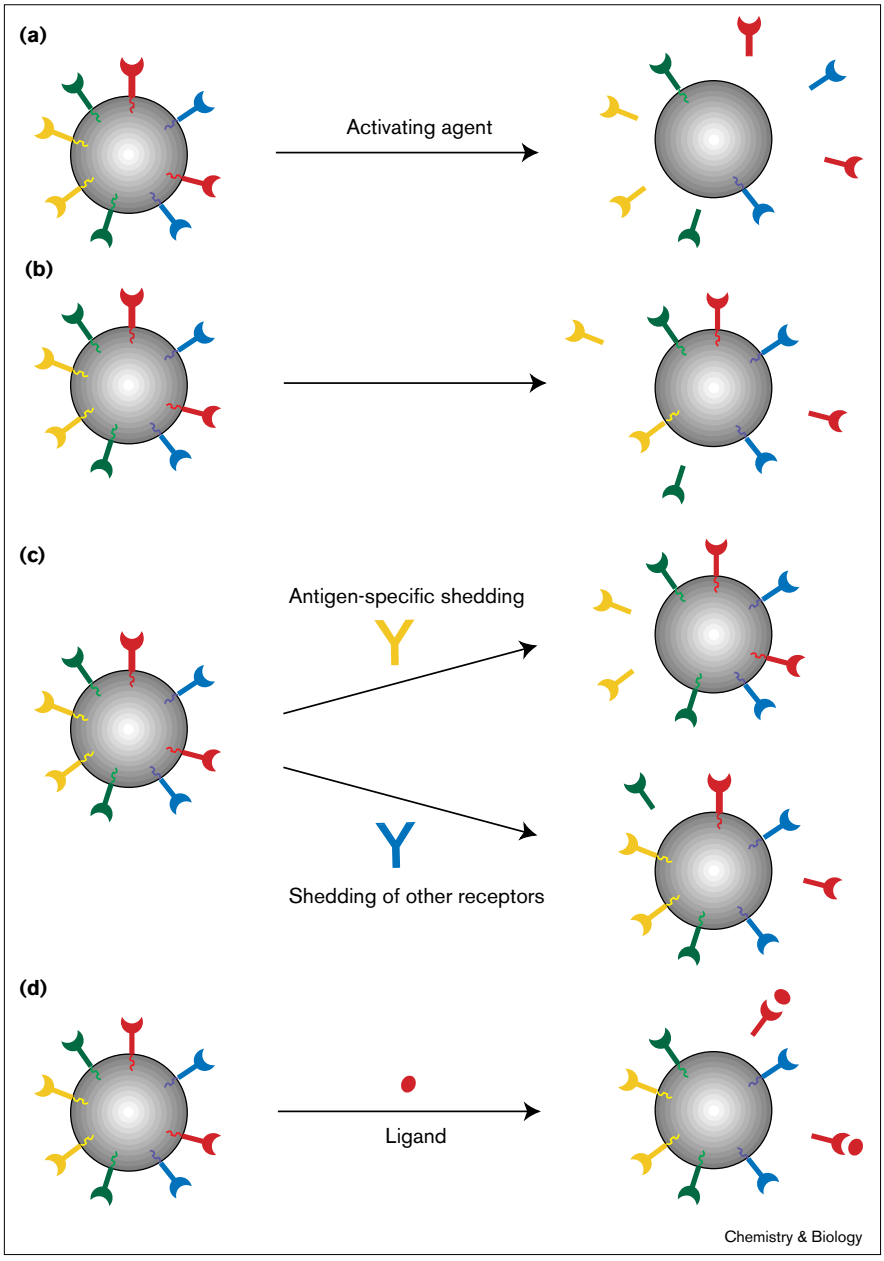
Cellular stimulants can induce the shedding of many proteins and also cause cellular activation and concomitant changes in cell behavior. The release of protein ectodomains appears to be one way for the cell to change the characteristics of its surface, eliminating activities that are no longer necessary or are counterproductive. Although the precise relationship between the new behaviour adopted by the cell and the effects of protein shedding remains to be determined, there is circumstantial evidence suggesting that they are functionally linked. For example, when leukocytes become activated they shed the leucocyte antigen CD43 from their surface, and also become more able to adhere to target cells. CD43 is a highly anionic sialomucin, proposed to function as an 'anti-adhesive' molecule. It is found in high concentrations on circulating white blood cells, and towers over the cell surface, extending ~45 nm beyond the membrane [7]. The negative charges on CD43 are believed to prevent leukocyte aggregation and undesired adhesion events by both electrostatic and steric repulsion. When leukocytes become activated, they shed CD43 from the surface, rendering the cell surface conducive to adhesive interactions suggesting that CD43 shedding is an important part of the activation process [8]. Similar persuasive explanations for the functional roles of other shedding events have been suggested, but, as it is currently difficult to separate individual shedding events and thus study the functional effect of shedding one protein at a time, it has been hard to provide rigorous tests of these explanations.

Little is known about the pathways by which cellular stimulants induce the release of extracellular proteins. Many different agents have this capacity, including phorbol esters, chemotactic peptides, calcium ionophores, cytokines and growth factors (Figure 2a). These agents all initiate signal transduction, but use a variety of different pathways, making it difficult to identify the branch of the pathway that triggers protein release. Most studies of activation-promoted protein shedding have used phorbol esters (such as phorbol myristate acetate [PMA]) to stimulate protein kinase C (PKC) activity and some information about how cell stimulation leads to shedding has emerged from these investigations.

