

## Analysis of ATF6 Activation in Site-2 Protease-deficient Chinese Hamster Ovary Cells

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**ABSTRACT.** Mammalian transcription factor ATF6 is constitutively synthesized as a type II transmembrane protein embedded in the endoplasmic reticulum (ER). It is activated when unfolded proteins are accumulated in the ER under ER stress through a process called regulated intramembrane proteolysis (Rip), in which ATF6 is transported from the ER to the Golgi apparatus where it undergoes sequential cleavage by Site-1 and Site-2 proteases. The cytosolic transcription factor domain of ATF6 liberated from the Golgi membrane enters the nucleus where it activates transcription of ER-localized molecular chaperones and folding enzymes, leading to the maintenance of the homeostasis of the ER. Here, we analyzed M19 cells, a mutant of Chinese hamster ovary cells deficient in Site-2 protease. It was previously shown that M19 cells are defective in the induction of mRNA encoding the major ER chaperone BiP. In M19 cells, ATF6 was not converted from the membrane-bound precursor form to the cleaved and nuclear form as expected. Moreover, some of the ATF6 was constitutively relocated to the Golgi apparatus, where it was cleaved by Site-1 protease, and remained associated with the Golgi apparatus, indicating that the ER of M19 cells was constitutively stressed. Consistent with this notion, the two other ER stress response mediators, IRE1 and PERK, were also constitutively activated in M19 cells. M19 cells showed inefficient secretion of a model protein. These results suggest that Rip-mediated activation of ATF6 is important for the homeostasis of the ER in not only ER-stressed but also unstressed cells.

**Key words:** endoplasmic reticulum/protein folding/molecular chaperone/transcription factor/proteolysis

### Introduction

Newly synthesized secretory and transmembrane proteins are folded and assembled in the endoplasmic reticulum (ER), where an efficient quality control system operates (Gething and Sambrook, 1992; Helenius *et al.*, 1992). Under a variety of conditions collectively termed ER stress, however, the quality control system is hampered, resulting

in the accumulation of unfolded proteins in the ER. Eukaryotic cells cope with ER stress and maintain the homeostasis of the ER by activating the unfolded protein response (UPR). In mammals, three transmembrane proteins in the ER have been identified as critical mediators of the UPR, namely IRE1, PERK and ATF6 (Harding *et al.*, 2002; Mori, 2000; Patil and Walter, 2001; Schroder and Kaufman, 2005).

Mammalian transcription factor ATF6 is synthesized as a type II transmembrane protein embedded in the ER (Haze *et al.*, 2001; Haze *et al.*, 1999). Upon ER stress ATF6 is cleaved via a process termed regulated intramembrane proteolysis (Rip), resulting in the release of the N-terminal region of ATF6 from the membrane (Ye *et al.*, 2000). This fragment, carrying the basic leucine zipper and transactivation domains, is translocated into the nucleus where it activates the transcription of ER-localized molecular chaperones and folding enzymes (collectively termed ER chaperones hereafter) by directly binding to the cis-acting ER

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Abbreviations: CFP, cyan-emitting green fluorescent protein; CHO, Chinese hamster ovary; eIF, eukaryotic initiation factor; ER, endoplasmic reticulum; ERSE, ER stress-response element; GFP, green fluorescent protein; PI, proteinase inhibitor; Rip, regulated intramembrane proteolysis; RT-PCR, reverse transcription-coupled polymerase chain reaction; S1P, Site-1 protease; S2P, Site-2 protease, SREBP, sterol-regulatory element binding protein; UPR, unfolded protein response; WT, wild-type.

stress-response element (ERSE) present in the ER chaperone promoters, in collaboration with the general transcription factor NF-Y (Okada *et al.*, 2002; Yoshida *et al.*, 1998; Yoshida *et al.*, 2000; Yoshida *et al.*, 2001b). Induced ER chaperones help maintain the homeostasis of the ER.

Rip relies on the sequential action of two proteases to cleave substrate protein, namely Site-1 and Site-2 proteases (S1P and S2P) in the case of ER membrane-bound transcription factors with type II topology such as ATF6 and sterol-regulatory element binding proteins (SREBPs); SREBPs are activated when cellular cholesterol levels decrease (Brown *et al.*, 2000). As both S1P and S2P are localized in the Golgi apparatus, these substrate transcription factors must move from the ER to the Golgi apparatus to be cleaved (DeBose-Boyd *et al.*, 1999; Okada *et al.*, 2003; Shen *et al.*, 2002). Although the mechanism of SREBP activation in response to cholesterol depletion has been subjected to extensive analysis, that of ATF6 activation remains largely unknown.

The M19 mutant of Chinese hamster ovary (CHO) cells is deficient in S2P (Hasan *et al.*, 1994; Rawson *et al.*, 1997). It was previously shown that M19 cells are unable to induce the expression of ERSE reporter gene or of the major ER chaperone BiP at either mRNA or protein levels in response to ER stress (Lee *et al.*, 2002). This provided us the opportunity to investigate the activation status of ATF6 in M19 cells. Results suggest the importance of ER stress-induced cleavage of ATF6 in not only ER-stressed cells but also unstressed cells.

