Rapid Turnover of Unspliced Xbp-1 as a Factor That Modulates the Unfolded Protein Response*

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The mammalian and yeast unfolded protein responses (UPR) share the characteristic of rapid elimination of unspliced Xbp-1 (Xbp-1u) and unspliced Hac1p, respectively. These polypeptides derive from mRNAs, whose splicing is induced upon onset of the UPR, so as to allow synthesis of transcription factors essential for execution of the UPR itself. Whereas in yeast translation of unspliced Hac1p is blocked, mammalian Xbp-1u is synthesized constitutively and eliminated by rapid proteasomal degradation. Here we show that the rate of Xbp-1u degradation approaches its rate of execution of the UPR itself. Whereas in yeast translation of unspliced Hac1p is blocked, mammalian Xbp-1u is synthesized constitutively and eliminated by rapid proteasomal degradation. Here we show that the rate of Xbp-1u degradation approaches its rate of synthesis. The C terminus of XBP-1u ensures its trafficking to the cytoplasm, and is sufficient to impose rapid degradation. Degradation of XBP-1u involves both ubiquitin-dependent and ubiquitin-independent mechanisms, which might explain its unusually rapid turnover. Xbp-1Δ7/3 mouse embryonic fibroblasts reconstituted with mutants of XBP-1u that show improved stability differentially activate UPR target genes. Unexpectedly, we found that one of the mutants activates transcription of both XBP-1-specific and non-XBP-1-dependent UPR targets in response to tunicamycin treatment, even more potently than does wild type Xbp-1. We suggest that the degradation of Xbp-1u is required to prevent uncontrolled activation of the UPR while allowing short dwell times for initiation of this response.

Protein folding in the endoplasmic reticulum (ER) is carried out under the constant scrutiny of the ER quality control machinery (1). The overall capacity of the ER to fold newly synthesized proteins must match the load of client proteins that emerge into the ER. When this amount exceeds the folding capacity of the ER, a signaling pathway emanates from the ER that controls gene transcription, as well as protein translation. This ER to nucleus signaling cascade is referred to as the unfolded protein response (UPR). The overall goal of the UPR is to enhance the clearance of misfolded proteins from the ER, and consequently the UPR alleviates ER stress (2).

In yeast, Ire1p is the only known transducer of the UPR. In response to ER stress conditions, Ire1p dimerizes and undergoes autophosphorylation. This event induces a conformational change that activates a nuclease activity, activated Ire1p splices the mRNA of Hac1, which in its spliced form encodes Hac1p, a potent transcription factor that induces transcription of many genes that encode ER chaperones, proteins that participate in ER to Golgi trafficking and components of the ER degradation machinery (2).

The mammalian UPR is minimally composed of three transducers: Perk, Atf6, and Ire1 (5). Ire1 is highly conserved from yeast to mammals, but the homolog of Hac1 eluded scientists for many years. The mammalian counterpart of Hac1 was identified as Xbp-1. Xbp-1, a member of the CREB/ATF family of transcription factors, does not share any significant sequence homology with Hac1. It is composed of a basic leucine zipper-containing DNA binding domain located at the N terminus. The C terminus of Xbp-1 operates as a transcription activation domain. In contrast to yeast, in which Ire1p removes a relatively large intron from Hac1 mRNA, only 26 bases are excised by splicing the mRNA of Xbp-1. This splicing induces a frameshift in the coding sequence, replacing the 105-amino acid C terminus of the unspliced Xbp-1 protein with a 226-amino acid domain (6, 7). The spliced C terminus potently activates transcription of downstream target genes. Similar to yeast, the specific target genes of Xbp-1 encode proteins that enhance the folding capacity of the ER and participate in the clearance of misfolded proteins from the ER (8).