Phorbol esters induce collective shedding of a wide variety of cell-surface molecules, resulting in the release of cytokines (e.g., TNF-α), growth factors (e.g., proTGF-α), receptors (e.g., TNF receptor, IL-2R), cell-adhesion

Figure 1

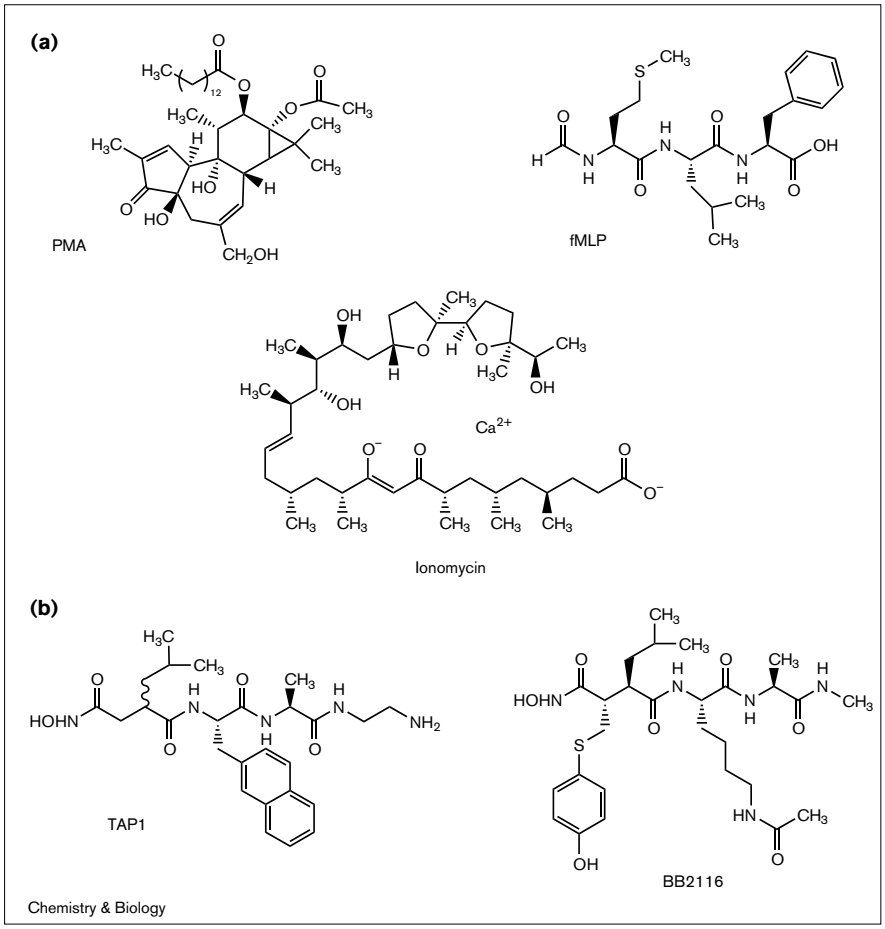
The liberation of the ectodomain of transmembrane proteins (protein shedding). **(a)** Activation-induced shedding. **(b)** Constitutive shedding. **(c)** Antibody-induced shedding. **(d)** Ligand-induced shedding.



molecules (e.g., L-selectin), enzymes (e.g., angiotensin converting enzyme) and proteins of unknown function (e.g., the amyloid precursor protein) [4,9,10]. The induction of shedding by phorbol esters suggests that PKC may act directly to cause protein shedding. Activated PKC phosphorylates serine and threonine residues in the cytoplasmic domains of selected proteins, but for many of the shed proteins the presence of a cytoplasmic domain, and therefore a potential intracellular signaling or recognition sequence, is not required for cleavage [11,12]. Additionally, many of the proteins released from the cell surface using phorbol esters are not phosphorylation substrates for PKC. Collectively, these results suggest that PKC does not directly promote protein shedding but is perhaps an upstream modulator of these proteolytic events.

The sequences of protease cleavage sites for several shed proteins have been determined and they have no obvious similarities. When the extracellular sequences of shed proteins are appended to proteins that are resistant to shedding, however, the resulting chimeric proteins are susceptible to proteolytic release [11,13], suggesting that proteins are released from the cell surface when they have either a sequence that adopts a specific conformation or

Figure 2



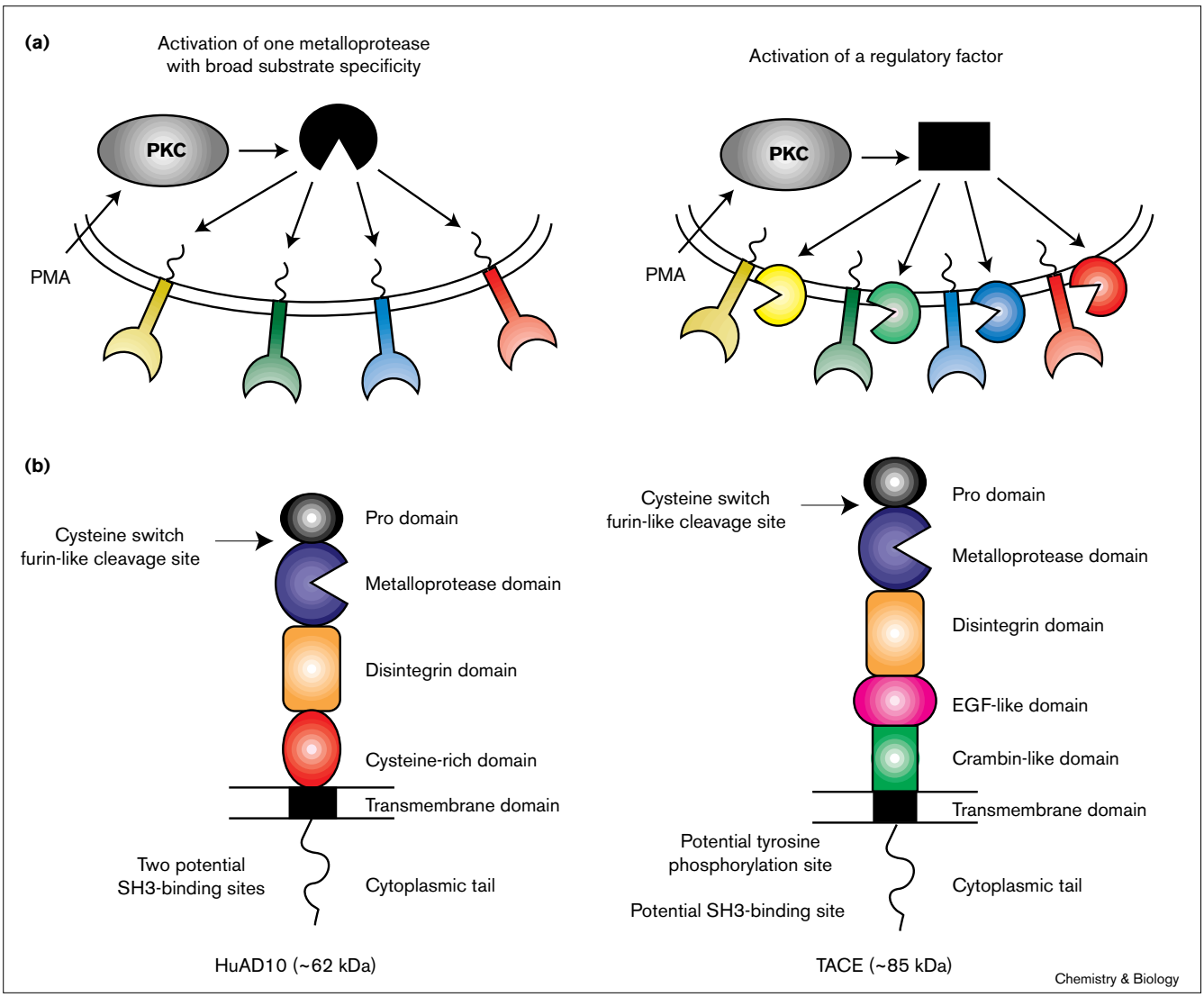
Chemical structures of molecules that promote or inhibit the shedding of cell-surface proteins. **(a)** The phorbol ester PMA, the chemotactic peptide fMLP and the calcium ionophore ionomycin are cellular activators that can promote the cleavage of a variety of cell-surface molecules. **(b)** Hydroxamic-acid-based peptide-like molecules, such as TAP1 and BB2116, inhibit the cell-surface proteolysis of many proteins.