## Materials and Methods

### Cell culture, transfection and luciferase assay

CHO wild-type and M19 (Hasan *et al.*, 1994) cells were grown in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin sulfate) in a 5% CO<sub>2</sub>, 95% air incubator at 37°C. Cells were transfected with plasmid DNA as described previously (Nadanaka *et al.*, 2004) using Superfect (Qiagen) basically according to the manufacturer's instructions and then incubated at 37°C for an appropriate time for expression of the transfected gene. The plasmid pECFP-N1-A1AT, used to express  $\alpha$ 1-PI-CFP fusion protein, was constructed previously (Nadanaka *et al.*, 2004). Reporter luciferase assay was carried out according to our published procedures (Yoshida *et al.*, 2000). pGL3-GRP78P(-132)-luc (Yoshida *et al.*, 1998) is called the ERSE reporter.

### Reverse transcription-coupled PCR (RT-PCR) and Northern blot hybridization

Total RNA was isolated from CHO cells by the acid guanidinium-phenol-chloroform method using Isogen (Nippon Gene). One

microgram of total RNA was treated with M-MLV reverse transcriptase (Invitrogen) and then amplified by PCR with Ex-Taq polymerase (Takara) using a pair of primers corresponding to the nucleotides 359–378 (GCTTGTGATTGAGAACCAGG) and 611–630 (CTGGACAAGTTGGACCCTGT) of rat XBP1 cDNA (GeneID: 289754; cDNA clone MGC: 94999 IMAGE: 7115933).

Total RNA was also analyzed by standard Northern blot hybridization (Sambrook *et al.*, 1989) using an Alkphos direct labeling and detection system (GE Healthcare). Chemiluminescence was visualized using an LAS-1000plus LuminImage analyzer (Fuji Film).

### Immunological techniques

Immunoblotting analysis was carried out according to the standard procedure (Sambrook *et al.*, 1989) as described previously (Okada *et al.*, 2002) using Western Blotting Luminol Reagent (Santa Cruz Biotechnology). Chemiluminescence was detected using an LAS-1000plus LuminImage analyzer (Fuji Film). ATF6 $\alpha$  was detected with rabbit anti-ATF6 $\alpha$  polyclonal antibody (Haze *et al.*, 1999). Goat anti-ribophorin I polyclonal antibody (C-15) was purchased from Santa Cruz. Mouse anti-GM130 monoclonal antibody and rabbit anti-phosphorylated eIF2 $\alpha$  polyclonal antibody were obtained from BD Transduction Laboratories and Cell Signaling Technology, respectively. Anti-KDEL monoclonal antibody, anti-calnexin carboxy terminus polyclonal antibody, anti-calreticulin polyclonal antibody, and anti-PDI antibody were purchased from Stressgen. A.v. peptide antibody against green fluorescent protein (GFP) was obtained from Clontech.

Immunoprecipitation and indirect immunofluorescence were carried out essentially as described previously (Nadanaka *et al.*, 2004). Anti-PERK antibody was the kind gift of Dr. D. Ron (New York University School of Medicine). Anti-GFP monoclonal antibody (mixture of clone 7.1 and 13.1) was purchased from Roche.

### Discontinuous sucrose gradient centrifugation

CHO cells were scraped after being washed once with 1 ml of PBS containing protease inhibitor cocktail (Nacalai Tesque) and 10 mM MG132. Cells of 8 dishes were pooled, suspended in 200 µl of sucrose buffer (10 mM HEPES-KOH, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA, protease inhibitor cocktail, and 10 mM N-ethylmaleimide), and homogenized with 30 strokes using a dounce tissue homogenizer (1-ml size, Wheaton). After the addition of 200 µl of sucrose buffer, cells were further homogenized with 20 strokes and then centrifuged at 1,000×g for 5 min at 4°C. Two hundred-microliter aliquots of the resulting supernatant were laid on the top of a discontinuous sucrose gradient consisting of five layers of 200 µl each with distinct sucrose concentrations (20%, 30%, 40%, 50%, and 60% from top to bottom) and centrifuged at 4°C at 50,000 rpm for 30 min in a S55S-1096 rotor (Hitachi) using a Himac CS 150 GXL microultracentrifuge (Hitachi), with deceleration performed without braking. Six fractions of 200 µl each were then collected from the top.

