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# Regulated Intramembrane Proteolysis: A Control Mechanism Conserved from Bacteria to Humans

Review

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Explosive advances in biological knowledge are often triggered by episodes of conceptual convergence—moments when scientists working on different experimental systems realize suddenly that they are studying the same mechanism. Knowledge explodes because lessons from one system quickly ignite sparks in the other systems. Notable examples include the discovery of introns in viruses and animal cells; the appreciation of GTP-binding proteins as central to vision, hormone action, cancer, and yeast mating; and the recognition of receptor-mediated endocytosis as essential for cellular nutrition, viral entry, and degradation of protein hormones.

In the field of cell signaling, the new millennium is witnessing a new conceptual convergence: the realization that transmembrane proteins can be cleaved within the plane of the membrane to liberate cytosolic fragments that enter the nucleus to control gene transcription. This mechanism, called regulated intramembrane proteolysis (Rip), influences processes as diverse as cellular differentiation, lipid metabolism, and the response to unfolded proteins. In addition to its presence in animal cells, Rip has been observed in bacteria, and, remarkably, the bacterial proteases are related evolutionarily to the ones used in animal cells. The past 3 months have witnessed no fewer than 12 papers describing Rip in cells from bacteria to humans (An et al., 1999; DeBose-Boyd et al., 1999; Haze et al., 1999; Katayama et al., 1999; Niwa et al., 1999; Ray et al., 1999; Rudner et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Brou et al., 2000; Mumm et al., 2000).

Figure 1 lists five proteins that are known or postulated to undergo Rip in animal cells. In some of these examples, the information is still fragmentary, and in no case do we have a complete picture of the processing events. Yet, the parallels evident to date allow certain generalizations to be drawn. The most recently recognized examples of Rip are Ire1 (Niwa et al., 1999) and ATF6 (Haze et al., 1999), two transmembrane proteins in the endoplasmic reticulum (ER) that release their cytosolic domains in response to the presence of unfolded secretory proteins in the ER. Another example is Notch, a plasma membrane receptor whose cytosolic domain is released in response to Delta, a protein that dictates cell fate decisions throughout development (Annaert and De Strooper, 1999; Chan and Jan, 1999). The list also includes the SREBPs (sterol regulatory element-binding proteins), transmembrane proteins of the ER whose cytosolic transcription factor domains are liberated when cells are deprived of sterols, thereby activating genes controlling lipid synthesis and uptake (Brown and Goldstein, 1997). A final example is the amyloid precursor protein (APP), a transmembrane protein of unknown function that is cleaved within the membrane to produce the extracellular amyloid β peptide that is suspected to cause Alzheimer's disease (Selkoe, 1996; Annaert and De Strooper, 1999). Although the fate of the liberated APP cytosolic domain is not known, the mechanism for intramembrane processing of the APP precursor is so similar to that of the other proteins that it warrants inclusion in this list.

### Common Features of Rip

The eukaryotic proteins of Figure 1 share several characteristics. They all span the membrane bilayer at least once, but they do so in different directions. Three of the proteins (Ire1, Notch, and APP) are type I membrane-spanning proteins oriented with their $NH_2$ termini in the lumen and their COOH termini in the cytosol. One protein, ATF6, is a type 2 membrane protein with its $NH_2$ terminus in the cytosol. The fifth protein, SREBP, contains two membrane-spanning regions that are inserted in the bilayer in a helical hairpin fashion. SREBP is first cleaved within the lumen, separating its two transmembrane helices. The $NH_2$-terminal fragment becomes a type 2 membrane protein, which undergoes intramembrane cleavage to release the $NH_2$-terminal piece into the cytosol.

For three of the proteins (SREBP, Notch, and APP), the cleaved peptide bonds have been identified, and a uniform pattern is observed (Annaert and De Strooper, 1999; Brown and Goldstein, 1999; Chan and Jan, 1999; Brou et al., 2000; Mumm et al., 2000). In each case, the intramembrane cleavage does not take place until the bulk of the protein on the extracytosolic (luminal or extracellular) face has been removed by a primary cleavage. This primary cleavage can occur in the lumen of the ER, in a post-ER compartment, or at the cell surface. Although the cleaved bond differs in the three cases, the net effect is the same: the cleavage shortens the extracytosolic segment to less than 30 amino acids, which is a prerequisite for the secondary intramembrane cleavage. For Notch and the SREBPs, the intramembrane cleavage occurs near the cytosolic end of the membrane-spanning segment, generating a cytosolic fragment that enters the nucleus with three or four amino acids from the membrane-spanning helix still attached. In contrast, APP is cleaved near the center of the membrane-spanning segment. APP came to attention because the intramembrane cleavage generates a luminal fragment that assumes a β-pleated sheet configuration, causing it to aggregate and form indigestible amyloid fibrils (Selkoe, 1996). The function of the cytosolic fragment is unknown. For Ire1 and ATF6, the cleaved bonds have not been identified, but parallels with the aforementioned proteins suggest that they, too, may undergo