one that is conformationally flexible. The apparent lack of selectivity has complicated the identification of protease(s) responsible for activation-induced shedding [9,10].

Most activation-induced shedding processes can be inhibited by metalloproteinase inhibitors, especially hydroxamic acid derivatives (Figure 2b). For example, these agents prevent the activation-induced shedding of several proteins, including TNF-α, p60 and p80 TNF-receptors, L-selectin, TGF-α, FasL, thyrotropin receptor, and angiotensin-converting enzyme [4]. The results suggest that at least one essential enzyme in the pathway is a Zn^{2+}-dependent metalloproteinase, or perhaps that the membrane-protein-solubilizing enzyme or enzymes themselves are metalloproteinases. Metalloproteinases that are known to act extracellularly include matrix metalloproteinases (MMPs), the astacin-related bone morphogenetic protein 1 (BMP-1)/tolloid family of metalloproteinases, and the adamalysin-related class of metalloprotease disintegrins, which are referred to as ADAMs (a disintegrin and metalloprotease domain) or MDCs (metalloprotease/disintegrin/cysteine-rich protein) [14–20]. These enzymes probably share structural features such as similar zinc-binding sites, and they have been classified into a superfamily termed 'metzincins' [21]. Given the structural similarities, it would be expected that a hydroxamic acid derivative could inhibit multiple zinc proteinases.

Although hydroxamic acids block activation-induced shedding, metalloproteinases are not necessarily the only proteases involved in the process. For example, many of the metalloproteinases are produced in pro-form, a state in which the active-site zinc ion is trapped by cysteine coordination in a catalytically inactive coordination state. Proteolysis of the pro-metalloenzymes results in the loss of the cysteine ligand (cysteine switch), making the zinc center catalytically active. Thus, these enzymes can be activated in a proteolytic cascade that could involve extracellular metallo-proteinases or serine proteases. Inhibition data, however, suggest that most PMA-promoted shedding events are not blocked by serine protease inhibitors, although a highly active protease at the cell surface could be inaccessible to the inhibitors [22]. Additionally, naturally occurring inhibitors of the MMPs (tissue inhibitors of

Figure 3



Cellular activation leads to the shedding of many cell-surface proteins. **(a)** Two models for the mechanism of PMA-promoted shedding. **(b)** Domain organization of two distinct metalloprotease/disintegrins that cleave pro-TNFa, HuAD10 (left) and TACE (right). HuAD10 and TACE have 49.8% overall similarity.

metalloproteinase, including TIMP-1, TIMP-2) do not diminish protein shedding so, although protease accessibility may also be a factor in these studies, the results do suggest that MMPs may not be the predominant agents causing activation-induced protein release.

Significant research efforts have focused on identifying the membrane-associated protease that mediates the proteolytic release of tumor necrosis factor-α (TNF-α), a cytokine involved in inflammation. This search has resulted in the identification of at least two distinct enzymes (Figure 3b), and the genes encoding them have been cloned. Two groups identified the same protease, termed TNF-α converting enzyme (TACE), which is a member of the ADAMs family of disintegrin metalloproteinases [23,24]. Disruption of the gene encoding TACE in mice resulted in lower levels of TNF-α, supporting the idea that the TACE enzyme is responsible for endogenous TNF-α processing [23]. The second protein that has been identified, HuAD10, can cleave TNF-α, but not other proteins that are shed, suggesting it acts specifically [25]. HuAD10 is also a member of the ADAMs family, which is intriguing because ADAMs family proteases have been implicated in important processes, including fertilization, muscle fusion and development [26,27]. Both TNF-α processing enzymes have a zinc metalloproteinase domain

and pro-domain, probably a cysteine switch, in addition to a disintegrin domain and a cysteine-rich domain in the extracellular region (Figure 3b). The functions of these regions are not clear, but the disintegrin domain probably serves as a site for recognition of either the substrate TNF-α or as a link to the integrins. Because integrins are cell-adhesion and signaling molecules involved in cell–cell and cell–matrix interactions, the adhesion domain could couple integrin function to receptor shedding. In addition, the cytoplasmic domains of TACE and HuAD10 contain proline-rich sequences that could bind to SH3-containing proteins. SH3-containing proteins are involved in cell signaling, suggesting that there may be a connection between signal transduction and shedding [28]. Both TACE and HuAD10 were identified from the same cell line (THP-1), so different ADAM metalloproteinases in the same cell can process a single substrate. This provides further impetus for elucidating the factors controlling protease activity.

