Regulated intramembrane proteolysis: from the endoplasmic reticulum to the nucleus

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Abstract

Regulated intramembrane proteolysis (Rip) is an ancient and widespread process by which cells transmit information from one compartment (the endoplasmic reticulum) to another (the nucleus). Two separate cleavages that are carried out by two separate proteases are required for Rip. The first protease cleaves its protein substrate within an extracytoplasmic domain; the second cleaves it within a membrane-spanning domain, releasing a functionally active fragment of the target protein. In eukaryotes, examples of Rip can be divided into two classes, according to the proteases that are involved and the orientation of the substrates with the membrane. Class 1 Rip involves type 1 transmembrane proteins and requires presenilin for cleavage within a membrane-spanning domain. In Class 2 Rip, the highly hydrophobic metalloprotease, site-2 protease, is required for cleavage within a membrane-spanning domain and substrates are type 2 transmembrane proteins. Both classes of Rip are implicated in diseases that are important in modern societies, such as hyperlipidaemias (via the sterol

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regulatory element binding protein pathway) and Alzheimer’s disease (via processing of the amyloid precursor protein.)

**Introduction**

In an ever-changing world, survival depends on the ability to assess quickly and respond appropriately to changes in the environment. At the cellular level, an appropriate response often includes altering the complement of cellular proteins, as new protein functions are required while old ones become unnecessary. Synthesis of macromolecules is a time-consuming process that requires minutes or hours to go from inactive gene to transcript to functional protein. One strategy employed to ensure a cell’s rapid response to environmental cues is the availability of previously synthesized transcriptional activators that are present outside the nucleus in an inactive form. Upon receipt of a specific signal, these pre-existing proteins are then activated and translocate to the nucleus where they direct the increased transcription of target genes. Many variations on this strategy have been identified and the activating events can include phosphorylation as in the case of the signal transducers and activators of transcription (STAT) factors. Signalling via STATs employs a mechanism whereby the latent, cytoplasmic transcription factors are phosphorylated by Janus family tyrosine kinases. Phosphorylation promotes dimerization of STATs and nuclear translocation, whereupon they modulate the expression of target genes [1]. In a different approach to keeping a pool of pre-existing factors out of the nucleus until needed, nuclear factor κB (NFκB)/Rel transcription factors are kept latent in the cytoplasm by interaction with the protein inhibitory κBα (IkBα). Receipt of an appropriate external signal causes IkBα to become phosphorylated, ubiquitinated, and then degraded by the proteasome (see Chapter 5). The free NFκB then translocates to the nucleus to activate its gene targets [2].

Recent investigations into several different systems have identified an unexpected role for proteolysis in the direct activation of pools of latent transcriptional activators that reside within the membrane compartment of the cell. These precursor proteins undergo proteolytic processing by two distinct enzymes in response to an intracellular signal. Different pairs of proteases are utilized to process different transcription factor precursors. The activity of the first enzyme in each pair is directly regulated, while the activity of the second enzyme is regulated indirectly by substrate availability dependent on the first, regulated cleavage. The second enzymes are particularly interesting because they cleave substrates within domains that are believed normally to lie within the plane of the membrane. In light of these observations, this phenomenon has been termed regulated intramembrane proteolysis (Rip) [3].
New appreciation for an old strategy

Rip arose early in evolution, with examples being found in organisms from prokaryotes to primates. Processes regulated by Rip range from control of metabolism to differentiation and development. In the bacterium *Bacillus subtilis*, Rip controls the differentiation of the endospore, a dormant form of the organism formed in response to nutrient deprivation. During this process, Rip-mediated release of the membrane-bound, transcriptional regulatory protein pro-α is necessary to complete the programme of sporulation [4]. In another prokaryote, *Enterococcus*, Rip is required to produce a pheromone that is needed to initiate aggregation behaviour [3]. The bacterial proteases involved in the generation of active transcription factors and signalling molecules are encoded by the genes SpoIVFB in *Bacillus* and eep in *Enterococcus* and are related in sequence to mammalian enzymes that catalyse the second cleavage event of Rip.

