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Review

Endoplasmic reticulum stress response and regulated intramembrane proteolysis in plants

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Abstract In the endoplasmic reticulum (ER) stress response, protein folding in the ER is disturbed, resulting in the induction of genes encoding proteins that facilitate correct folding of proteins. Unique mechanism of signaling pathway in the ER stress response has been studied in yeast and animals. In animals, several bZIP type transcription factors with a transmembrane domain controlled by regulated intramembrane proteolysis (RIP) have been reported to be involved in the signaling pathway. Very recently, AtbZIP60, which is a bZIP type transctiption factor with a transmembrane domain, was identified to be involved in the ER stress response in Arabidopsis. Several other transctiption factors that seem to be regulated by RIP have been also reported in Arabidopsis. Analysis of the regulation mechanism of AtbZIP60 will contribute to understanding of the ER stress response and RIP in plants.

Key words: *Arabidopsis*, endoplasmic reticulum, regulated intramembrane proteolysis, transcription factor, tunicamycin.

The ER stress response

Protein translation results in functional proteins, which are capable of catalyzing various reactions in cellular responses. After translation in the ribosome, nascent proteins are further modified, folded correctly, and assembled to function properly. If such maturation processes fail, the resulting misfolded proteins may aggregate causing serious damage to cells. To avoid such situations, misfolded proteins are monitored, correctly folded by various chaperones, or eventually degraded if the correct structure is not achieved. The importance of such quality control after translation has been recognized and the molecular mechanism, the secretory pathway, for regulating protein quality control has been extensively studied, especially for the proteins synthesized in the endoplasmic reticulum (ER).

Proteins for the secretory pathway (approximately one third of total protein) are synthesized in the ER. Proper folding and assembly necessary for their transportation are ensured by the protein quality control in the ER. Perturbations that alter ER homeostasis often disrupt protein folding and lead to the accumulation of unfolded proteins and protein aggregates that are detrimental to cell survival. More specifically, disturbances in calcium homeostasis or redox status in the ER, increased demand for protein folding due to elevated synthesis of secretary proteins, and lack of asparagine-linked glycans that facilitate protein folding prevent the correct folding or assembly of proteins. Such perturbations preventing protein maturation have been referred to as ER stress. The ER quality control process that alleviates ER stress is called the ER stress response. In mammals, the ER stress response consists of three major mechanisms; attenuation of protein synthesis to prevent supplying additional unfolded proteins, induction of chaperones and folding enzymes to facilitate protein folding (unfolded protein response; UPR), and degradation of unfolded proteins in proteasomes after retrotranslocation to the cytoplasm (ER-associated protein degradation; ERAD) (Kaufman et al. 2002; Mori 2000; Patil and Walter 2001; Rutkowski and Kaufman 2004). In yeast, attenuation of protein synthesis has not been observed; however, both UPR and ERAD mechanism are found to be conserved, thereby suggesting their functions to be similar to those in humans. In UPR, the gene expression for ER-resident chaperones such as BiP, calreticulin (CRT), calnexin (CNX), and protein disulfide isomerase (PDI) was highly induced. In particular, the mRNA induction of BiP, an ER-resident Hsp70, has been considered an indication of UPR. Induction of these genes implies the involvement of a signaling pathway

Abbreviations: bZIP, basic leucine zipper; ER, endoplasmic reticulum; RIP, regulated intramembrane proteolysis; S1P, site-1 proteinase; S2P, site-2 proteinase; TMD, transmembrane domain

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from the ER where misfolded proteins are generated and transported to the nucleus where gene expression occurs.

Studies conducted in yeast and mammalian cells have shown that the ER stress response plays essential roles not only under specific stresses but also under normal growth conditions (Harding et al. 2001; Iwakoshi et al. 2003; Reimold et al. 2001; Scheuner et al. 2001). For instance, an ER stress response is required for terminal differentiation of B lymphoid cells to plasma cells, where the ER compartment expands five fold to accommodate the large increase in immunoglobulin synthesis. Pancreatic β-cells are also reported to require proper function of the ER stress response.