There are some common features in the activation-induced release of proteins. Arribas *et al.* [9] obtained genetic evidence to suggest a common pathway for PMA-induced shedding of protein ectodomains. They identified mutant Chinese hamster ovary (CHO) cell lines that did not release membrane-anchored transforming growth factor α (TGF-α) upon treatment with phorbol ester, and found that these mutant cell lines were unable to release the unrelated protein ectodomains from L-selectin, the β-amyloid precursor protein and the interleukin-6 receptor. Two different possibilities could account for these results. First, one protease of broad specificity can cleave a wide range of cell-surface proteins; second, several members of a family of related proteases, which cleave several substrates, are activated by a specific signaling event (Figure 3a). If one protease could cleave many cell-surface proteins, disruption of a gene encoding a protease that has an unusually broad substrate specificity could account for the lack of activity. Although this is possible, the metalloproteinases, which act extracellularly, have been shown to be selective for particular protein substrates *in vitro*. If many proteases were activated by a specific signalling event (the second possibility) mutations in a gene that encodes a single regulatory factor would prevent the regulatory factor from activating a variety of proteases. The factor could be a protease itself, as many proteases are activated by proteolytic cleavage.

Most investigations into protein shedding have involved cellular activation using phorbol esters, but there is some indication that the activation-induced shedding of certain receptors may occur by more than one mechanism, which is not surprising as different stimulants can trigger distinct activation events. For example, the cleavage of the receptors for interleukin-6 and lipopolysaccharide (CD14) is induced by PMA or pore-forming toxins [29]. Although both processes are prevented by metalloproteinase inhibitors, the toxin-dependent cleavage site was mapped to a different position than the site of cleavage induced by phorbol ester, suggesting either the involvement of different proteases in the cleavage or that alterations of the cell membrane can cause changes in the cleavage site. Thus, studies of protein shedding that involve different activation methods will illuminate the mechanistic pathways available for regulating the display of cell-surface proteins.

Constitutive proteolytic release

Several proteins, including the TNF receptors, the β-amyloid precursor protein, syndecan, L-selectin and the thyrotropin receptor, appear to be shed constitutively from the surface of cells without cellular stimulation by outside agents [4] (Table 1). The mechanism(s) of continuous or spontaneous shedding is not known, although, in several instances, the process can be inhibited by serine and/or metalloproteinase inhibitors, characteristics suggesting that constitutive shedding may occur by the same, or related, proteases used in activation-induced shedding. Spontaneous shedding of receptors may result from the action of constitutively active proteases and may be a necessary part of the cellular turnover process for cell-surface molecules. In the normal cellular aging process, some loss of cell-surface proteins could occur, and this change in protein display could arise, in part, from constitutive shedding [30].

Investigations of the extracellular proteolysis of ErbB-4, a growth factor receptor tyrosine kinase that undergoes constitutive shedding, reveal an interesting connection between extracellular and intracellular proteolysis [31,32]. A soluble fragment of ErbB-4 is generated by spontaneous shedding or by phorbol-ester-induced proteolysis and the resulting membrane-anchored kinase domain can no longer respond to its ligand, but it remains catalytically active. Under these circumstances, however, the growth factor fragment is ubiquitinated and targeted to the proteasome for degradation. The intact ErbB-4 receptor is not subject to ubiquitination, but extracellular proteolysis generates a substrate for polyubiquitination and subsequent intracellular proteolysis. Unregulated kinase domains could function as renegade signaling molecules, but this series of proteolytic events eliminates such substrates. The Met tyrosine kinase appears to use a similar pathway, suggesting that this may be a general mechanism for eliminating unresponsive signaling molecules [33]. As with activation-dependent protein release, an increased understanding of the regulatory processes governing proteolysis will facilitate our understanding of the role of spontaneous shedding.

Antibody-induced shedding

Treatment of cells with antibodies to specific cell-surface molecules promotes receptor shedding in some cases. For example, antibodies to the lipopolysaccharide receptor CD14 [34], the sialomucin CD43 [8], the hyaluronate receptor CD44 [35] and the lymphocyte homing receptor

L-selectin (CD62L) [36–38] can promote the shedding of their respective antigens. The mechanism of antibody-induced shedding has been suggested to be dependent on the ability of the antibody to cluster its receptor, analogous to ligand engagement. In such a model, proteolysis would be induced by receptor clustering, but antibodies can trigger other effects besides simple receptor clustering. Consequently, it has been difficult to separate the roles of receptor clustering and of various signaling processes in antibody-promoted shedding events.

Several examples in the literature demonstrate that antibodies to specific receptors can promote the shedding of other surface molecules. For instance, treatment of lymphocytes with antibodies to the signal transduction molecule Leu-13 [37], the protein tyrosine phosphatase CD45 [39,40] or the FcγRIII CD16 [41] promotes cleavage of L-selectin. Monoclonal antibodies that bind to CD95 (APO-1/Fas, a member of the tumor necrosis receptor family) promote the shedding of L-selectin, the hyaluronate receptor CD44 [42,43] and several other proteins. Similarly, anti-CD20 antibodies induce cleavage of the IgE receptor CD23 [44]. These data emphasize that antibodies function as more than simple cross-linking agents; they can activate additional processes, such as signal transduction pathways, that result in the shedding of other receptors. Antibody-mediated release of some proteins is inhibited by protein tyrosine kinase inhibitors, but many of these shedding events resemble those promoted by cellular activation. Given the possibilities, it can be difficult to definitively interpret the mechanism(s) of antibody-promoted receptor shedding.

Ligand-induced proteolysis

There have been several reports describing the proteolytic cleavage of proteins in response to ligand engagement. Proteins susceptible to this type of proteolysis belong to a wide variety of unrelated receptor families, including G-protein-coupled receptors, cytokine and growth factor receptors and cell-adhesion molecules. Because ligand binding can cause signal transduction, the molecular events involved in some ligand-induced proteolytic processes may overlap with those that are caused by cellular stimulants. Alternatively, ligand binding could cause changes in protein multimerization or conformation, increasing the susceptibility of a receptor to proteolysis. Consequently, ligand-promoted proteolysis may be a general regulatory mechanism (Figure 4) that links protein cleavage to protein function. Several proteins that appear to be proteolytically cleaved in response to ligand binding are discussed in more detail below.