Despite high levels of the unspliced Hac1 mRNA, no unspliced Hac1 protein is detectable, caused by selective inhibition of its synthesis. The mechanism of the arrest in translation of Hac1 mRNA involves base pairing interactions between the intron and the 3′ untranslated region (9). In contrast to Hac1, unspliced Xbp-1 (Xbp-1u) is continuously synthesized, but it is unstable and quickly degraded (10). Once Xbp-1 mRNA is spliced, the encoded protein gains stability and allows activation of transcription.

Lee et al. (10) previously demonstrated that an N-terminal segment of Xbp-1, the domain shared between the unspliced and the spliced forms, is relatively stable and exerts dominant-negative activity for expression of genes activated by spliced Xbp-1 (Xbp-1s) targets. This is probably because of direct competition between the N-terminal segment of Xbp-1 and Xbp-1s for the available DNA binding sites. Furthermore, overexpression of a mutant of Xbp-1u, which cannot be spliced because of point mutations in the intron (referred to here as “unspliceable”), also displays dominant-negative characteristics, when assayed under ER stress conditions that robustly generate Xbp-1. Finally, replacement of lysine residues with arginines at the C terminus of Xbp-1u increases its stability and potentiates its dominant negative activity. These observations implicate the ubiquitin-proteasome system as responsible for Xbp-1u degradation (10). Overall, these data suggest that Xbp-1u is a potential inhibitor of the Xbp-1 pathway of the UPR, and its removal by proteolysis is a prerequisite for proper activation of the UPR.

Here, we examined the localization and stability of Xbp-1u and its C terminus. We show that Xbp-1u is an intrinsically unstable protein, which is degraded in living cells at a rate comparable with its rate of
synthesis. The C terminus of Xbp-1u is sufficient to mediate this exceedingly rapid degradation, which occurs predominantly in the cytoplasm involving ubiquitin-dependent and -independent pathways. Against expectation, improvement of the stability of Xbp-1u augmented the transcription of Xbp-1-specific and -nonspecific target genes under conditions that robustly induce the UPR. We conclude that the rapid degradation of Xbp-1u is required to prevent uncontrolled activation of the UPR.

EXPERIMENTAL PROCEDURES

In Vitro Transcription and Translation—pcDNA3.1-encoded Xbp-1
N terminus, Xbp-1u, and Xbp-1s were translated using the TNT coupled reticulocyte lysate system (Promega) according to manufacturer’s instructions, in the presence of [35S]methionine (PerkinElmer Life Sciences). To initiate the chase period, we added RNase (1 mg/ml final concentration, Roche) together with excess unlabeled methionine. Equal aliquots were taken at the indicated time points, diluted in reducing sample buffer, boiled, and analyzed by SDS-PAGE (12%) followed by fluorography.

Cell Lines—HeLa and Xbp-1−/− mouse embryonal fibroblasts (MEFs) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. ts20 cells were cultured at 32 °C in α-minimal essential medium supplemented with 10% fetal calf serum (11).

Epifluorescence Imaging—HeLa cells were seeded on glass coverslips 18 h before transfection. Vectors encoding GFP fused at its C terminus to the specified constructs were transfected using the calcium phosphate precipitation method (CalcPhos, BD Biosciences). 24 h after transfection, nuclei were labeled with Hoechst 33342 (blue fluorescence, Invitrogen). Images were obtained with a Spot RT digital camera mounted on a TE300 Nikon microscope at ×40 magnification.

Retrovirus Production—Wild type Xbp-1 and Xbp-1 mutants were cloned into the pMiG MSCV vector harboring an internal ribosomal entry site-GFP element to allow sorting of infected cells. Viral particles were made in 293T cells by triple transfection of the retroviral vector (2 μg), pMD-gag-pol (2 μg), and pVSV-G (2 μg) using Effectene (Qiagen). Cells were infected as previously described (12).