In plants, the ER stress response was observed in the *floury-2* endosperm mutant of maize (Boston et al. 1991; Fontes et al. 1991). This mutant produces an aberrant 24 kDa α-zein storage protein with a defective signal peptide processing site. As a result, the defective storage protein accumulates as a membrane-anchored protein in the ER and in ER-derived protein bodies. The seeds show the ER stress response with dramatically increased levels of BiP and other ER-resident chaperones (Coleman et al. 1995; Gillikin et al. 1997). BiP expression is also regulated during development and by the environment. This regulation was observed during seed development in soybean, rice, pumpkin and Douglas fir, where high amounts of seed storage proteins are folded and assembled in the ER (Forward and Misra 2000; Hatano et al. 1997; Kalinski et al. 1995; Muench et al. 1997). It was also observed that this expression was regulated by various environmental conditions such as salt/osmotic stress (Koiwa et al. 2003). The induction of BiP and other ER chaperones has also been observed in the presence of an artificial stressor such as tunicamycin, a potent inducer of ER stress that inhibits asparagine-linked glycosylation (Vitale and Ceriotti 2004). Treatment with tunicamycin stimulates the expression of BiP and other ER-resident chaperones in several plant systems (Cascardo et al. 2000; Denecke et al. 1991; Koizumi 1996; Okushima et al. 1999; Wrobel et al. 1997). However, the significance of the ER stress response in cellular processes remains to be clarified.

Molecular mechanisms of the ER stress response

The mechanism of signal transduction for the ER stress response has been extensively characterized in yeast and mammalian cells (Figure 1). In yeast cells, IRE1, an ER membrane-located protein kinase/ribonuclease, plays a pivotal role in perception of ER stress (Cox et al. 1993; Mori et al. 1993). Sensing ER stress, IRE1 dimerizes, trans-autophosphorylates, and activates its ribonuclease activity (Bertolotti et al. 2000; Shamu and Walter 1996). Activated IRE1 catalyzes the spliceosome-independent splicing of *Hac1* mRNA, encoding a basic leucine zipper (bZIP) transcription factor. HAC1 protein is efficiently synthesized from spliced *Hac1* mRNA and binds to a *cis*-element, UPRE (CAGCGTG), resulting in induction of downstream chaperone genes such as *BiP* (Kohno et al. 1993; Mori et al. 1996; Mori et al. 1992).

Mammalian cells have multiple ER stress response pathways in contrast to yeast cells, which have a linear pathway consisting of IRE1, HAC1, UPRE, and induction of chaperone genes. In mammals, at least two bZIP transcription factors, XBP1 and ATF6, which function in the ER stress response, have been identified. The *XBP1* mRNA is spliced by IRE1α through