G-protein-coupled receptors

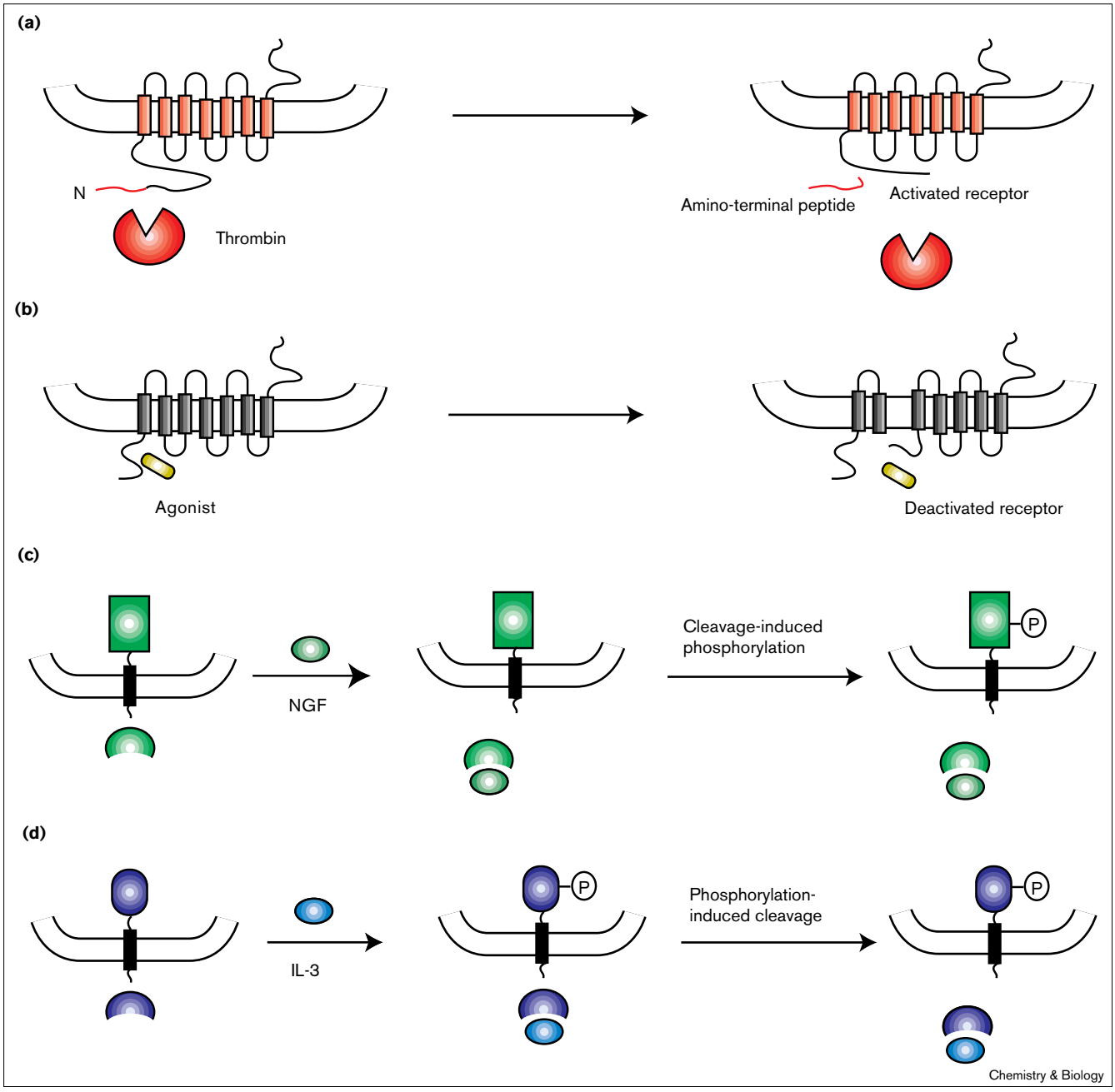
Some G-protein-coupled receptors, termed protease-activated receptors (PARs) can be stimulated by proteolytic cleavage (Figure 4a). G-protein-coupled receptors, characterized by their seven helical transmembrane domains, generally transmit information by undergoing a conformational change induced by ligand binding. The conformational change in PARs is triggered by the binding of ligand proteases, such as thrombin (PAR-1, PAR-3) or trypsin (PAR-2). The protease initiates signaling by binding to its target receptor, and cleaving the receptor near the amino terminus, causing a conformation change that creates a new site for intramolecular interaction. Investigations of PARs have revealed an elegant mode by which regulated proteolysis can control signal transduction (Figure 4a) [1].

It could be imagined that a similar covalent-bond change induced by a ligand could also be used for deactivation of a receptor. One feature of the G-protein-coupled signal transduction system is that cells tend to become desensitized after prolonged exposure to ligand. Receptor internalization is one consequence of ligand engagement but receptor shedding could also play a role in desensitization. Circumstantial evidence indicates that several G-protein-coupled receptors are subject to limited proteolysis, including the β_1-adrenergic receptor [45], the endothelin ET_B receptor [46,47], the V_2 vasopressin receptor [48] and the thyrotropin receptor (TSHR) [49,50]. Two examples from this receptor class in which ligand-induced shedding has been proposed to play an important role are the V_2 vasopressin receptor and the thyrotropin receptor.

Kojro *et al.* [48] used receptor-containing bovine kidney membranes to demonstrate that the V_2 vasopressin receptor, which mediates the antidiuretic action of the hormone vasopressin, is cleaved upon exposure to a vasopressin agonist. They proposed that ligand engagement exposes the cleavage site, allowing cleavage between the second transmembrane domain and the first extracellular loop, which contains the ligand-binding site. The enzyme, an unidentified metalloproteinase, leaves a truncated, membrane-bound receptor that is no longer able to bind ligand. The authors propose that ligand-induced proteolysis can attenuate signal transmission between vasopressin and the vasopressin receptor (Figure 4b).