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| Membrane Protein | Orientation in Bilayer | Protein Required for Rip | Cleavage Organelle | Regulator of Cleavage | Sites of Cleavage | | Nuclear Gene Targets |
|---|---|---|---|---|---|---|---|
| | | | | | Primary | Secondary | |
| | | | | | Extracytosolic | Membrane | Cytosolic |
| SREBP | Helical Hairpin → Type 2 | S2P | *cis*-Golgi | Sterols | 20aa | | Lipid Metabolism |
| APP | Type 1 | Presenilin-1 | Golgi/Endosomes | ? | 28aa | | ? |
| Notch | Type 1 | Presenilin-1 | Golgi/Plasma Membrane | Delta | 12aa | | Differentiation |
| Ire1 | Type 1 | Presenilin-1 | ? ER | Unfolded Proteins | ? | ? | ER Chaperones |
| ATF6 | Type 2 | ? | ? ER | Unfolded Proteins | ? | ? | ER Chaperones |

Figure 1. Membrane Proteins that Are Postulated to Undergo Regulated Intramembrane Proteolysis in Animal Cells

For simplicity, we do not show the alternate pathway for APP processing, which involves cleavage by $\alpha$-secretase. For Notch, we show only the transmembrane subunit. For Ire1 and ATF6, intramembrane proteolysis has not yet been demonstrated, but its existence seems likely (see text). aa, amino acids.

sequential cleavages, first in the lumen and then within the membrane.

In four of the eukaryotic Rip systems, a protein has been identified that is required for intramembrane cleavage, and a remarkable pattern has been observed (Figure 1). In the case of SREBP, a type 2 membrane protein, intramembrane cleavage requires Site-2 protease (S2P), a polytopic membrane protein with the characteristics of a membrane-embedded zinc metalloprotease (Rawson et al., 1997; Zelenski et al., 1999). Proteins that resemble S2P have also been implicated in intramembrane cleavage of type 2 transmembrane proteins in bacteria (discussed below). In contrast, all three proteins of the type 1 class require presenilin-1, a polytopic membrane protein that is postulated to be an aspartyl protease (Wolfe et al., 1999). The role of presenilin-1 in Rip was first identified in the APP system. Presenilin-1 has been shown recently to be required for Notch signaling in animal cells (De Strooper et al., 1999) as well as in *Drosophila* (Struhl and Greenwald, 1999; Ye et al., 1999). In mammalian cells, presenilin-1 is also required for cleavage of Ire1 (Niwa et al., 1999). These data, although limited, suggest a general principle: S2P-like proteins are required for intramembrane cleavage of type 2 proteins and presenilin-1 is required for type 1 proteins (Figure 1). The validity of this generalization will be tested as further examples of Rip are discovered.

### The SREBP Pathway: Control by Rip

The SREBP pathway was the first system of Rip for which the luminal and intramembrane proteases were identified. As shown in Figure 1, SREBP is inserted in ER membranes in a helical hairpin fashion (Hua et al., 1995). The cytosolic $NH_2$-terminal domain is a transcription factor of the basic-helix-loop-helix-leucine zipper family. The COOH-terminal domain, also cytosolic, forms a complex with SREBP cleavage-activating protein (SCAP), a transmembrane protein with eight membrane-spanning regions (Nohturfft et al., 1998; Sakai et al., 1998a). The first (luminal) cleavage of SREBP is catalyzed by Site-1 protease (S1P), a membrane-bound serine protease of the subtilisin family that is oriented with its active site in the lumen (Sakai et al., 1998b). S1P is synthesized in the ER as an inactive precursor that is activated autocatalytically by the removal of an $NH_2$-terminal propeptide (Espenshade et al., 1999). The activated S1P is located in a post-ER compartment that has the property of the *cis* or medial Golgi complex. The substrate, SREBP, is transported to this organelle by SCAP (DeBose-Boyd et al., 1999; Nohturfft et al., 1999). In mutant cells that lack SCAP, there is no transport of SREBP, and cleavage does not occur (Rawson et al., 1999; DeBose-Boyd et al., 1999). The membrane-embedded portion of SCAP contains a sterol-sensing domain that regulates this transport (Brown and Goldstein, 1999). When cells are deprived of sterols, the SCAP/SREBP complex moves to the cleavage compartment. When cells are overloaded with sterols, the SCAP/SREBP complex is trapped in the ER and cleavage cannot occur (DeBose-Boyd et al., 1999; Nohturfft et al., 1999). Inasmuch as SREBPs activate genes encoding enzymes of lipid synthesis, this sterol-mediated block in transport allows cholesterol to inhibit its own synthesis in a classic feedback fashion (Brown and Goldstein, 1997).