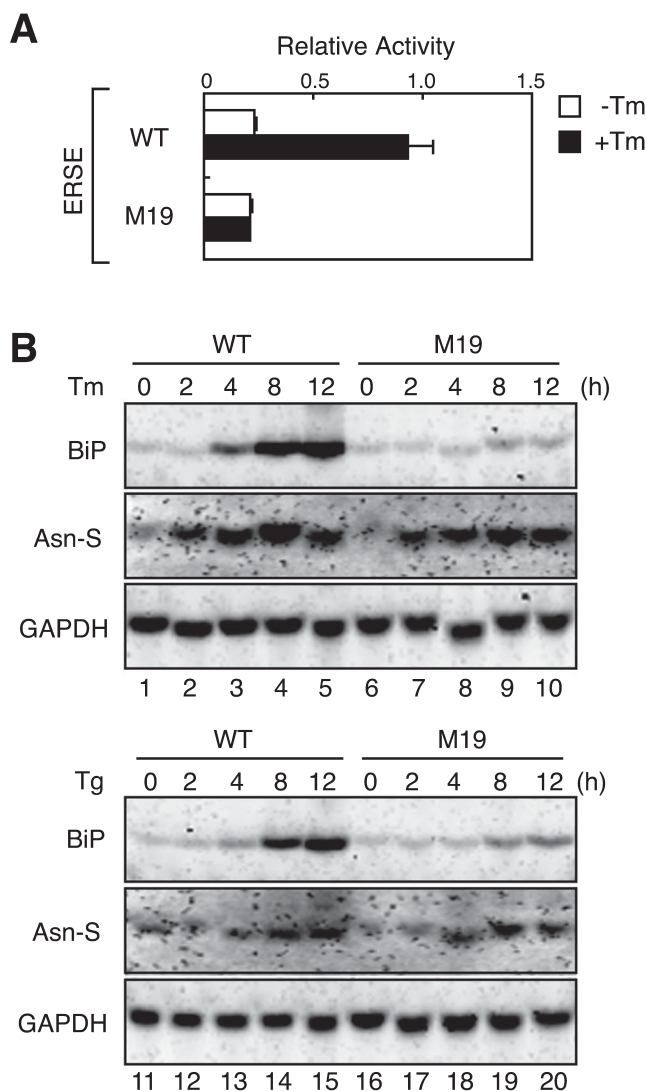
## Results

### Defective induction of the ER chaperone BiP in M19 cells

Transcriptional induction of ER chaperones in response to ER stress is mediated by the cis-acting ERSE present in their promoter regions (Yoshida *et al.*, 1998). The degree of ERSE-mediated transcription has been monitored using human BiP promoter carrying three functional ERSEs fused to firefly luciferase gene as a reporter. As shown in Fig. 1A, luciferase activity expressed from the ERSE reporter was induced in wild-type (WT) CHO cells treated with tunicamycin, which causes ER stress by inhibiting protein N-glycosylation (Kaufman, 1999). In contrast, luciferase activity was not induced at all in M19 CHO cells deficient in S2P. Accordingly, the induction of BiP mRNA in response to treatment with tunicamycin or thapsigargin (which causes ER stress by inhibiting ER  $\text{Ca}^{2+}$ -ATPase) (Kaufman, 1999) observed in WT cells was abolished almost completely in M19 cells (Fig. 1B). These results are consistent with those reported previously (Lee *et al.*, 2002) and suggest the necessity of Rip-mediated activation of ATF6 in ERSE-mediated transcriptional enhancement. In Fig. 1B, mRNA encoding asparagine synthetase served as a control (Okada *et al.*, 2002); the expression of asparagine synthetase is regulated by the PERK pathway of the UPR and was induced similarly in WT and M19 cells. These results prompted us to examine the activation status of ATF6 in M19 cells, which to our knowledge has yet to be investigated.

### Activation status of ATF6 in M19 cells

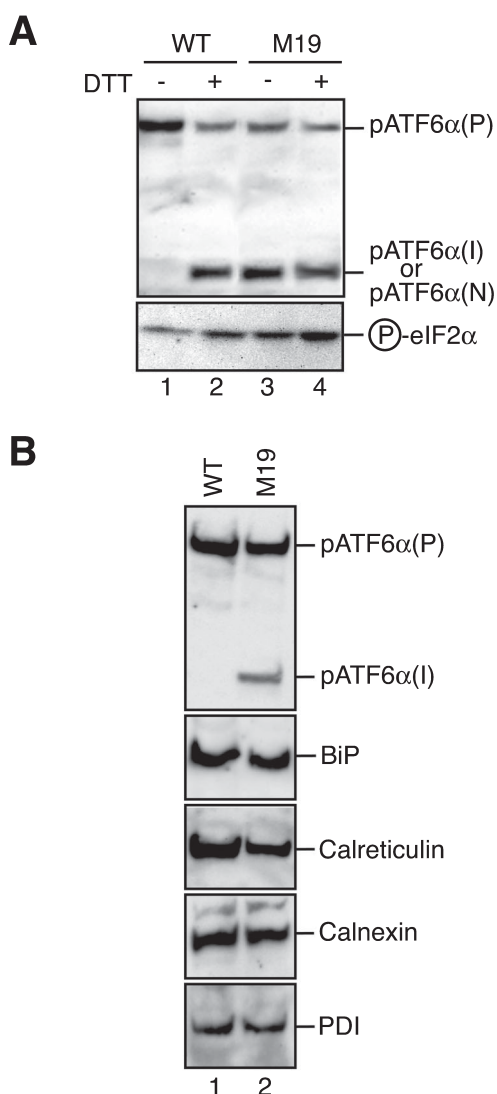
Mammalian ER expresses two types of ATF6 ubiquitously, designated ATF6 $\alpha$  and ATF6 $\beta$  (Haze *et al.*, 2001; Haze *et al.*, 1999). Although ATF6 $\beta$  appears less active in transcription than ATF6 $\alpha$  (Thuerauf *et al.*, 2004), no significant difference in activation process has been noticed between them. We therefore focused on ATF6 $\alpha$  in this report. The activation status of ATF6 $\alpha$  was checked by immunoblotting. As shown in Fig. 2A, ATF6 $\alpha$  was expressed as a membrane-bound precursor form, pATF6 $\alpha$ (P), in unstressed WT cells (lane 1) and was cleaved to produce pATF6 $\alpha$ (N), a nuclear form, in response to dithiothreitol treatment (lane 2), which causes ER stress by reducing disulfide bridges (Kaufman, 1999). In contrast, a cleaved form of ATF6 $\alpha$  was detected in M19 cells in both the absence (lane 3) and presence (lane 4) of dithiothreitol, suggesting that the ER of M19 cells are constitutively stressed, leading to constitutive cleavage of ATF6 $\alpha$ . Consistent with this notion is the phosphorylation status of the  $\alpha$  subunit of eukaryotic initiation factor 2 (eIF2 $\alpha$ ). eIF2 $\alpha$  is known to be rapidly phosphorylated upon ER stress to attenuate translation (Ron, 2002) and this was indeed the case in WT cells (Fig. 2A, compare lane 2 with lane 1). In contrast, the level of phosphorylated