In eukaryotes, examples of Rip fall into two distinct classes (Table 1). Class 1 Rip involves type-1 transmembrane proteins (N-terminus extracytoplasmic, C-terminus cytoplasmic) whose cleavage is dependent upon γ-secretase activity. This activity requires two or more proteins acting either as the γ-secretase or as cofactors. Genetic and biochemical evidence indicates that γ-secretase activity involves the presenilin polytopic membrane proteins [5] and the transmembrane glycoprotein nicastrin [6]. Proteolysis appears to take place at or near the plasma membrane. Proteins known to undergo Class 1 Rip include the developmental signalling molecule Notch [7], the human epidermal growth factor receptor-like protein ErbB-4 [8], and the amyloid precursor protein (APP) (the proteolysis of APP is discussed in more detail in Chapter 4).

Intramembranous cleavage of APP came to attention owing to the fact that one of the products of this cleavage, the amyloid-β peptide, forms the amyloid plaques that are believed to cause neuronal death in Alzheimer’s disease [9]. Recent investigation into the function of the cytoplasmic domain of APP supports the notion that Rip is a mechanism for controlling gene expression. This domain of APP forms a multimeric complex composed of the nuclear adaptor protein Fe65 and the histone acetyltransferase Tip60 [10]. This complex in turn stimulates mRNA synthesis via interaction with transcription factors that have DNA-binding domains. While the latter proteins have not yet been identified, this result indicates that proteolytic release of the cytoplasmic domain of APP by Rip may regulate gene expression [10].

Recent results indicate that some instances of intramembranous cleavage of type-1 transmembrane proteins do not require γ-secretase and do not release a transcription factor domain to the cytosol [11,12]. In *Drosophila*, soluble Spitz (a protein homologous with vertebrate transforming growth factor α) is a ligand for the *Drosophila* epidermal growth factor receptor. Spitz is synthesized as a type-1 transmembrane protein with the ligand portion facing the lumen of the endoplasmic reticulum (ER). Genetic evidence indicates that processing of Spitz depends
Table 1. Eukaryotic proteins that undergo Rip

*For APP and ErbB-4, the *in vivo* targets of transcriptional activation are unknown, although a role for ErbB-4 in growth and differentiation has been suggested. †It is not known whether nicastrin is required for cleavage of ErbB-4 or which of the presenilins may be normally involved. For further discussion of these proteins and for a brief description of a potentially new class of intramembrane proteolysis involving the Star–Spitz–Rhomboid-1 pathway in *Drosophila*, see text.

<table>
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| Class 2         |                       |                                             |                              |            |
| SREBP-1 and -2  | Type 2                | S2P                                         | Transcriptional regulation   | [3,13]     |
|                 |                       |                                             | (lipid metabolism)           |            |
| ATF6α and ATF6β | Type 2                | S2P                                         | Transcriptional regulation   | [3,26]     |
|                 |                       |                                             | (ER stress response)         |            |
on both Star and Rhomboid-1 proteins. Lee et al. [11] demonstrated that the role of Star in Spitz processing is to escort newly-synthesized Spitz from the ER to the Golgi. At the Golgi, Rhomboid-1 is required for the proteolytic release of soluble Spitz into the lumen of the ER and its subsequent secretory pathway. Urban, Lee and Freeman [12] provide evidence that Spitz is cleaved within its transmembrane domain and that Rhomboid-1 may be the prototype for a novel family of serine proteases involved in this intramembrane proteolysis. The Star–Spitz–Rhomboid-1 pathway therefore points to expanded roles for intramembrane proteolysis in eukaryotes, beyond the direct control of transcription.

Proteins involved in Class 2 Rip in eukaryotes are type-2 transmembrane proteins (N-terminus cytoplasmic, C-terminus extracytoplasmic) (Figure 1a). The best understood example of Class 2 Rip is the membrane-bound transcription factor sterol regulatory element binding protein (SREBP). The first cleavage of SREBP produces a type-2 transmembrane protein that is the substrate for the second protease. Recently, proteolytic release of another type-2 mem-

![Figure 1. Proteins processed by Class 2 Rip](image)

(a) Schematic diagram of the orientation of SREBP and ATF6 within the membrane of the ER. The sites of cleavage and the position of the basic helix-loop-helix leucine zipper (bHLH-zip) transcription factor domains are indicated. (b) Sequence comparison of the membrane-spanning and luminal domains of SREBP and ATF6. The positions of site 1 and site 2 cleavage are indicated. The membrane-spanning domain is indicated by grey shading. Residues that are critical for site 1 or site 2 cleavage of ATF6 [26] and SREBP-2 [27] are indicated by blue shading. Identical residues in SREBP-1 and ATF6β are also indicated. The GenBank accession numbers for the human protein sequences shown are: SREBP-1, P36956; SREBP-2, A54962; ATF6α, AAB64434; ATF6β, NP_004372.