Cleavage by S1P separates the two transmembrane helices of SREBP, but the $NH_2$-terminal fragment remains membrane bound until it is cleaved by S2P at a site that is three residues within the transmembrane segment (Duncan et al., 1998). Even though the activity of S2P is not regulated directly by sterols, S2P cannot act until the two transmembrane segments have been separated by S1P, which effectively brings this reaction under cholesterol control.

The human gene encoding S2P was isolated by complementation cloning in mutant CHO cells that were auxotrophic for cholesterol, owing to a deletion in the *S2P* gene (Rawson et al., 1997). Similar genes were identified in published DNA sequences from *Drosophila*, *C. elegans*, and from the archean *Sulfolobus solfataricus* (Rawson et al., 1997). These findings were extended by Lewis and Thomas (1999), who found other apparent homologs in eubacteria, including *E. coli* and *B. subtilis*. The encoded proteins share a similar hydrophobicity

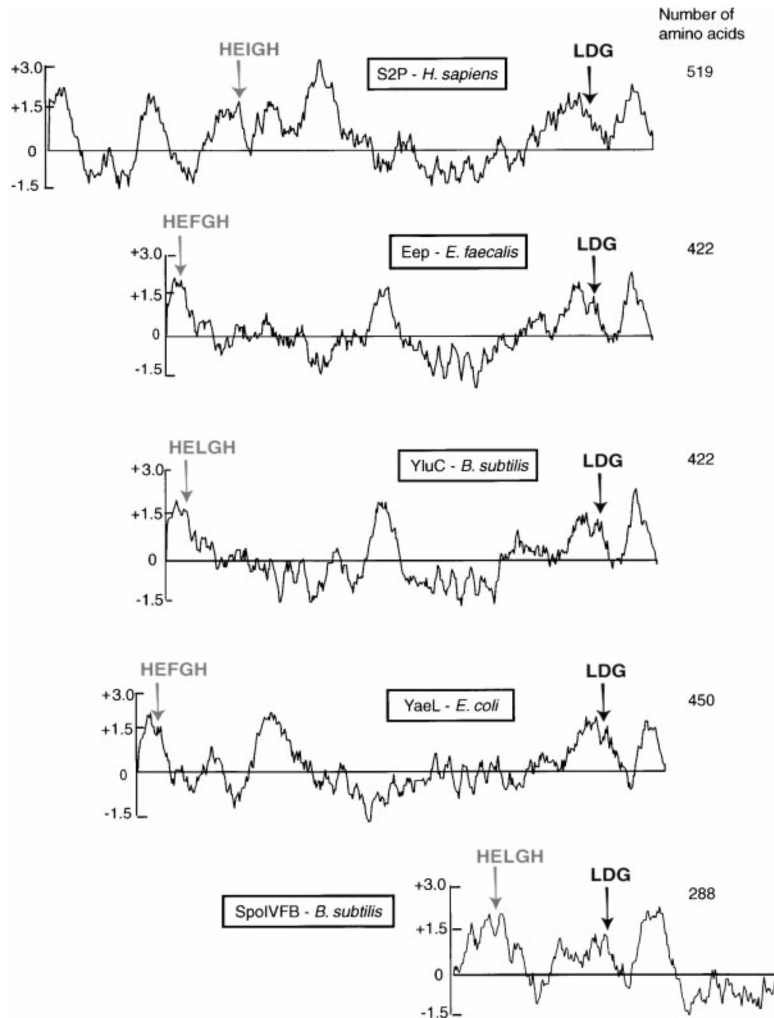


Figure 2. Hydropathy Plots of Human S2P and Its Bacterial Homologs

The residue-specific hydropathy index was calculated over a window of 20 amino acids by the method of Kyte and Doolittle (1982). The HExxH and LDG motifs (discussed in the text) are highlighted in red and blue, respectively. Each plot is drawn on the same scale and aligned vertically by the position of the LDG motif. GenBank Accession Numbers for the amino acid sequences are as follows: S2P, AAC51937; Eep, AAD47948; YluC, CAB13529; YaeL, AAC73287; and SpoIVFB, p26937.