**Fig. 1.** Comparison of induction of ERSE reporter and BiP genes in WT and M19 cells. (A) WT and M19 cells were transfected with the ERSE reporter gene carrying human BiP promoter (−132 to +7) fused to firefly luciferase gene together with reference plasmid carrying Renilla luciferase. Twelve hours later transfected cells were incubated in the presence (closed boxes) or absence (open boxes) of 10  $\mu\text{g}/\text{ml}$  tunicamycin (Tm) for 16 h. Luciferase activity was determined and is expressed as relative activity, namely the ratio of firefly luciferase activity to Renilla luciferase activity. (B) WT and M19 cells were treated with 10  $\mu\text{g}/\text{ml}$  tunicamycin (Tm, upper panel) or 1  $\mu\text{M}$  thapsigargin (Tg, lower panel) for the indicated periods. Total RNA was isolated and analyzed by Northern blot hybridization using a cDNA probe specific to BiP, asparagine synthetase (Asn-S) or GAPDH.

eIF2 $\alpha$  in unstressed M19 cells was already comparable with that in ER-stressed WT cells (compare lane 3 with lane 2) and was further increased after dithiothreitol treatment (compare lane 4 with lane 3).

Ectopic expression of pATF6 $\alpha$ (N) has been shown to induce transcription of various ER chaperone genes in the



**Fig. 2.** Comparison of activation status of ATF6α in WT and M19 cells. (A) WT and M19 cells were treated with (+) or without (–) 1 mM dithiothreitol (DTT) for 0.5 h. Cell lysates were prepared and analyzed by immunoblotting using anti-ATF6α or anti-phosphorylated eIF2α antibody. The migration positions of pATF6α(P), pATF6α(I) or pATF6α(N), and phosphorylated eIF2α are indicated. (B) Lysates of uninduced WT and M19 cells were prepared and analyzed by immunoblotting using anti-ATF6α, anti-KDEL, anti-calreticulin, anti-calnexin or anti-PDI antibody. Anti-KDEL and anti-PDI antibodies recognize BiP and protein disulfide isomerase, respectively.

absence of ER stress (Okada *et al.*, 2002). However, the levels of various ER chaperones constitutively expressed in M19 cells were similar to those expressed in WT cells (Fig. 2B). Thus, the cleaved form of ATF6α detected in M19 cells was not active in transcription. We reasoned that the cleaved form of ATF6α detected in M19 cells represented an intermediate form of ATF6α, pATF6α(I), which had been cleaved by the first Rip protease, S1P, but not yet by

the second Rip protease, S2P, which is deficient in M19 cells. We also reasoned that because the S1P cleavage site is close to that of S2P, the size of pATF6α(I) is similar to that of pATF6α(N). If this is the case, pATF6α(I) would be expected to be localized in the Golgi apparatus, given that S1P and S2P are considered to be present in the Golgi apparatus and come into contact with their substrates only after the substrates relocate from the ER to the Golgi apparatus (DeBose-Boyd *et al.*, 1999).