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brane-spanning transcription factor, activating transcription factor 6 (ATF6) (see below), has been shown to require the same proteases that process SREBP. These examples of eukaryotic Class 2 Rip are involved in transmitting signals from the ER to the nucleus. This essay focuses on the regulation of Class 2 Rip and the mechanisms by which control is accomplished.

### Class 2 Rip: the processing machinery

At present, three different loci are known to comprise the processing machinery required for Class 2 Rip in mammalian cells: SREBP cleavage activating protein (SCAP; Figure 2a), site-1 protease (S1P; Figure 2b) and site-2 protease (S2P; Figure 2c). The genes that encode these proteins were isolated by expression and complementation cloning using mammalian cells with defects in the feedback regulation of cholesterol biosynthesis owing to disrupted processing of SREBPs [13]. S1P and S2P were the first Rip proteases whose encoding cDNAs were cloned.

S1P (also known as SKI-1; subtilisin/kexin-like isoenzyme [14]) is a subtilisin-like, membrane-bound serine protease that is located in the ER and Golgi. It is a type 1 membrane protein, with the bulk of the protein, including the active site, in the lumen and only a short cytoplasmic C-terminal tail (Figure 2b). S1P contains a classic serine protease catalytic triad (His\(^{218}\), Asp\(^{249}\) and Ser\(^{414}\)) and, like other subtilases, it is synthesized as an inactive proenzyme that undergoes autocatalytic processing prior to activation [15]. Active S1P resides in the Golgi apparatus. S1P/SKI-1 is crucial for Rip involving ATF6 and SREBP as substrates. The protein may also play other roles in cellular physiology. It has been shown to cleave a number of other substrates and may be active when secreted into the medium (see Chapter 7).

S2P contains an HEXXH zinc-binding motif that is characteristic of several families of metalloproteases, although its sequence is otherwise unlike any previously described enzyme. The residues of the putative zinc-binding site are essential for S2P function; substitutions in the HEXXH signature sequence abolish its ability to restore SREBP cleavage in mutant cells that lack S2P [16]. The glutamate residue within this motif is thought to act as a nucleophile in peptide bond cleavage. In contrast with many other metalloproteases, the substitution of the glutamate residue with aspartate reduces, but does not eliminate, S2P activity. In a distant S2P homologue, the SpoIVFB protease of *Bacillus*, the analogous substitution also permits significant activity. These results emphasize one of several differences between S2P-like enzymes and other zinc-binding metalloproteases and render the proteins sufficiently distinct to be included as a separate subfamily (M50, [17]).

S2P is a hydrophobic protein with several putative membranous domains that are separated by distinct hydrophilic domains and are accessible to the glycosylation machinery of the ER and Golgi [18]. The hydrophobic regions
that separate the hydrophilic domains do not span the membrane but appear to be embedded within it (Figure 2c).

The third component of the processing machinery of Class 2 Rip is the large polytopic membrane protein SCAP, which is necessary for cleavage of SREBP [13]. Six of the eight N-terminal, membrane-spanning helices of SCAP comprise a sterol-sensing domain (Figure 2a). This designation rests on two
lines of evidence. (1) Similar domains are found in a region of 3-hydroxy-3-methyl glutaryl-CoA reductase that controls sterol-regulated degradation of the protein. Related domains are also found in the gene that is defective in Niemann–Pick Type-C disease, a cholesterol storage disorder, and in the Patched and dispatched gene products of the hedgehog signalling pathway. The hedgehog protein is modified covalently with a cholesterol moiety [19]. (2) Four independently isolated mutations that reduce or abolish sterol regulation of SCAP function occur at two residues within the sterol-sensing domain [13].