profile that predicts a highly hydrophobic structure with multiple membrane-spanning regions (Figure 2). The $NH_2$-terminal portion of all of these proteins contains a sequence conforming to the HExxH consensus that is found in a large subfamily of zinc metalloproteases (where X is typically a noncharged amino acid) (Rawlings and Barrett, 1995). In these proteins, the two histidines of the HExxH motif coordinate with a zinc atom, and the glutamate activates a water molecule, allowing it to make a nucleophilic attack on the peptide bond. Unlike its location in the classic zinc metalloproteases (such as thermolysin) (Matthews, 1988), in S2P the HExxH sequence is embedded in a highly hydrophobic segment of the protein (Figure 2).

In addition to the HExxH motif, thermolysin and other classic zinc metalloproteases contain a remote residue (tyrosine or aspartate) that provides an additional coordination bond for the zinc. In the S2P-like proteins, this function is believed to be filled by the aspartate of the sequence LDG, which resides in a hydrophobic segment that shows a characteristic double peak in hydrophobicity plots and is located near the COOH terminus (Figure 2). When either of the histidines or the glutamate of the HExxH consensus or the aspartate of the LDG sequence is mutated, the S2P cDNA loses its ability to rescue SREBP cleavage in mutant animal cells that lack S2P (Rawson et al., 1997; Zelenski et al., 1999). These findings suggest strongly that S2P is a zinc metalloprotease. The hydrophobic nature of S2P is consistent with its postulated role in cleaving a peptide bond that is located within a membrane bilayer. It seems likely that this cleavage will require at least a partial unfolding of the $\alpha$ helix. It should be noted that thus far it has not been possible to show directly that isolated S2P or any of its bacterial relatives can cleave peptide bonds in vitro.

### The Unfolded Protein Response: Control by Rip?

Among the transmembrane proteins in Figure 1, the closest parallel to SREBP is ATF6, a type 2 membrane protein of the ER whose $NH_2$-terminal cytosolic domain is a transcription factor of the bZIP family (Yoshida et al., 1998). The $NH_2$-terminal domain of ATF6 was identified by virtue of its ability to bind to ER stress response elements in the promoter regions of the genes encoding BiP/GRP78, GRP94, and calreticulin (Yoshida et al., 1998). When mammalian cells produce unfolded ER proteins, ATF6 undergoes proteolysis, and the $NH_2$-terminal domain enters the nucleus where it activates transcription of *BiP/GRP78* and other genes whose products

assist the folding of ER proteins (Haze et al., 1999). This regulatory pathway, called the unfolded protein response (Gething and Sambrook, 1992; Chapman et al., 1998), helps cells to survive conditions of ER stress. It must be emphasized that the protease(s) that process ATF6 have not yet been identified, and it has not yet been shown formally that ATF6 is cleaved within the membrane-spanning helix.

Ire1, the other transcriptional mediator of the unfolded protein response in mammalian cells, also undergoes regulated proteolysis to release its cytosolic domain. Ire1p was first identified in the yeast *Saccharomyces cerevisiae* (Cox et al., 1993; Mori et al., 1993), and its mammalian orthologs, Ire1α and Ire1β, have recently been cloned (Tirasophon et al., 1998; Wang et al., 1998). Ire1p is a type 1 membrane protein of the ER. Its COOH-terminal cytosolic sequence contains two domains. One of these domains is a protein kinase that becomes activated and phosphorylates adjacent Ire1p molecules when unfolded proteins are present in the ER. Sidrauski and Walter (1997) made the remarkable discovery that the second domain in yeast Ire1p is an endonuclease that cleaves the mRNA encoding Hac1p, a bZIP transcription factor (Cox and Walter, 1996). In the uninduced state, the *HAC1* mRNA fails to be translated because it contains an unspliced intronic sequence that attenuates translation. Unfolded ER proteins activate the endonuclease function of Ire1p, which precisely cleaves *HAC1* mRNA at the borders of the intron sequence. The cleaved mRNA is religated by tRNA ligase, generating a functional *HAC1* mRNA. The resulting Hac1p activates transcription of genes encoding BiP/GRP78 and other proteins that assist the folding of ER proteins (Chapman et al., 1998).