The thyrotropin receptor (TSHR), involved in regulation of thyroid cell growth and function and implicated in certain autoimmune diseases, also seems to be regulated by ligand-induced shedding [50]. TSHR is shed spontaneously from human thyroid cells and transfected cell lines, and the shedding is increased upon exposure to the ligand, thyrotropin (TSH), as well as PMA or calcium ionophores such as A23187 or ionomycin. TSHR is unique in that it is expressed as a heterodimer at the cell surface where the extracellular α subunit and the transmembrane spanning β subunit are held together through disulfide bonds. It was discovered recently that the proteolytic release of the α subunit is dependent on metalloprotease-mediated cleavage followed by a subsequent

Figure 4



Several receptors appear to be proteolytically released from the cell in response to ligand binding. **(a)** Thrombin receptor. **(b)** Vasopressin receptor. **(c)** TrkA receptor. **(d)** mIL-3 receptor.

disulfide bond reduction [49]. Release of the α subunit may prevent signal transmission through the TSHR, whereas the soluble α subunit may inhibit binding of TSH to intact receptors on the cell surface. Although the relative importance of ligand-induced proteolysis of TSHR and ligand-induced receptor downregulation has not been determined, the proteolytic shedding may modulate receptor–ligand interactions in this system.

Cytokine and growth factor receptors

Several cytokine and growth factor receptors, including the tumor necrosis factor receptors (TNFRs), the TrkA neurotrophin receptor and the murine IL-3 receptor (mIL-3R), are proteolytically released from the cell surface upon exposure to either their cognate ligands or agonists (Table 1). The mechanisms of ligand-induced shedding of these receptors are not well understood; conformational

change, receptor dimerization or receptor clustering upon ligand binding may contribute to the signal for proteolytic cleavage. There is also evidence that phosphorylation is involved in the ligand-induced shedding of some of these receptors, either as a signal for cleavage, as in the mIL-3R, or as a consequence of cleavage, as in the TrkA receptor.

Soluble forms of TNF-α and its two receptors (p55 TNFR and p75 TNFR) can be generated by proteolytic cleavage [51–54]. High levels of soluble TNFR can be found in patients who have elevated TNF levels, suggesting a link between the formation of soluble TNFR and TNF [55,56]. Although the majority of studies on the shedding of the p55 TNFR and p75 TNFR have investigated the cleavage induced by phorbol ester stimulants [57–60], several reports suggest that TNF-α promotes the shedding of its receptors. Although some have suggested only the p75 TNFR is susceptible to ligand-promoted shedding [61,62], others indicate that TNF-α can stimulate the release of both p55 and p75 TNFRs [58,63,64]. Analysis of the complex of the related ligand TNF-β and p55 TNFR revealed that the trimeric ligand was bound to three copies of the receptor [65]. Thus, it appears that the trimeric TNF ligands induce a change in receptor organization. Whether this altered conformation is recognized by a protease or whether the signals transduced by the receptor lead to protease activation is not known.

The observations that the shedding of TNF-α and TNFR appear to be connected and the uncharacterized enzyme that proteolytically releases TNFR is similar to that responsible for TNF-α shedding have important therapeutic ramifications. Protease inhibitors that block production of TNF-α may simultaneously inhibit TNFR shedding, increasing the cellular response to low levels of TNF-α. Manipulation of the inflammatory response through these receptor–ligand pairs requires selectivity, provided by, for example, agents that inhibit the release of TNF-α but do not prevent the release of its receptors. Alternatively, the discovery of molecules that promote the cleavage of the TNFRs without affecting TNF-α could be useful modulators of the inflammatory response, but, to date, no such molecules have been described.

The receptor tyrosine kinase TrkA, which is involved in the development and survival of neural cells, appears to undergo ligand-promoted shedding (Figure 4c). A TrkA ligand, nerve growth factor (NGF), was found to promote ectodomain proteolysis of the TrkA receptor, which results in the production of a fragment composed of a transmembrane region and an intracellular kinase domain [66]. The remaining membrane-bound fragment had a greater phosphotyrosine content than its intact counterpart, and the increase was attributed to autophosphorylation. Interestingly, the augmented level of kinase activity was probably a consequence of the cleavage, not the cause of it, as TrkA isozymes with defective kinase domains were also shed. Like the growth factor ErbB-4 discussed previously, the kinase domain of the cleavage product is active but no longer subject to regulation. In various tumor cell lines, constitutively active mutants of receptor tyrosine kinases are often truncated cell-associated receptors that lack their extracellular domains. Thus, the release of the extracellular domain, and therefore the signal triggered by the kinase-active fragment must be regulated to prevent unwanted signaling events. As with many other shed proteins, the soluble fragment generated upon release could modulate the effects of the ligand.

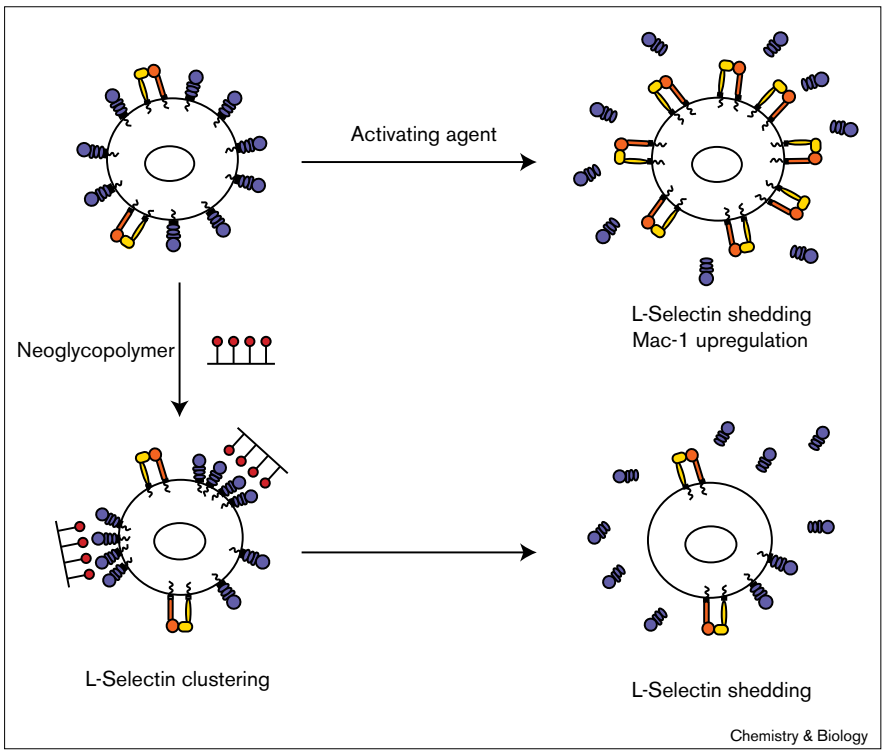
Although some receptor tyrosine kinases, such as the TrkA receptor, are activated upon release of their extracellular domain, receptor cleavage may also be induced by phosphorylation. The murine IL-3 receptor (IL-3R) is phosphorylated and then shed upon binding of IL-3 (Figure 4d) [67]. Mui *et al* found that receptor phosphorylation accompanied proteolytic release of the β subunit, but that unmodified IL-3R is not a substrate for cleavage. The authors propose that ligand binding to the IL-3R results in phosphorylation of its intracellular domain, which triggers its cleavage. In analogy to the proposed situation for TNF binding by TNF-R, the extracellular domain could be downregulated on the cell surface, and the soluble form of the receptor could regulate IL-3-mediated signal transduction.