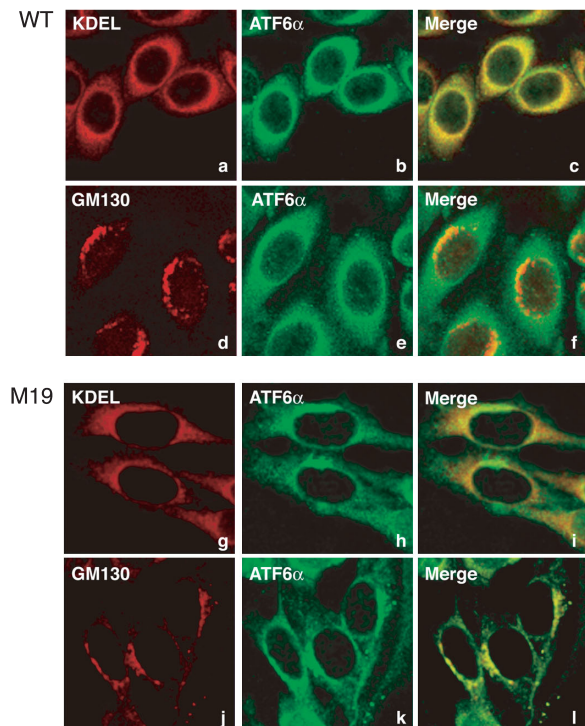
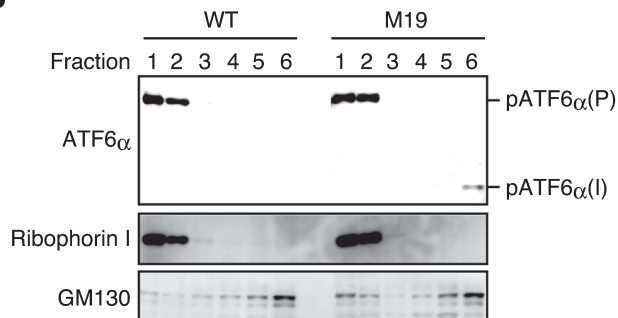
We then determined the localization of ATF6α by immunofluorescence analysis using anti-ATF6α antibody, as follows. As shown in Fig. 3A, WT cells showed perinuclear staining for ATF6α (panel b), which overlapped with the staining with anti-KDEL antibody: anti-KDEL antibody recognized some of the ER chaperones such as BiP and GRP94 (panel a), but this staining for ATF6α differed from staining with anti-GM130 antibody specific to a protein in the Golgi apparatus (panel d). M19 cells also showed perinuclear staining for ATF6α (panel h); however, part of this staining did not overlap with that obtained with anti-KDEL antibody (panel g) but rather merged with that with anti-GM130 antibody (panel j). These results indicate that ATF6α was localized exclusively in the ER in WT cells but was present in both the ER and Golgi apparatus in M19 cells. To confirm this finding and to determine which form is localized in the Golgi apparatus, we fractionated WT and M19 cells by sucrose density gradient centrifugation (Fig. 3B). Ribophorin I, an ER marker, was recovered in the heavy fractions (1 and 2) while the Golgi marker GM130 was recovered mainly in the lightest fractions (6). Under the conditions used, pATF6α(P) was detected in the heavy fractions in both WT and M19 cells, whereas pATF6α(I) was detected in the lightest fractions. These results demonstrate that some ATF6α molecules are translocated to the Golgi apparatus, cleaved by S1P, and remain associated with the Golgi apparatus in unstressed M19 cells.

As pATF6α(I) is larger than pATF6α(N) by approximately 40 amino acids (Ye *et al.*, 2000), pATF6α(I) should migrate more slowly than pATF6α(N) on SDS-PAGE with better resolution, as was achieved in Fig. 4. In WT cells, pATF6α(N) was produced only after treatment with tunicamycin (lanes 1–5) or thapsigargin (lanes 11–15). In M19 cells, pATF6α(I) was constitutively produced (lanes 6 and 16), consistent with the results shown in Fig. 2, whereas pATF6α(N) was not produced at all even after treatment with ER stress inducers. The inability of ER-stressed M19 cells to produce pATF6α(N) due to the absence of S2P is considered to result in the defective induction of ERSE reporter and ER chaperone genes as shown in Fig. 1.

### Constitutive ER stress in M19 cells

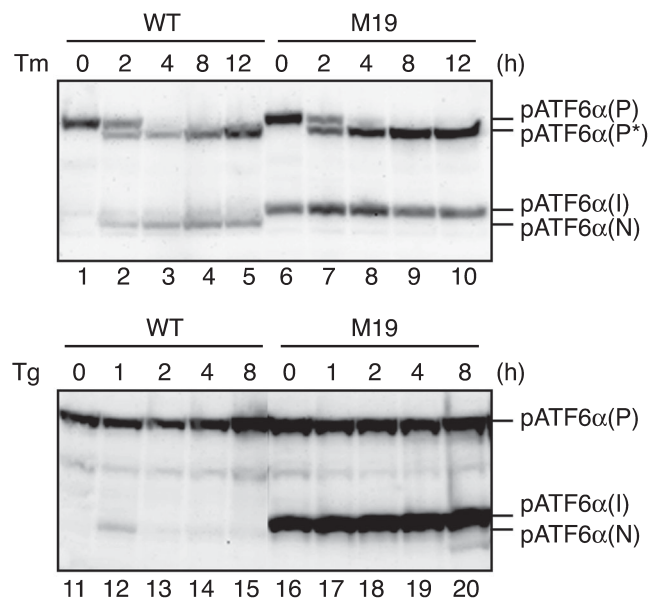
We next examined the activation status of IRE1 and PERK in M19 cells, which are also activated in response to ER stress. IRE1 is a transmembrane protein in the ER which