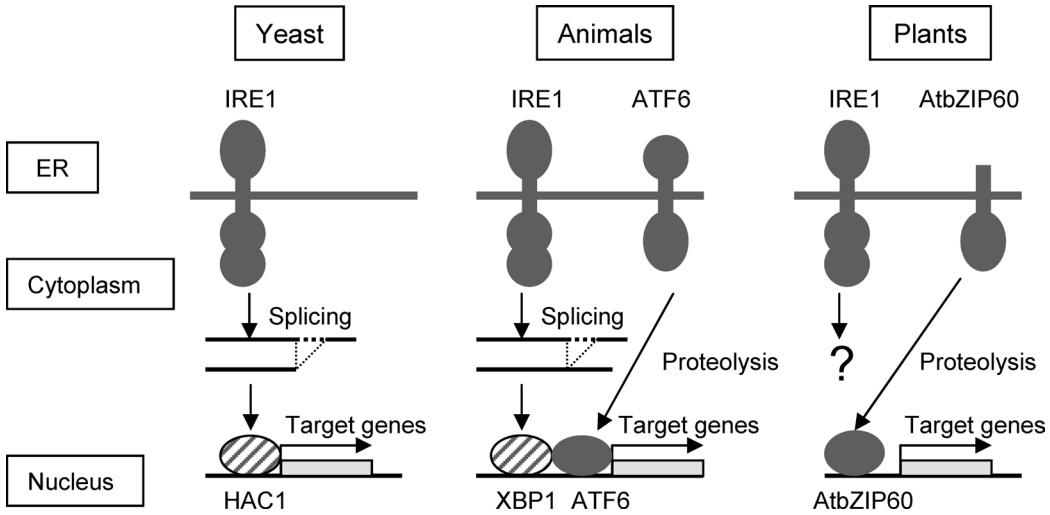

Figure 1. Overview of molecular mechanisms of the ER stress response among yeast, animals and plants. In yeast, IRE1 monitors ER stress and transmits the signal by splicing of HAC1 mRNA that produces active form of HAC1 protein. HAC1 activates transcription of target genes such as ER chaperone genes. In animals, IRE1 catalyzes splicing of XBP1 mRNA in similar with yeast. In addition, ATF6 anchored to the ER membrane becomes active form by proteolysis. In plants, target of IRE1 has not been identified yet. AtbZIP60 is cleaved in response to ER stress converting to a soluble and active transcription factor. It should be noted that Hac1, XBP1 and ATF6 are all bZIP transcription factors.

unconventional splicing, similarly to yeast *Hac1* (Yoshida et al. 2001). This splicing removes 26 nucleotides from authentic *XBP1* mRNA, resulting in a frameshift. XBP1 protein, with an activation domain at the C-terminus, is synthesized after splicing and enhances target gene expression through the *cis*-elements ERSE (CCAAT-N9-CCACG), ERSE-II (ATTGG-N-CCACG), or XBP1-BS (GA-TGACGT-G(T/G)) (Kokame et al. 2001; Shen et al. 2001; Wang et al. 2000; Yamamoto et al. 2004; Yoshida et al. 1998). Another protein, ATF6, is a transmembrane protein located in the ER membrane with a bZIP domain on the cytoplasmic side. In response to ER stress, ATF6 protein is translocated to the Golgi and processed by site 1 (S1P) and site 2 (S2P) proteases in the transmembrane domain (Haze et al. 1999; Lee et al. 2002). The processing localizes the cytoplasmic bZIP domain to the nucleus that activates downstream genes through ERSE or ERSE-II cooperating with the NF-Y transcription factor complex (Yoshida et al. 2000; Yoshida et al. 2001). The active form of ATF6 is produced prior to that of XBP1 in response to ER stress, since the former is derived from a preexisting precursor protein, whereas the latter needs to be newly translated from transcriptionally induced mRNA and then processed by IRE1-dependent splicing (Yoshida et al. 2003; Yoshida et al. 2001). Because *XBP1* contains ERSE in its promoter, ER stress signaling can be amplified through the transcription of *XBP1* as long as IRE1 is activated.

Plants also show a clear ER stress response (Boston et al. 1991; Jelitto-Van Dooren et al. 1999; Koizumi 1996; Koizumi et al. 1999; Leborgne-Castel et al. 1999; Martinez and Chrispeels 2003), although knowledge of the molecular mechanism for the response is limited. Until date, IRE1 homologs have been isolated in *Arabidopsis thaliana* and rice (*Oryza sativa*) (Koizumi et al. 2001; Okushima et al. 2002). A *cis*-element, P-UPRE (plant UPR element), responsible for the ER stress response was identified in the *BiP2* (AGI code; At5g42020) promoter of *Arabidopsis* (Oh et al. 2003). Interestingly, P-UPRE consisted of two *cis*-elements identified in the mammalian ER stress response, ERSE-II and XBP1-BS. In addition to the *BiP2* promoter, P-UPRE was found in the promoters of other ER-chaperone genes including *BiP1* (AGI code; At5g28540). A transcriptomic approach using microarrays showed that ERSEs were also found in promoters of several genes induced by ER stress (Martinez and Chrispeels 2003; Noh et al. 2003). Further, the third BiP, *BiP3* (AGI code; At1g09080) was also found to contain two functional ERSEs, as a mutation in ERSE in the *BiP3* promoter abolishes induction in response to ER stress (Noh et al. 2003). Therefore, *cis*-elements are conserved between mammals and plants. However, a database search for *Arabidopsis* genomic information did not succeed in finding possible homologs of XBP1 or ATF6.