In a recent paper dealing with mammalian cells, Niwa et al. (1999) showed that unfolded ER proteins activate the proteolytic cleavage of one isoform of Ire1, designated Ire1α. The liberated cytosolic domain of Ire1α enters the nucleus where it presumably encounters a mammalian version of the unspliced yeast *HAC1* mRNA. As with ATF6, the protease(s) that cleave Ire1α and the peptide bonds that are cleaved have not been identified.

Presenilin-1 appears to be required for the intramembrane cleavage of Ire1α, but its precise function remains to be elucidated. Niwa et al. (1999) found that unfolded proteins failed to induce Ire1α cleavage in mutant cells that lack presenilin-1. In contrast, Katayama et al. (1999) reported that the unfolded protein response is diminished in cells that express mutant forms of presenilin-1 that are superactive, at least with regard to the cleavage of APP. Whether this block in the unfolded protein response results from a failure of Ire1α cleavage is unknown.

The findings on the proteolysis of mammalian Ire1α are quite new, and many questions remain to be answered. First, proteolytic cleavage of Ire1p has not been demonstrated in yeast (Niwa et al., 1999), suggesting that cleavage may not be universal. Second, proteolytic processing of mammalian Ire1 was not observed in earlier studies, nor was Ire1 visualized in the nucleus by immunofluorescence (Tirasophon et al., 1998; Wang et al., 1998). These apparent discrepancies may be traced to the lability of the nuclear fragment. If the experiments with SREBPs are a guide, then the nuclear forms of Ire1 may be rapidly degraded. Indeed, visualization of the nuclear fragments of SREBPs is difficult unless the cells have been pretreated with proteasome inhibitors (Wang et al., 1994). Consistent with this hypothesis, Niwa et al. (1999) found it necessary to harvest mammalian cells with hot SDS solution in order to prevent degradation of the nuclear form of Ire1. Rapid degradation of the nuclear fragments may be a general feature of Rip. Inasmuch as the proteolytic process of Rip is irreversible, rapid degradation may allow the nuclear signals to be turned off rapidly once Rip is inhibited.

In addition to problems in detecting the nuclear fragments, the study of Rip is rendered difficult by the necessity to avoid experiments based on overexpression of transfected cDNAs. For example, overexpression of SREBPs leads to adventitious cleavages that obscure the regulated physiologic cleavage (Hua et al., 1995). This problem has been circumvented in transfection experiments by expressing SREBP under control of the weak HSV thymidine kinase promoter, which produces levels of protein that are approximately equivalent to normal endogenous levels (Hua et al., 1995).

A further unresolved issue relates to the second mammalian isoform of Ire1, designated Ire1β (Niwa et al., 1999). This protein can also cleave yeast *HAC1* mRNA, but unlike Ire1α, Ire1β appears to undergo constitutive proteolytic cleavage and to enter the nucleus even when unfolded proteins are not being overproduced (Niwa et al., 1999). Why does this nuclear form of Ire1β not lead to a constitutive unfolded protein response? It should also be pointed out that the mammalian *HAC1* gene has not yet been identified. The observation that mammalian Ire1α and Ire1β can cleave yeast *HAC1* mRNA when the mammalian Ire1s are expressed in yeast (Niwa et al., 1999) suggests that a mammalian *HAC1* will be found.

**Processing of APP: Rip of Unknown Function**

Recent progress has been made in identifying the proteases that process APP to generate the toxic amyloid β peptide. One of the enzymes was identified independently by three groups of scientists using different approaches (Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). This protease, called β-secretase, carries out the first (luminal) cleavage of APP at a site that is 28 amino acids from the transmembrane helix. Although β-secretase is a membrane-bound aspartyl protease, the cellular compartment that houses the active enzyme has not been identified.