**A****B**

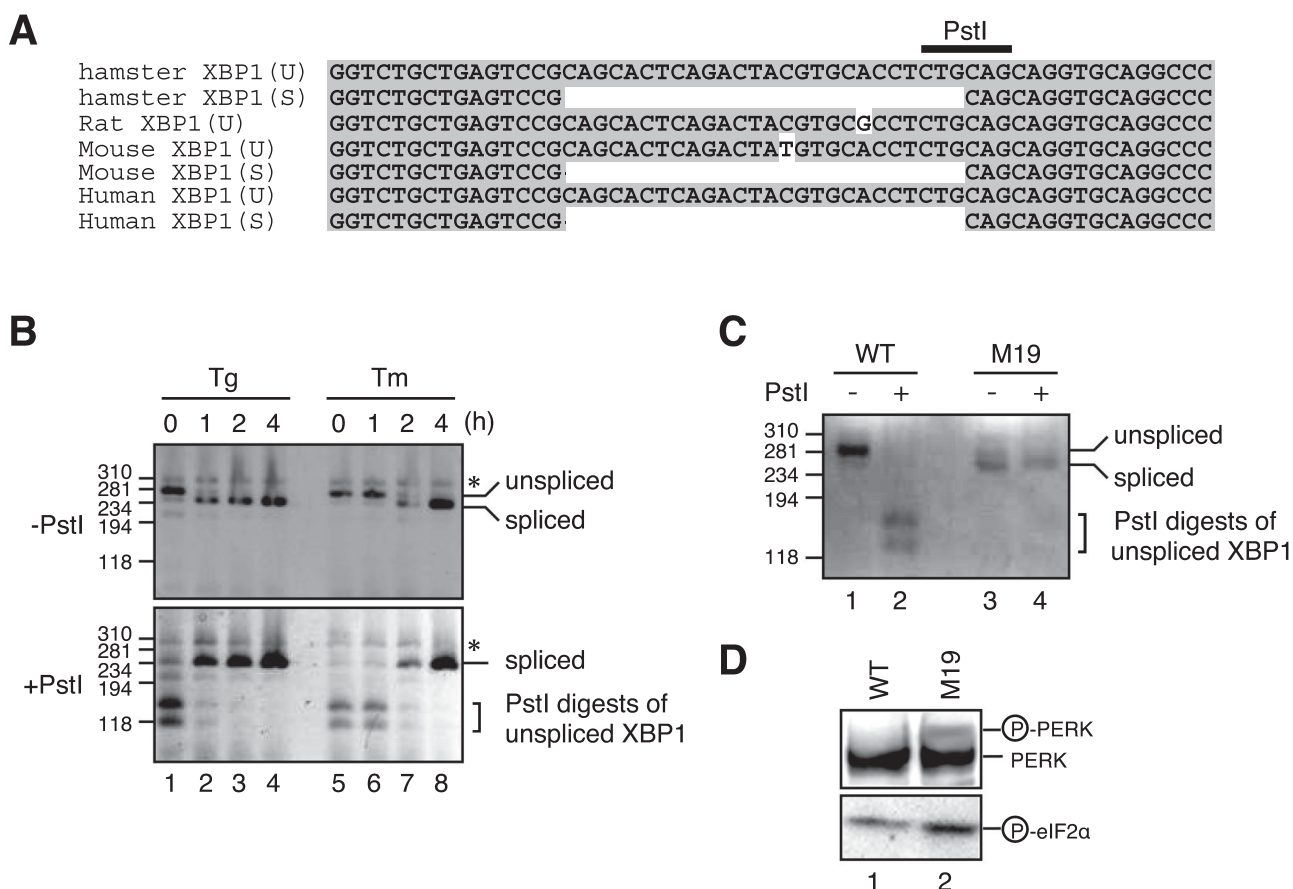
**Fig. 3.** Comparison of localization of ATF6 $\alpha$  in WT and M19 cells. (A) Unstressed WT and M19 cells were fixed and stained with anti-KDEL (panels a and g), anti-ATF6 $\alpha$  (panels b, e, h, and k) or anti-GM130 (panels d and j) antibody. (B) Unstressed WT and M19 cells were homogenized and fractionated by sucrose density gradient centrifugation. Aliquots of the resulting six fractions were analyzed by immunoblotting using anti-ATF6 $\alpha$ , anti-ribophorin I or anti-GM130 antibody.

possesses protein kinase and endoribonuclease activity on its cytosolic side (Mori, 2003). ER stress-induced oligomerization and autophosphorylation of IRE1 result in the initiation of unconventional (frame switch) splicing, whose substrate in mammalian cells is XBP1 mRNA encoding the UPR-specific transcription factor XBP1 (Yoshida *et al.*, 2001a). Because the nucleotide sequence of hamster XBP1 gene and its cDNA have not been deposited in the data bank, we amplified a fragment encompassing the putative



**Fig. 4.** Comparison of ATF6 activation in WT and M19 cells. WT and M19 cells were treated with 10  $\mu$ g/ml tunicamycin (Tm, upper panel) or 1  $\mu$ M thapsigargin (Tg, lower panel) for the indicated periods. Lysates were prepared and analyzed by immunoblotting using anti-ATF6 $\alpha$  antibody. pATF6 $\alpha$ (P\*) denotes the non-glycosylated form of pATF6 $\alpha$ (P).

splice site of hamster XBP1 mRNA by RT-PCR using RNA isolated from unstressed WT cells as template and a pair of primers corresponding to the rat XBP1 mRNA sequence. Sequences around the splice sites of rat, mouse and human XBP1 cDNA are shown in Fig. 5A, where U and S stand for unspliced and spliced, respectively. On RT-PCR analysis, a band of expected size (272 bp) was obtained from unstressed cells (Fig. 5B, upper panel, lanes 1 and 5). In contrast, a smaller band was amplified (Fig. 5B, upper panel, lanes 2–4 and 6–8) when RT-PCR analysis was carried out on RNA isolated from WT cells which had been treated with thapsigargin or tunicamycin. Sequencing analysis showed that this smaller band lacked 26 bp present in the 272 bp band, as shown in Fig. 5A (compare hamster XBP1(U) with XBP1(S)). Thus, hamster XBP1 mRNA is spliced in response to ER stress, and its exon-intron boundary is identical to those of mouse and human XBP1 mRNA. It should be noted that, as with these other species, the 3' intron-exon boundary of hamster XBP1 contains a PstI site (Fig. 5A). Accordingly, the RT-PCR product amplified from unspliced XBP1 mRNA was digested with PstI to produced 2 fragments, whereas that from spliced XBP1 mRNA was not (Fig. 5B, bottom panel). These findings allowed us to determine the activation status of IRE1 in M19 cells. Results of RT-PCR analysis showed that almost all RT-PCR product amplified from RNA in unstressed WT cells corresponded to unspliced XBP1 mRNA (Fig. 5C, lane 1) and was digested with PstI (lane 2). In contrast, a majority