The C-terminal portion of SCAP is located in the cytoplasm and consists of five copies of a motif designated WD repeats (owing to the presence of tryptophan and aspartate residues) that mediate protein–protein interactions [20]. In order for the first (site 1) cleavage event to occur within SREBP, SCAP and SREBP must form a complex via their C-termini. Formation of this complex is not regulated by sterols; however, the budding of the heterodimer into vesicles is [21]. The role of SCAP in Class 2 Rip appears to be limited to cleavage of SREBPs, as the molecule does not appear to be required for cleavage of ATF6.

**Rip and the control of lipid biosynthesis: SREBPs**

There are three distinct SREBP proteins, the products of two separate genes, that play differential but overlapping roles in the transcriptional regulation of lipid metabolism. SREBP-1a and SREBP-1c mainly activate the transcription of genes that are required for fatty acid synthesis. SREBP-2 principally activates the transcription of genes that are required for cholesterol biosynthesis and uptake [22]. The SREBPs are synthesized as large precursors that are inserted into the membrane of the ER in a hairpin fashion, with both the N-terminal transcription factor domain and the C-terminal regulatory domain located within the cytoplasm. Two membrane-spanning domains, which are separated by a short luminal loop, anchor the precursor to the membrane and produce the hairpin orientation (Figure 1a).

SREBPs are cleaved in response to cellular demand for sterols, and the sterol-sensing domain of SCAP is central to this feedback regulation. When this domain of SCAP is overexpressed, sterols no longer regulate cleavage of SREBPs, the active form of which is then produced constitutively [23]. The sterol-sensing domain is thought to compete with full-length SCAP for interaction with an unidentified protein that normally retains it within the ER in the presence of sterols. When this interaction is blocked by a truncated sterol-sensing domain, the full-length SCAP–SREBP complex is free to travel from the ER to a post-ER/Golgi compartment in an unregulated fashion. This results in constitutive cleavage of SREBPs.

Once the SREBP–SCAP complex reaches the Golgi, active S1P cleaves SREBP within its luminal loop, releasing the N-terminal transcription factor domain from the C-terminal SCAP-binding domain. Both halves of the protein remain bound to the membrane owing to the presence of one membrane-span-
ning domain in each. This domain in the N-terminal half is the site of the second cleavage by S2P, which releases the active transcription factor from the membrane, thereby enabling movement to the nucleus and gene activation. The overlapping transcriptional specificity of SREBP-1 and SREBP-2, coupled with the processing of both proteins via Rip, allows animal cells to co-ordinate the biosynthesis of the principal membrane components, cholesterol and fatty acids.

**Rip and the unfolded protein response: ATF6**

ATF6 (also known as ATF6α, [24]) is one of two closely related type-2 transmembrane proteins that are involved in the transcriptional response to ER stress (also known as the unfolded protein response). The other protein is ATF6β (formerly G13, [24]). Like the SREBPs, the cytoplasmic, N-terminal domains of ATF6α and ATF6β encode transcription factors of the basic helix-loop-helix leucine zipper family (Figure 1b). When unfolded proteins accumulate in the ER, transcription of genes encoding folding enzymes and chaperone proteins is upregulated owing to ATF6 binding to ER stress response elements in their promoters. ATF6 is also cleaved proteolytically to free the active factor from the membrane and allow movement to the nucleus [25]. Like SREBP, two separate cleavages are required for this release: the first, luminal cleavage largely depends on S1P, while the second cleavage requires S2P [26].

**Class 2 Rip: the substrates**

The amino acid sequences recognized and cleaved by S1P at site 1 were first identified for SREBP-2 [13]. S1P cleaves the Leu522–Ser523 bond in the sequence RSVL↓S (Figure 1b). Arg519 and Leu522 are both important for recognition by S1P. When Arg519 is replaced with alanine (Arg519→Ala), cleavage at site 1 is abolished. Even the conservative substitution Arg519→Lys greatly reduces the efficiency of cleavage. Similarly, the requirement for Leu522 is very stringent; substitution of this residue by valine completely abolishes cleavage [27]. These same requirements are observed for cleavage at the Leu419–Gly420 bond in the sequence RHLL↓G in the luminal domain of ATF6α. The mutations Arg416→Ala or Leu419→Val abolish site 1 cleavage of ATF6α by S1P [26]. It is likely that ATF6β is also a substrate for S1P as it shares an identical luminal sequence (R437HLLG) with ATF6α (Figure 1b).