AtbZIP60 was identified as a bZIP transcription factor in *Arabidopsis* that caused an increase in transcripts when treated with tunicamycin (Iwata and Koizumi 2005). Since three representative transcription factors, HAC1 in yeast and XBP1 and ATF6 in animals, are bZIP, it is interesting whether plants also have bZIP transcription factors functioning in the ER stress response pathway. AtbZIP60 (295 amino acids) contains a bZIP domain followed by a putative transmembrane domain (TMD: 218–240 amino acids). Thus, it was presumed that AtbZIP60 protein might be activated in the ER stress response by an activation mechanism similar to that of ATF6, namely proteolysis. In fact, a truncated form of AtbZIP60 (AtbZIP60ΔC: 1-216 amino acids) that lacked a C-terminal region including TMD was localized to the nucleus when it was fused with GFP and transiently expressed in *Arabidopsis* protoplasts. In transient assay of protoplasts using luciferase as a reporter, AtbZIP60ΔC activated promoters of BiP and calnexin genes that are induced under the ER stress response. It also indicated that AtbZIP60ΔC increased reporter activities driven by P-UPRE and ERSE suggesting activation of BiP and calnexin promoters through such *cis*-elements. The transcription of AtbZIP60 is activated by tunicamycin as described above and the promoter of AtbZIP60 contains an ERSE-like sequence essential for activation of the AtbZIP60 promoter by AtbZIP60ΔC in transient assay. Based on these findings, it has been hypothesized that the AtbZIP60 protein localizes to the ER membrane under unstressed conditions and it is cleaved by a specific protease under ER stress. The cleaved form, probably similar to AtbZIP60ΔC, is thought to be translocated to the nucleus and activate the transcription of target genes such as BiP and AtbZIP60 itself. In fact, protein cleavage and translocation of AtbZIP60 was confirmed using a specific antibody against AtbZIP60ΔC (Iwata et al. unpublished result).

Regulated intramembrane proteolysis

Regulated intramembrane proteolysis (RIP) is one of the posttranslational modifications in proteins by which membrane anchored proteins are released from membranes to become active forms. Proteolysis of membrane anchored transcription factors regulated by RIP on specific stimuli has been reported (Brown et al. 2000; Hoppe et al. 2001). The cleaved forms of transcription factors translocate to the nucleus and activate the transcription of target genes. The first digestion of such transcription factors occurs at the luminal or extracellular side of the protein near the TMD sensing signals. Subsequently the second digestion takes

place in the TMD, namely in the lipid bilayer, releasing the cytoplasmic domain of the transcription factor from the membrane (Figure 2). A considerable number of transcription factors and RIP proteases have been identified to date for which the molecular mechanisms and physiological functions have been clarified. Representative RIP regulated transcription factors in eukaryotes are shown in Table 1.

SREBP involved in lipid metabolism was first identified as a transcription factor regulated by RIP (Sakai et al. 1996). The molecular mechanism of RIP regulation has been extensively analyzed in SREBP (Brown and Goldstein 1997, Brown and Goldstein 1999, Goldstein et al. 2006). SREBP has two TMD and localizes to the ER membrane showing a hairpin structure in which both the N- and C-termini are in the cytoplasmic region. The N-terminal domain of SREBP is a transcription factor and the C-terminal domain is bound to the SREBP cleavage-activating protein (SCAP) that is a sensor protein for levels of membrane sterols. When levels of membrane sterols decrease, SCAP binds to COPII proteins resulting in translocation of SREBP to the Golgi apparatus. SREBP translocated to the Golgi apparatus is digested on the luminal side by S1P in the Golgi membrane (Sakai et al. 1998) and then in TMD by S2P (Zelenski et al. 1999). The released N-terminal domain translocates to the nucleus as a transcription factor that induces genes involved in lipid metabolism.

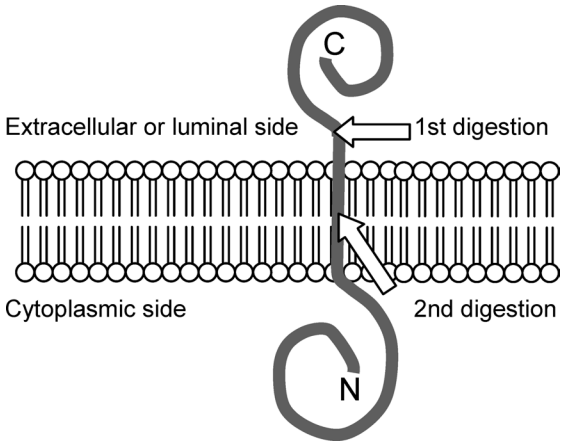

Figure 2. Schematic presentation of regulated intramembrane proteolysis (RIP). Many of proteins digested by RIP proteases are type II membrane proteins as shown. The first digestion of protein occurs in extracellular or luminal side. The second digestion occurs in the membrane domain releasing cytoplasmic domain.