The β-secretase shears off the bulk of the luminal domain of APP, which then allows the protein to be cleaved within the membrane. The intramembrane cleavage of APP is complex since it can occur at either of two sites separated by two amino acids, leading to two different amyloid β peptides, designated $A\beta_{1-40}$ and $A\beta_{1-42}$. The enzyme that carries out these cleavages is designated γ-secretase, but it is unclear whether a single enzyme cuts at both sites or whether two enzymes are involved. Presenilin-1 is closely involved in this intramembrane cleavage reaction. Human presenilin-1 was originally identified by gene mapping in patients with an autosomal dominant form of early-onset Alzheimer's disease (Sherrington et al., 1995). In these patients, the

disease is caused by amino acid substitutions in presenilin-1 that exert a dominant effect, most likely a gain-of-function that leads to overproduction of $A\beta_{1-42}$, which is more neurotoxic than the $A\beta_{1-40}$ variety. At the other extreme, knockout of the *presenilin-1* gene in mice leads to a failure of $\gamma$-secretase cleavage with preservation of cleavage at the luminal $\beta$ site (De Strooper et al., 1998).

Wolfe et al. (1999) noted that presenilin-1 contains two aspartate residues that are located in two transmembrane helices at positions that are predicted to place them at the same depth in the membrane. These residues are conserved in presenilin-1 orthologs from all species, including fish, worms, and flies. This finding is reminiscent of aspartyl proteases, which contain two closely apposed aspartates that are required for activity. When either of the two intramembrane aspartates of presenilin-1 was replaced with alanine, in transfected cells the production of $A\beta_{1-40}$ and $A\beta_{1-42}$ was reduced to levels seen in cells lacking presenilin-1. These findings are compatible with the hypothesis that presenilin-1 is an aspartyl protease that catalyzes cleavage at the $\gamma$ site(s), but final proof awaits demonstration of cleavage activity using purified proteins in vitro.

In addition to its cleavage by $\beta$-secretase, APP can be cleaved by $\alpha$-secretase, which cuts the extracytosolic domain even closer to the membrane, leaving only 12 amino acids on the external surface (Selkoe, 1996). Like the $\beta$-secretase cleavage, $\alpha$-secretase cleavage is followed by $\gamma$-secretase cleavage. In this case, the liberated fragment is too short to form an amyloid deposit, and thus cleavage by $\alpha$-secretase does not lead to Alzheimer's disease. The protease that carries out $\alpha$ cleavage is TACE, a membrane-bound metalloprotease whose active site faces the extracellular surface (Buxbaum et al., 1998). Remarkably, TACE also carries out the extracytosolic cleavage of Notch (see below), and it also cleaves this protein at a site that is 12 residues outside of the membrane.

## Notch and Differentiation: Control by Rip

The cell surface receptor Notch is synthesized as a 300 kDa precursor that is processed constitutively by a furin-like enzyme in the secretory pathway. The enzyme cleaves the precursor to generate two subunits: an extracellular subunit and a transmembrane subunit. The two subunits of Notch remain associated as a noncovalent heterodimer (Annaert and De Strooper, 1999). The heterodimer travels to the cell surface and remains intact until it binds Delta, a protein that resides on the surface of an adjacent cell. Binding leads to cleavage of the transmembrane subunit of Notch at a point that is 12 amino acids outside of the membrane (Mumm et al., 2000). Recent in vitro studies show that this cleavage can be mediated by TACE, the same enzyme that can carry out cleavage at the $\alpha$ site of APP in vitro (Buxbaum et al., 1998; Brou et al., 2000). TACE cleavage releases most of the extracellular portion of the transmembrane subunit of Notch along with the attached extracellular subunit. The shortened transmembrane subunit is then cleaved within the membrane-spanning helix, liberating a cytosolic fragment. The cytosolic fragment translocates to the nucleus where its presence leads to activation of several genes, including those that encode a family of bHLH transcription factors. These factors, in turn, regulate other genes whose net effect is to influence the fate of cells during development (Chan and Jan, 1999).

The involvement of presenilin-1 in Notch processing was first suggested by experiments in *C. elegans*. SEL-12, the worm ortholog of human presenilin-1, was identified in developmentally defective roundworms (Levitan and Greenwald, 1995). Loss-of-function mutations in *sel-12* attenuate signaling by LIN-12, the worm ortholog of *Drosophila* Notch. Later, it was shown that mammalian Notch proteins also fail to undergo intramembrane cleavage in mouse cells that lack presenilin-1 (De Strooper et al., 1998). Ray et al. (1999) recently extended these findings by showing that in mammalian cells Notch and presenilin-1 form a complex in the ER and move together to the cell surface where Notch is cleaved. They also studied the effect of substituting one of the two intramembrane aspartates of presenilin-1 that are required for APP cleavage. The mutant presenilin-1 formed the normal complex with Notch and escorted it to the cell surface, but intramembrane cleavage failed to occur. These data are consistent with the suggestion that presenilin-1 is indeed the protease that catalyzes the intramembrane cleavage of Notch and that the two aspartates are required for cleavage, but not for complex formation. However, the alternate possibility that presenilin-1 is a required cofactor for another protease cannot be ruled out with certainty.