In mammalian cells that lack S1P, processing of SREBPs cannot occur. As a result, these cells cannot upregulate transcription of the genes of cholesterol and fatty acid synthesis and are therefore auxotrophic for these lipids. Processing of ATF6α is also greatly reduced in these cells. Interestingly, when fed free cholesterol and unsaturated fatty acid, the mutant cells grow at a rate that is indistinguishable from that of their wild-type counterparts. Similarly, in cholesterol auxotrophic cells that lack S2P, no ER-stress-related growth or
survival phenotype is observed under standard culture conditions, even though processing of ATF6α is completely abolished [26].

Studies of wild-type ATF6α in mutant cells that lack S2P confirm the involvement of this protease in the ER stress response. Both ATF6 cleavage and induced transcription of the ATF6 target gene, which encodes Bip (Grp78; glucose-regulated protein of 78 kDa), are profoundly deficient in cholesterol auxotrophic mutant cells lacking S2P [26]. These results are complemented by experiments with point mutants of ATF6α in wild-type cells. In order for a type 2 transmembrane protein to serve as a substrate for S2P, the substrate protein must have a luminal domain of no more than 25 amino acids and an asparagine/proline motif within its adjacent membrane-spanning helix [28]. Both SREBP and ATF6 are cleaved by S1P at a site C-terminal of the membrane-spanning domain (by 20–23 amino acids) (Figure 1b). The second requirement for asparagine or proline within the membrane-spanning domain is also observed in both SREBP and ATF6. When either the asparagine or proline residues in the first membrane-spanning domain of SREBP-2 are mutated to phenylalanine or leucine respectively, site 2 cleavage still occurs [28]. Simultaneous substitution of both residues, however, eliminates cleavage at site 2 and leads to the accumulation of the membrane-bound intermediate formed by site 1 cleavage [28]. This result holds for an asparagine/proline motif in the membrane-spanning domain of ATF6α as well (N391YGp; Figure 1). Single substitutions of either Asn391→Phe or Pro394→Leu do not abolish cleavage of ATF6α by S2P. By contrast, the double substitution (Asn391→Phe and Pro394→Leu) completely blocks production of the nuclear form of ATF6α and leads to increased accumulation of a membrane-bound intermediate form [26]. Again, it seems likely that ATF6β shares these same requirements for processing, as the protein contains an N410FGP motif in a membrane-spanning domain (Figure 1b).

The asparagine/proline motif is common at the ends of α-helices in globular proteins where it is thought to cap the N-termini [29], but it is rarely found in membrane-spanning domains. In a hydrophobic, membrane-spanning helix, the asparagine/proline motif may facilitate a transition from the usual α-helical conformation to a more extended conformation, which may be more susceptible to proteolytic attack by S2P than an α-helix would be [28]. Unfolding of the membrane-spanning α-helix in this scenario need not be an abrupt transition from a hydrophobic environment within the membrane to a hydrophilic one in the cytoplasm. Considering the unusually hydrophobic nature of S2P, some of its membranous sequences could conceivably stabilize the substrate in an extended conformation prior to proteolysis.

In contrast with cells that lack S1P or S2P, processing of ATF6α is normal in mutant cells that lack SCAP [26]. This conclusion is further supported by the fact that levels of cellular sterol that strongly induce or suppress cleavage of SREBPs have no effect on the processing of ATF6. Reciprocally, treatments that induce ER stress, and therefore ATF6 processing, have little effect on
SREBP cleavage. Since active S1P, and presumably S2P, resides in a post-ER/Golgi compartment, it is likely that ATF6 must also exit the ER in order to be processed. It is conceivable that an unidentified factor performs a function analogous to that of SCAP as an ER to Golgi escort for ATF6.