Cell-adhesion molecules

Soluble forms of several cell-adhesion molecules are detected in circulation (Table 1). Many are shed constitutively or upon cell activation, but there is increasing evidence that ligand binding may promote the shedding of at least some of these proteins. In addition to the notion that antibody-induced shedding of CD43 [8], CD44 [35] and L-selectin (CD62L) [36–38] is analogous to ligand-induced shedding, some reports imply that shedding may be associated with cell adhesion [68] or migration [69]. Ligand-dependent shedding of cell-adhesion molecules may function in part to promote the de-adhesion process, a necessary component of cell–cell interactions.

L-Selectin is a cell-adhesion molecule involved in the inflammatory response, and the shedding of L-selectin is unusual because it can occur by at least two apparently independent pathways: by induction with cellular stimulants and with multivalent L-selectin ligands (Figure 5). L-Selectin mediates an early step in leukocyte recruitment to the endothelium — the rolling of leukocytes along the endothelial cell wall. L-Selectin is shed rapidly from neutrophils and lymphocytes upon treatment with phorbol esters, an event that can be blocked using zinc metalloproteinase inhibitors (hydroxamic acid derivatives). The activation-induced shedding of L-selectin coincides with the appearance of increased levels of the

Figure 5



The proteolytic release of L-selectin can be induced with cellular stimulants or multivalent L-selectin ligands. These processes are distinct: cellular stimulants cause upregulation of the β2-integrin Mac-1, but synthetic ligands do not.

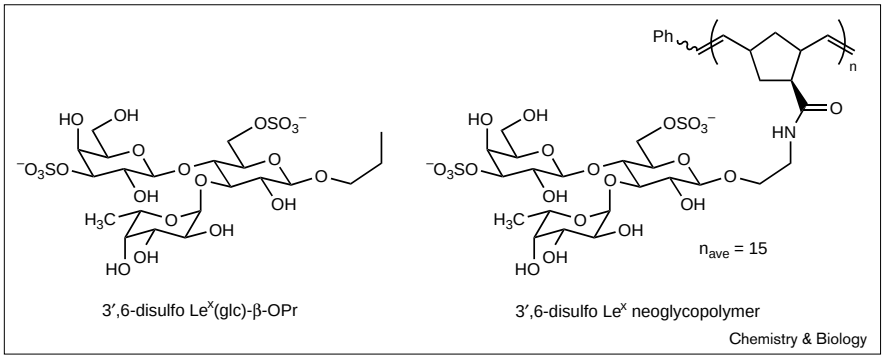
β2-integrin Mac-1 in neutrophils. β2-integrins also function in the leukocyte recruitment process, mediating the firm leukocyte–endothelium adhesion that follows rolling. The concomitant stimulant-promoted downregulation of L-selectin and upregulation of the β2-integrins suggests that the coupling of events is not merely circumstantial. Anti-L-selectin antibodies can also promote L-selectin shedding [36–38], a result that suggests ligand binding and or receptor clustering might regulate the display of L-selectin on the cell surface. Because antibody engagement of other cell-surface molecules like CD45 also triggers L-selectin release [39,40], the antibody-promoted shedding experiments are difficult to interpret.

To gain insights into the relationship between L-selectin binding, clustering, and proteolytic release, Gordon *et al.* [70] directly investigated the effects of L-selectin ligands on the cell-surface presentation of the protein. The known physiological L-selectin ligands, GlyCAM-1 [71], CD34 [72], and PSGL-1 [73], are mucin-like glycoproteins that display multiple copies of an L-selectin binding epitope (derivatives of the tetrasaccharide sialyl Lewis X) [74,75] on their surface. The development of methods to synthesize monovalent and multivalent derivatives provided the means to investigate whether L-selectin ligands could promote shedding and, if so, to ascertain which structural features are required. Monovalent derivatives of the trisaccharide Lewis X, which are L-selectin inhibitors (IC_{50} values of 1–3 mM) [76], did not cause L-selectin shedding. In contrast, the synthetic multivalent ligands (termed neoglycopolymers) induced L-selectin shedding from human leukocytes in a dose-dependent manner (Figure 6).

Intriguingly, the proteolytic cleavage of L-selectin induced by the neoglycopolymers was quite distinct from that observed with other agents. One important difference is that the polymer-promoted cleavage appears to selectively initiate L-selectin shedding (E.J.G. and P.A. Mowery, unpublished observations), whereas the activation-promoted process results in the cleavage of a number of surface molecules. In addition, the neoglycopolymer-treated leukocytes showed no signs of activation, as determined by the lack of increase in the surface concentration of the β2-integrin Mac-1 (Figure 5). Thus, integrin upregulation and L-selectin downregulation are not necessarily coupled events, which is important as it may be therapeutically useful to minimize cell-surface levels of L-selectin without simultaneously initiating other signaling pathways. The polymer-induced shedding was not inhibited by the PKC inhibitor staurosporine, which blocked the PMA-stimulated cleavage. Surprisingly, a hydroxamic-acid based metalloproteinase inhibitor, which diminishes activation-induced shedding, also had no effect on activity of the neoglycopolymer [70]. These results demonstrate the multivalent, but not monovalent, synthetic ligands

Figure 6

Synthetic models designed to mimic physiological L-selectin ligands. Only the multivalent molecule, 3′,6-disulfo Le^x neoglycopolymer (right) induces the cleavage of L-selectin from the surface of leukocytes.



can initiate L-selectin shedding, and they suggest that the ligand-induced shedding process has unique features that can be exploited.