Presenilin, a representative RIP protease, was identified as a product of a gene where mutation causes Alzheimer's disease and has been extensively characterized (see review Landman and Kim 2004; Koo and Kopan 2004; Tabaton and Tamagno 2007). Presenilin is a component of the γ-secretase complex that catalyzes intramembrane digestion of the amyloid β precursor protein (APP) where mutation may also result in Alzheimer's disease. APP is a type I membrane protein that has the C-terminal domain in cytoplasm and is sequentially digested by β-secretase and γ-secretase releasing amyloid β. If presenilin has mutated, abnormal amyloid β aggregates causing apoptosis of nerve cells, although the physiological function of authentic amyloid β has not been elucidated. Presenilin also digests Notch, a type I membrane protein localized to the plasma membrane with a TMD. If ligands bind to the receptor domain of Notch, it is digested sequentially by metalloproteases and by presenilin in the γ-secretase complex. The cytoplasmic domain of released Notch functions in the nervous system.

The ER stress response controlled by RIP

ATF6 was isolated to interact with ERSE and then demonstrated to be processed at the protein level to function in induction of UPR related genes (Yoshida et al. 1998). It was shown that ATF6 was processed by S1P and S2P as same as SREBP. From this finding, RXXL, a

Table 1. Transcription or co-transcription factors controlled by RIP.

Factors	1st protease	2nd (RIP) protease	Related phenomenon
Animals			
SREBP	S1P	S2P	Cholesterol homeostasis
APP	β-secretase	Pserenilin-1	Alzheimer's disease
Notch	metalloprotease	Pserenilin-1	Neurogenesis etc.
ATF6	S1P	S2P	ER stress response
OASIS	S1P	S2P	ER stress response
CREB-H	S1P	S2P	acute phase response /ER stress response
CREB4	S1P	S2P	ER stress response
Luman	S1P	S2P	ER stress response
BBF2H7	S1P	S2P	ER stress response
Plants			
AtbZIP60	not identified	not identified	ER stress response
NTM1	not identified	not identified	Inhibition of cell growth
NTL8	not identified	not identified	Late flowering
AtbZIP17	not identified	not identified	Salt stress response

consensus sequence of S1P recognition, was determined (Ye et al. 2000). However, the recognition mechanism of the stimulus differs between ATF6 and SREBP. ATF6 protein lacking the C-terminal luminal domain associated with BiP was constitutively translocated to the Golgi apparatus. This experiment suggested that ATF6 localizes into the ER membrane under unstressed conditions and it is translocated to the Golgi by the ER stress. The Golgi localization signal in the luminal domain of ATF6 is considered to be masked by BiP under unstressed conditions and to become active by ER stresses since BiP dissociates from ATF6 (Shen et al. 2002).

Transcription factors regulated by RIP in a similar manner as ATF6 have been recently reported to be involved in the ER stress response in animal cells (Kondo et al. 2005; DenBoer et al. 2005; Nagamori et al. 2005; Stirling et al. 2006; Zhang et al. 2006; Liang et al. 2006; Kondo et al. 2007). Relations of these factors with the ER stress response have been studied since their protein structure is similar to ATF6. Namely, all of them are type II membrane proteins having a bZIP domain in the cytoplasmic N-terminal region, as does ATF6. In addition, they have the S1P recognition sequence RXXL or RXL near the transmembrane domain.

OASIS is one of these transcription factors. Sensitivity to ER stress differs among various cell types in the nervous system and astrocytes show stronger resistance to ER stress than neurons. OASIS is structurally similar to ATF6 and is specifically expressed in astrocytes. It is processed by ER stress, translocated to the nucleus, and it activates the BiP promoter. Cultured cells overexpressing OASIS showed stronger resistance to ER stress while knock down of OASIS by RNAi showed promotion of cell death. From these observations, OASIS is considered to suppress ER stress-dependent apoptosis in astrocytes (Kondo et al. 2005).