## Bacterial Versions of Rip

Two versions of the Rip process have been found in eubacteria, and the parallels with Rip in animal cells are striking. *Enterococcus faecalis*, a gram positive bacterium, secretes an 8 amino acid peptide pheromone called cAD1, which induces a mating response in other enterococci that harbor a plasmid called pAD1 (Dunny and Leonard, 1997). Genomic sequencing revealed that the octapeptide pheromone is derived from a 143 amino acid precursor that contains a signal peptide at the $NH_2$ terminus and is inserted into the plasma membrane with a type 2 transmembrane orientation (An et al., 1999). Figure 3 shows a model to illustrate how the pheromone is cleaved from the membrane precursor. The pheromone corresponds to the COOH-terminal eight amino acids of the signal peptide (Firth et al., 1994; An et al., 1999). The octapeptide is generated by cleavage of the precursor at two sites, at the extracellular side of the membrane in a reaction carried out by signal peptidase, and at a site in the middle of the transmembrane signal peptide (Figure 3).

The two-step proteolysis that produces the enterococcol pheromone is analogous to the primary and secondary cleavages of the animal cell proteins shown in Figure 1. Recently, An et al. (1999) identified an *Enterococcus faecalis* gene that is necessary for the production of cAD1. Remarkably, this gene encodes a protein, designated Eep, that resembles the metalloprotease that processes SREBPs within the membrane at Site-2 (Figure 2). Eep also resembles proteins of previously unknown function in *E. coli* and *B. subtilis* (designated YaeL and YluC, respectively, in Figure 2). Although the

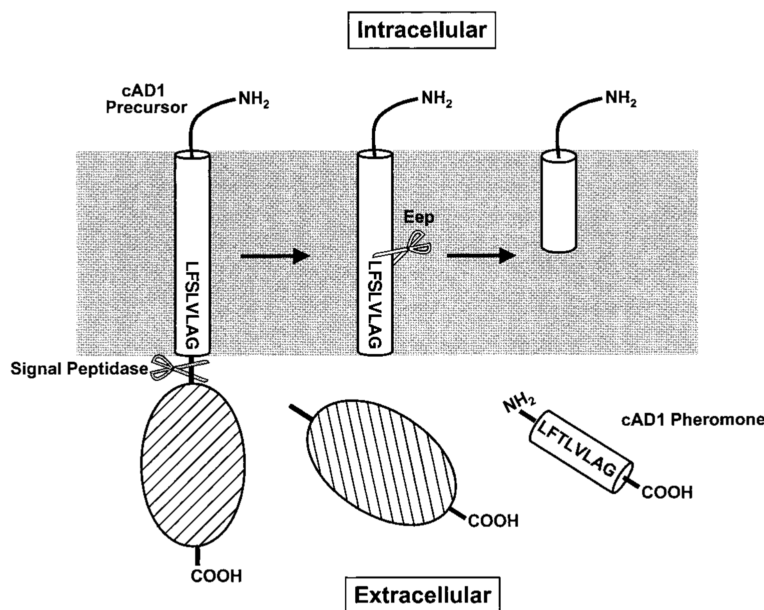


Figure 3. Rip in Bacteria: Intramembrane Cleavage of the Signal Peptide of the cAD1 Precursor to Generate the Mature cAD1 Pheromone

This two-site model is based on the data reported by An et al. (1999), Firth et al. (1994), and Dunny and Leonard (1997). The first cleavage is mediated by signal peptidase and occurs extracellularly. The second cleavage is mediated by the S2P-like protein, Eep, and occurs within the transmembrane signal peptide. See text for details.

amino acid identities between human S2P and the bacterial proteins are rather low (∼28% for the core domains), the structural similarities are striking. All of these proteins display similar hydrophobicity profiles, suggesting multiple membrane-spanning regions. They also share a putative zinc binding motif, HE(I/F/L)GH, that is embedded in a hydrophobic environment toward the $NH_2$-terminal end of the protein. Furthermore, all four proteins contain the sequence LDG in a segment with a nearly identical twin-peak hydrophobicity profile near the COOH terminus (Figure 2). As discussed above, the aspartate of the LDG sequence in S2P is required for the activity of this protein.