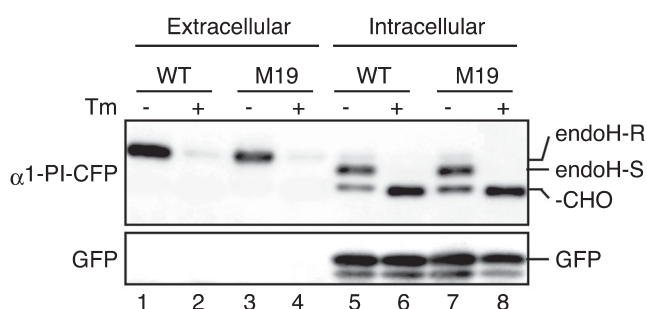
**Fig. 5.** Comparison of activation status of IRE1 and PERK in WT and M19 cells. (A) XBP1 cDNA sequences of various species around the splice sites are aligned with identical nucleotides (shaded). XBP1(U) and XBP1(S) represent unspliced and spliced XBP1, respectively. The position of the PstI site is indicated. (B) WT cells were treated with 1  $\mu$ M thapsigargin (Tg, lanes 1–4) or 2  $\mu$ g/ml tunicamycin (Tm, lanes 5–8) for the indicated periods. Total RNA was isolated and subjected to RT-PCR analysis to amplify a fragment encompassing the splice site. Resulting products were digested with (lower panel) or without (upper panel) PstI and then separated by 4% agarose gel electrophoresis. The migration positions of unspliced and spliced fragments as well as PstI digests of unspliced fragment are indicated. The asterisk denotes a fragment amplified non-specifically. (C) Total RNA was isolated from unstressed WT and M19 cells and subjected to RT-PCR analysis. Resulting products were analyzed as in (B). (D) Unstressed WT and M19 cells were lysed and subjected to immunoprecipitation using anti-PERK antibody. The immunoprecipitates were analyzed by immunoblotting using the same antibody. The migration positions of PERK and phosphorylated PERK are indicated. Lysates of unstressed WT and M19 were also analyzed by immunoblotting using anti-phosphorylated eIF2 $\alpha$  antibody.

of the RT-PCR product amplified from RNA in unstressed M19 cells corresponded to spliced XBP1 mRNA (lane 3) and was not cleaved by PstI (lane 4), suggesting that M19 cells are ER stressed constitutively.

PERK is a transmembrane protein kinase in the ER. When activated by ER stress-induced oligomerization and autophosphorylation, PERK phosphorylates eIF2 $\alpha$ , leading to a general attenuation of translation and a reduction in the burden incurred by the ER when the folding process is disturbed (Ron, 2002). We thus checked the activation status of PERK by detecting the phosphorylated form of PERK. As the expression level of PERK is low, lysates of WT and M19 cells were subjected to immunoprecipitation using anti-PERK antibody, and then the immunoprecipitates were analyzed by immunoblotting using the same antibody. As

shown in Fig. 5D, PERK was inactive in WT cells (lane 1) but some PERK molecules were phosphorylated and thus activated in M19 cells (lane 2). Accordingly, the level of phosphorylated eIF2 $\alpha$  was elevated in M19 cells (lane 2) as compared with that in WT cells (lane 1), consistent with the results shown in Fig. 2A.

We then examined whether the synthesis and secretion of secretory proteins are affected in M19 cells using  $\alpha$ 1-proteinase inhibitor ( $\alpha$ 1-PI, also called  $\alpha$ 1-antitrypsin) as a model protein. We showed previously that this glycoprotein was secreted into medium in unstressed CHO cells but retained in the ER when cells were treated with tunicamycin (Nadanaka *et al.*, 2004). WT and M19 cells were transfected with plasmid to express  $\alpha$ 1-PI fused to cyan-emitting green fluorescent protein (CFP) together with plasmid to express



**Fig. 6.** Comparison of synthesis and secretion of  $\alpha 1$ -PI-CFP in WT and M19 cells. WT and M19 cells were transfected with plasmid to express  $\alpha 1$ -PI-CFP together with plasmid to express GFP alone. Five hours later transfected cells were incubated with (+) or without (–) 2  $\mu$ g/ml tunicamycin (Tm) for 6 h.  $\alpha 1$ -PI-CFP and GFP were immunoprecipitated from both cell lysates and medium using anti-GFP antibody. The immunoprecipitates were analyzed by immunoblotting using anti-GFP antibody. Extracellular  $\alpha 1$ -PI-CFP migrated more slowly than intracellular  $\alpha 1$ -PI-CFP due to the conversion of its carbohydrate moieties from endoglycosidase H-sensitive form (endoH-S) to endoglycosidase H-resistant form (endoH-R). –CHO denotes the non-glycosylated forms of  $\alpha 1$ -PI-CFP.