Another RIP-regulated transcription factor CREB-H is a member of the CREB/ATF family and specifically expresses in liver cells (Omori et al. 2001; Chin et al. 2005). It has been reported that CREB-H is digested by S1P and S2P in the ER stress dependent manner and is translocated to the nucleus where it activates transcription of target genes through the *cis*-element responsible for the ER stress response. In addition, CREB-H activates genes expressed in the acute phase response by inflammatory cytokine or ER stresses, indicating linkage of the ER stress and inflammatory responses (Zhang et al. 2006).

RIP in plants

Until very recently, there has been no report of RIP in plants. In addition to presenilin and S2P, the signal peptide peptidase (SPP) and rhomboid families are known to be RIP proteases in prokaryotes and eukaryotes (Weihofen and Martoglio 2003). Similar genes for each family are found in the *Arabidopsis* genome but most of them have not been functionally characterized. It has been shown that the product of Rhomboid-like 2 (AtRBL2) digested Spits and Keren, substrates of Drosophila Rhomboid (Rho1), in animal cultured cells. This is the first report showing that plants also have RIP protease activity (Kanaoka et al. 2005). RIP is observed in both prokaryotes and eukaryotes, but the RIP-regulated transcription factor has been mostly studied in animals. Recently, RIP-regulated transcription factors have been reported in plants (table 1).

NAC with transmembrane motif 1 (NTM1) was a NAC-type transcription factor identified in *Arabidopsis* mutant *ntm1-D* that shows an abnormal developmental pattern (Kim et al. 2006). NTM1 protein contains a NAC domain in the N-terminal region followed by a TMD. In *ntm1-D*, translation of NTM1 terminates before a TMD resulting in production of truncated NTM1 that lacks the C-terminal domain (NTM1ΔC). Since genes for inhibition of cyclin-dependent kinases were up-regulated in *ntm1-D*, NTM1 was considered to negatively regulate cell division. Both full length and truncated NTM1 proteins are constitutively observed and cytokinin increases their stability. Taken together, it was hypothesized that cytokinin keeps the balance of cell growth by promoting cell division and by negatively regulating cell division through NTM1 at same time (Kim et al. 2006).

Besides NTM1, a NAC type transcription factor NTL (NTM1-like) 8 with a TMD has been reported to be regulated by RIP (Kim et al. 2007a). Expression of NTL8 increases under salt stress and decreases expression of FT. Overexpression of NTL8ΔC suppressed flowering genes such as FT, and flowering time was delayed. These results indicated that NTL8 affects flowering time under salt stress conditions by suppressing expression of FT (Kim et al. 2007a; Kim et al. 2007b).

It was recently shown that an *Arabidopsis* knock out mutant of a gene similar to S1P is more sensitive to salt stress (Liu et al. 2007). They postulated that RIP-regulated transcription factors are involved in the induction of genes for salt resistance. From an analogy with mammalian transcription factors, bZIP-type transcription factors with TMD in the C-terminal region were analyzed as possible targets of S1P. Among three such bZIPs containing a TMD with RXXL S1P recognition sequence in *Arabidopsis*, AtbZIP17 was digested by S1P *in vitro* and *in vivo*. In addition, *atbzip17* a null mutant of AtbZIP17 showed a sensitive phenotype to salt stress similar to *s1p*. AtbZIP17 protein localizes to the ER without stress and translocates to the nucleus by salt stress with protein processing.

Microarray analysis showed that induction of several genes by salt stress was suppressed in *s1p* as opposed to the wild type. Induction of these genes was also suppressed in *atbzip17*. These results indicated salt specific proteolysis of AtbZIP17 by S1P and induction of salt resistance related genes by the processed AtbZIP17 (Liu et al. 2007).

The first report of possible RIP regulation of transcription factors in plants is AtbZIP60. As described above, AtbZIP17 is involved in the salt stress response. In contrast to AtbZIP17, AtbZIP60 does not contain the S1P recognition sequence. Moreover, the luminal domain of AtbZIP60 is much smaller than that of ATF6. Thus, a different molecular mechanism from the S1P/S2P system may operate processing of AtbZIP60. The discovery of RIP protease processing of AtbZIP60 would provide a better understanding of ER stress response and RIP regulation in plants.

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