Another prokaryotic relative of mammalian S2P is a *B. subtilis* protein called SpoIVFB (Rudner et al., 1999). This protein also contains an HExxH motif in a hydrophobic segment near the $NH_2$ terminus and an LDG motif in a double-peaked hydrophobic segment toward the COOH terminus (Figure 2). However, the overall hydrophobicity profile of SpoIVFB differs from those of the other family members. SpoIVFB is a much smaller protein, and it contains a hydrophilic extension on the COOH-terminal side of the hydrophobic LDG segment. Compelling genetic evidence indicates that SpoIVFB is the protease that removes a membrane-embedded $NH_2$-terminal hydrophobic peptide from a transcription factor, pro-$\sigma^K$, thereby releasing the factor into the cytosol and allowing it to activate gene transcription (Stragier and Losick, 1996). This process is necessary for the completion of spore formation in response to nutrient deprivation. When the *B. subtilis pro-*$\sigma^K$ gene and the *SpoIVFB* gene were expressed together in *E. coli*, pro-$\sigma^K$ was processed normally, a finding that strongly suggests that SpoIVFB is the responsible protease (Stragier and Losick, 1996). This conclusion is supported by the finding that cleavage of pro-$\sigma^K$ is abolished when either the HELGH sequence or the LDG sequence is mutated (Rudner et al., 1999). Interestingly, the membrane orientation of pro-$\sigma^K$ is opposite to the type 2 orientation of SREBP and cAD1. In order to cleave a type 1 protein, SpoIVFB may require a different membrane orientation or other assisting proteins, and this may explain the difference in hydrophobicity profiles between SpoIVFB and the other enzymes in Figure 2.

## Rip: Unanswered Questions

Although the data reviewed here are incomplete and still emerging, they have clearly shown that Rip is used in signaling pathways in organisms ranging from bacteria to humans. It seems likely that the list of Rip systems will expand beyond the seven described to date (five in animal cells and two in bacteria). A crucial unresolved problem relates to the mechanism by which proteases cleave peptide bonds within a membrane bilayer. Membrane-spanning segments are generally felt to form $\alpha$ helices. The peptide bonds in $\alpha$ helices are protected from protease action because they are stabilized by hydrogen bonds that preclude attack by proteolytic enzymes (Paetzel et al., 1998). The two putative proteases that are implicated in Rip, i.e., S2P and presenilin-1, may have the ability to unfold the $\alpha$ helices, turning them into random coils or segments of extended $\beta$ strand–like conformation, both of which are more susceptible to proteolysis (Hubbard et al., 1994; Iwata et al., 1998; Matthews, 1988). Indeed, the products of one such intramembrane cleavage, the A$\beta$ peptides, are already known to form $\beta$-pleated sheets, a property that leads to aggregation and neurotoxicity (Selkoe, 1996). If S2P is a metalloprotease and presenilin-1 is an aspartyl protease, then nature has solved the problem of cleaving intramembrane peptide bonds at least twice, and in both cases highly hydrophobic proteins have been used to do the job.

A second unanswered question relates to the recognition signals that allow the proteases to cleave only selected transmembrane proteins. Their catalytic activity does not depend on the precise residues surrounding the cleaved peptide bond, which is unlike the usual case

for proteases that cleave nonmembrane sequences. A clue to specificity may lie in the observation that the extracellular part of the target protein must be reduced to less than 30 amino acids before the intramembrane cleavage can occur. Perhaps these enzymes do not need a specific recognition sequence. Rather, they will attack any transmembrane protein that lacks a bulky sequence on its luminal or extracelluar surface. Regulation is achieved indirectly by controlling the protease that removes the bulky luminal or extracellular sequence. This is clearly the case for SREBP and Notch, and it may turn out to be true for the other proteins of Figure 1.

A final question relates to the evolutionary advantage that is gained from using Rip for signaling in preference to more classical second-messenger systems. It is possible that intramembrane proteolysis evolved as a means for degrading the membrane-spanning stubs that are generated when the extracellular portions of transmembrane proteins are shed. Intramembrane proteolysis would allow the selective destruction of individual transmembrane fragments without the necessity for destroying whole patches of membrane. Once this mechanism was established, it would be a small step to engraft regulatory controls that could trigger the process in response to ligands. Now that Rip is a recognized phenomenon, it seems clear that these unresolved problems will be solved, and we will not have to wait until the next millennium for the answers.

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