GFP alone, which served as a control because GFP is not secreted.  $\alpha 1$ -PI-CFP and GFP were immunoprecipitated from cell lysates and medium using anti-GFP antibody, and the immunoprecipitates were analyzed by immunoblotting using anti-GFP antibody. Results showed that the levels of intracellular  $\alpha 1$ -PI-CFP and GFP were similar in WT and M19 cells (Fig. 6, compare lanes 7 and 5), while the level of extracellular  $\alpha 1$ -PI-CFP in M19 cells was nearly half of that in WT cells (Fig. 6, compare lane 3 with lane 1), suggesting that the folding and maturation of secretory proteins are compromised in the stressed ER of M19 cells. Importantly, however, secretion of  $\alpha 1$ -PI-CFP was blocked when WT and M19 cells were treated with tunicamycin (Fig. 6, lanes 2 and 4) and  $\alpha 1$ -PI-CFP was accumulated intracellularly as the unglycosylated form (Fig. 6, lanes 6 and 8), indicating that a quality control system operates normally in M19 cells even though the ER of M19 cells are stressed constitutively.

## Discussion

Rip is a mechanism conserved from bacteria to humans. A functional protein is synthesized as part of a large transmembrane protein and utilized by excision when its function is required by the cell (Brown *et al.*, 2000). In mammals, Rip substrates are often ER membrane-bound transcription factors. SREBPs with a hairpin structure are responsible for cholesterol homeostasis (Brown and Goldstein, 1999). On the other hand, transmembrane proteins with type II topology, such as ATF6, OASIS (Kondo *et al.*, 2005), and CREB-H (Zhang *et al.*, 2006), are involved in the UPR where they maintain the homeostasis of the ER. Interestingly, these ER membrane-bound transcription factors are

cleaved by the same two proteases localized in the Golgi apparatus, S1P and S2P, even though their activation is triggered by different stimuli (Ye *et al.*, 2000). SREBPs are translocated from the ER to the Golgi apparatus to be cleaved when cellular cholesterol levels decrease (DeBose-Boyd *et al.*, 1999), whereas ATF6, OASIS and CREB-H relocate from the ER to the Golgi apparatus when unfolded proteins accumulate in the ER (Kondo *et al.*, 2005; Okada *et al.*, 2003; Shen *et al.*, 2002; Zhang *et al.*, 2006). By these means, diverse cellular function can be regulated by a single Rip mechanism.

Although the role of ATF6 in the UPR is not fully understood, its importance is supported by the following observations. First, overexpression of a dominant negative form of ATF6 blocked the induction of mRNA encoding the major ER chaperone BiP in response to ER stress (Haze *et al.*, 2001). Second, ectopic expression of pATF6(N), a cleaved and nuclear form of ATF6, was sufficient for transcriptional induction of several ER chaperones in the absence of ER stress (Okada *et al.*, 2002). Third, the protease inhibitor AEBSF can block ER stress-induced cleavage of ATF6 by inhibiting S1P and the induction of BiP mRNA in response to ER stress (Okada *et al.*, 2003).

The availability of a CHO mutant cell deficient in S2P, the M19 cell, has provided the opportunity to determine the role of Rip in the UPR. In WT cells, ATF6 $\alpha$  was cleaved by S1P and S2P sequentially to produce pATF6 $\alpha$ (N) in response to ER stress (Fig. 4). Accordingly, ERSE-mediated transcription was activated in the nucleus (Fig. 1A) and BiP mRNA was well induced (Fig. 1B). In marked contrast, ATF6 $\alpha$  was cleaved by S1P but not by S2P, and the resulting pATF6 $\alpha$ (I) therefore remained associated with the Golgi apparatus in M19 cells (Figs. 3 and 4), leading to no transcriptional activation through ERSE and little induction of BiP mRNA (Fig. 1). Because other UPR regulators controlled by mammalian Rip exhibit tissue-specific expression, e.g. OASIS in astrocytes (Kondo *et al.*, 2005) and CREB-H in liver (Zhang *et al.*, 2006), these results strongly suggest that ATF6 is indispensable to the induction of ER chaperones in response to ER stress. Furthermore, even if they were expressed in CHO cells, their contribution to the ERSE-mediated induction of ER chaperones would be marginal because CREB-H enhances UPR element-mediated transcription but not ERSE-mediated transcription (Zhang *et al.*, 2006) and because OASIS prefers cAMP-response element to ERSE (Kondo *et al.*, 2005). Nonetheless, a final conclusion must await the construction and characterization of ATF6-knockout mice, which is in progress in our laboratory.

It is noteworthy that IRE1 and PERK in addition to ATF6 were constitutively activated, and that thus the ER of M19 cells are stressed constitutively (Fig. 5). Indeed, secretion of  $\alpha 1$ -PI-CFP was less efficient in M19 cells (Fig. 6). This suggests that ATF6-mediated induction of ER chaperones is required to maintain folding capacity in the ER even under

normal conditions. Without ATF6 activation, ER chaperone levels are not adjusted to the needs of the ER, and the productive folding process is compromised. This leads us to suggest that ATF6-mediated transcriptional induction of ER chaperones is important not only in ER-stressed but also unstressed cells.

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