To date, ligand-induced shedding of cell-adhesion molecules other than L-selectin has not been explored widely. Nevertheless, there is some evidence that suggests that the hyaluronic acid receptor, CD44, may also be proteolyzed upon ligand binding. CD44 is implicated in various cellular processes, including lymphocyte binding to HEV, T cell activation, monocyte stimulation to IL-1 production and lymphopoiesis [77]. It is interesting to note that, like L-selection, CD44 can mediate lymphocyte rolling, and therefore it may have a similar biological role [78]; CD44, again like L-selectin, binds a saccharide derivative—hyaluronic acid, a linear, anionic polysaccharide that could engage CD44 in multivalent binding. This process could cause receptor clustering, which might trigger the proteolytic release of soluble CD44. In a recent report by Friedl *et al.* [69] the migration of highly aggressive MV3 melanoma cells was shown to be accompanied by shedding of CD44. Loss of CD44 from the surface of the cells subsequent to ligand engagement could serve to assist the migration of cells by promoting their detachment, suggesting that the shedding of CD44, induced by adhesion, may not only assist in normal cell migration, but may also contribute to the metastasis of cancer cells.

The mechanism of receptor shedding

Given the number of proteins that can be shed, elucidating the mechanisms that control protein shedding is an important goal. The widespread shedding induced by phorbol esters is intriguing as it suggests there may be a general control mechanism to render a cell resistant to a multitude of extracellular signals. Alternatively, the ligand-induced processes may represent specific cases in which selected receptors are released preferentially in response to particular activation pathways. Ligand-induced shedding could result in a conformational change, dimerization or clustering of the receptor, thereby generating a recognition site for a protease. In this case, the protease itself is constitutively active, and is only dependent on the generation of a recognition site for shedding to occur. This would explain how some receptors are shed constitutively at low levels, whereas the shedding can be upregulated after exposure to ligand. Interestingly, some of the metalloproteinases are thought to act in multimeric form [20].

To focus on various events that lead to protein ectodomain release, we have divided different shedding processes into categories. These divisions provide a context in which to introduce specific examples, but little is known about the regulatory mechanisms governing the proteolytic release of ectodomains. Moreover, it often is difficult to distinguish among activation-, antibody- or ligand-induced shedding for a given set of experimental conditions. For example, ligands binding to receptor tyrosine kinase proteins will activate specific signaling pathways. Thus, shedding could be brought about by a change in the state of the receptor, such as oligomerization and/or phosphorylation, or an alteration of the protease, such as activation by a signal transduction cascade or a change in localization. The cell type, culture conditions, isolation/purification procedures, and shedding agents used, as well as the signal transduction pathways initiated can all dramatically affect cell behavior so comparing and analyzing results from different experiments is extremely complex and it is difficult to draw conclusions.

Future directions

The proteolytic release of a protein ectodomain can have several consequences. The cell-surface concentration of the protein decreases, and, if the protein is a receptor, ligand binding at the cell surface will also decrease, desensitizing the cell to the effects of the ligand. Simultaneously, the concentration of the soluble and potentially biologically active form of the receptor in circulation will be increased. Thus, the soluble form of the receptor can function as an inhibitor of receptor–ligand interactions or as a transporter of the ligand, removing the ligand from the cell surface and enhancing its stability in circulation. For proteins that have intracellular phosphorylation sites

or intrinsic kinase activity, receptor shedding may result in activation or deactivation of a signaling pathway. Alternatively, the shed molecules themselves can activate signaling pathways in other cells, as is observed with cytokines. Collectively, these events have the potential to dramatically alter the extracellular environment and affect cell behavior at adjacent and distant sites. Despite the widespread occurrence of protein shedding, however, little is known about the molecular events that regulate the process. The development of molecules that can either inhibit or promote the release of cell-surface proteins provides new opportunities for initiating or preventing specific extracellular recognition and signaling events. Such agents can be used to illuminate the events leading to shedding in addition to serving as new therapeutic leads.

Soluble forms of many receptor proteins are present in circulation in normal, healthy individuals but, in many diseases, concentrations of these receptors are altered, indicating that the ability to manipulate receptor shedding may have significant therapeutic potential [56,79]. For example, increased concentrations of TNF-α may contribute to the pathogenesis of several diseases, such as rheumatoid arthritis, insulin-dependent diabetes mellitus, HIV and sepsis. Strategies to prevent the cell surface release of TNF-α, including the use of several metalloproteinase inhibitors, are under development [80]. As many of the agents that block the activation-induced release of TNF-α also inhibit the shedding of other proteins, in addition to leading to new anti-inflammatory treatments, these studies will give an insight into the physiological roles of shedding and its importance. Blocking the release of other proteins may uncover additional therapeutic applications.

Protein activity can potentially be controlled by initiating the shedding of particular proteins but this method is largely unexplored. Selective agents that induce the proteolytic release of a specific receptor can protect cells from the actions of a deleterious ligand. In addition, the soluble form of the receptor may act to sequester the damaging ligand. Moreover, selective activation of a protease could have significant therapeutic advantages. For example, the β amyloid (Aβ) peptide (the major protein component of the amyloid plaques found in Alzheimer's patients) is generated by proteolytic processing of the β-amyloid precursor protein (βAPP). Interestingly, a protease, known as α-secretase, cleaves the βAPP within the Aβ sequence, precluding the formation of Aβ and its subsequent aggregation that can lead to plaque formation [81,82]. Increased α-secretase cleavage could prevent the accumulation of Aβ, which may be beneficial in Alzheimer's disease.

An important factor in designing molecules that promote shedding is that, because an endogenous enzyme promotes the cleavage, substoichiometric amounts of the shedding agent could be used. Not only does the proteolytic shedding of proteins therefore have the potential to occur catalytically, but, unlike noncovalent binding, it is also irreversible. These are inherent advantages that are absent from other strategies. The discovery that compounds based on glycoproteins selectively promote the shedding of L-selectin demonstrates that synthetic ligands can be used to modulate cell-surface display through proteolytic release. This result suggests new directions for controlling both the display of cell-surface proteins and the concentration of soluble proteins in circulation.

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