In both known examples of Class 2 Rip, a central feature of the process is the initial ER localization of the membrane-bound transcription factor. Release of the cytoplasmic domain depends on events in the ER that initiate transport of the substrate from the ER to another compartment where it can be cleaved. Thus, the S1P and S2P proteolytic machinery has been put into the service of communication between the ER (where sterols are synthesized and many proteins are folded) and the nucleus. By means of this seemingly roundabout mechanism, events in the ER result in alteration of a cell’s transcriptional programme.

Communication between the ER and nucleus without Rip: yeast

All prokaryotic and metazoan genomes that have been sequenced to date encode homologues of S2P. The chromosomes of higher plants also contain clear orthologues of both S1P and S2P, as revealed by database searches (R.B. Rawson, unpublished work). It thus seems reasonable to speculate that Rip may occur in this important group of eukaryotes as well, although no examples are yet reported. Given the widespread occurrence of S1P- and S2P-like genes, it is surprising that no examples of Rip (and no S2P homologues) are found in fungi.

An alternative strategy for the processing of membrane-spanning transcription factors occurs in fungi. This regulatory pathway is termed regulated ubiquitin/proteasome-dependent processing (RUP; [30].) In the yeast Saccharomyces cerevisiae, the membrane-bound transcription factors SPT23 and MGA2 (which are NFκB homologues) are processed in a ubiquitin-dependent manner to release a transcriptionally active fragment to the nucleus. Intriguingly, SPT23 and MGA2 are also necessary for the production of unsaturated fatty acids in yeast [30]. Having membrane-spanning transcription factors controlling aspects of lipid metabolism in response to specific proteolysis of precursors makes RUP logically similar to Rip; however, the two processes differ in their mechanisms. RUP depends on component proteases of the proteasome rather than a pair of membrane-bound proteases. Perhaps yeast, having no S2P-like enzymes, employ a different mechanism for transmitting information about the lipid status of the cell from the ER to the nucleus.

Rip in disease

Examples of both Class 1 and Class 2 Rip play or may play central roles in diseases that afflict large numbers of people in Western societies. For example, misregulation of SREBP cleavage and the consequent overproduction of lipids contribute to hyperlipidaemias and, therefore, to vascular disease, strokes and heart attacks. Such misregulation may also contribute to fatty liver pathologies.
On the other hand, the unfolded protein response can be triggered by many different disease states, including viral infection, in which the synthesis of viral proteins overwhelms the ER folding machinery. Disruptions in the ER stress response may thus be involved in the pathology of such infections.

For Class 1 Rip, the most dramatic disease known is Alzheimer’s. It is likely that this debilitating neurodegenerative condition results from the deposition of amyloid plaques, which are composed of a direct product of Rip [3]. Some of the mutations that are responsible for inherited forms of early onset Alzheimer’s disease are point mutations within APP itself. The majority, however, are mutations in the presenilins that are required for intramembranous cleavage of substrates in Class 1 Rip. All of these mutations result in increased production of the amyloidogenic amyloid β₁-₄₂ fragment [31] (see Chapter 4). This biochemical phenotype indicates the crucial role of Rip in a very common disease. More broadly, the role of the Notch signalling pathway during development suggests the plausible hypothesis that perturbations in Class 1 Rip could be a cause of certain birth defects.

**Conclusions**

Appreciation of Rip is recent, many details of the phenomenon are incompletely understood and some intriguing questions remain unanswered. For example, how did such an involved system come into being? Why are two cleavages necessary when it would appear that one would suffice? What other proteins are involved? Because of a proven or potential involvement in common diseases, it is clear that understanding more about the mechanisms of Rip will contribute to our knowledge of both cellular signalling and pathological states.

**Summary**

- **Rip is an ancient and widespread process for controlling gene expression.**
- **Two separate cleavages by two separate proteases are required, one in the extracytoplasmic space and one within a membrane-spanning domain.**
- **In eukaryotes, Rip falls into two classes that are defined by the enzymes involved and the nature of their substrates**
- **Class 1 Rip depends on a complex containing presenilin for intramembranous cleavage. Class 2 Rip depends on an integral membrane metalloprotease, S2P.**
• Both classes of Rip play crucial roles in diseases common in modern societies such as hyperlipidaemias and atherosclerosis (via the SREBP pathway) and Alzheimer’s disease (via APP processing.)

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References