Metabolic Labeling, Pulse-Chase Analysis, and Immunoprecipitation—Metabolic labeling, pulse-chase analysis, detergent solubilization, and immunoprecipitation were performed as described (13, 14). Polyclonal rabbit anti-Xbp-1 was purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-GFP antibody was raised against the recombinant GFP protein. ts20 cells were transfected with pcDNA3.1 that encodes an unsplicable Xbp-1u using Lipofectamine 2000 (Invitrogen). After 24 h, when indicated, cells were switched to 42 °C for 12 h. Pulse-chase analysis was performed at 32 or 42 °C. Ikbα was immunoprecipitated with a polyclonal rabbit anti-Ikbα antibody (sc-371, Santa Cruz Biotechnology).

Real-time PCR Analysis—Total RNA was isolated from tissues using TRIzol reagent (Invitrogen). cDNA was synthesized from RNA samples using the iScript cDNA synthesis kit containing oligo(dT) and random hexamer primers (Bio-Rad). Quantitative real-time PCR employing SYBR green fluorescent reagent were run in an ABI PRISM 7700 system (Applied Biosystems). The relative amounts of mRNAs were calculated using the comparative threshold cycle (Ct) values using β-actin as control. Primer sequences were designed by Primer Express software (Applied Biosystems). The following primers were used: Bip forward, TCATCGAGACGGACTTTGGA; Bip reverse, CAACACCTTGGAATGGCAAGA; Erdj4 forward, GCTGCTGATCACTTCTGC; Erdj4 reverse, GCCGTCAACATGCCACTA; Chop forward, GTCCCTAGTGTTG; β-actin forward, GCTTGGCTCTGACCATCCT; β-actin reverse, GCCACCAGTCCACACCGCT.

RESULTS

Xbp-1u Is an Intrinsically Unstable Protein—Previous studies demonstrated rapid turnover of Xbp-1u in different cell types (7, 10). To test whether Xbp-1u is an intrinsically unstable protein, we examined its stability in a cell-free assay. Using the T7 TNT in vitro translation system, we generated Xbp-1u, Xbp-1s, and the region of Xbp-1 shared between Xbp-1u and Xbp-1s, amino acids 1–161 (referred to as Xbp-1N terminus). This approach yields radiochemically pure product under native conditions. The chase period was initiated by inclusion of RNase and excess unlabeled methionine into the reaction mixture. As seen in intact cells, the N terminus of Xbp-1 was stable throughout the chase period (Fig. 1A, upper panel). In contrast, Xbp-1u decayed rapidly. Over 80% of the initial Xbp-1u was degraded within the first hour of chase, the remainder was stable till the end of the experiment (Fig. 1A, middle panel). The percentage of stable material varied from experiment to experiment, but never exceeded 20% of the initial amount of Xbp-1u. We speculate that this material represents Xbp-1u that, by means unknown, is sequestered from the degradation machinery. Xbp-1s decayed more gradually than Xbp-1u (Fig. 1A, lower panel). Inclusion of proteasome inhibitors, such as ZL3VS or MG132 had little if any effect on the degradation observed in vitro (data not shown).

In addition, we looked for the appearance of polypeptides that might indicate their modification by ubiquitin. Longer exposures of the autoradiogram showed the presence of additional polypeptides for Xbp-1u, arranged in a ladder reminiscent of modification by ubiquitin. Note that the reticulocyte lysate used for the generation of in vitro translation products is competent for ubiquitination reactions. These additional polypeptides decayed over time (Fig. 1B, middle panel, labeled by asterisk). Of note, they were observed in the absence of E-ethylmaleimide or proteasome inhibitors, commonly added to enrich for ubiquitinated proteins (15). We neither observed this pattern for the N terminus of Xbp-1 nor for Xbp-1s (Fig. 1B, left and right panels).

To confirm that the multiple bands, which appear at higher molecular weight than Xbp-1u, indeed correspond to products modified by ubiquitin, we translated Xbp-1u in the presence of recombinant HA-tagged ubiquitin. The HA tag increases the molecular mass of Ub by 3 kDa, and thus alters the mobility of the ubiquitinated polypeptides. We indeed observed a shift in the mobility of the high molecular weight polypeptides, indicating modification of Xbp-1u by ubiquitin (Fig. 1C). The instability of Xbp-1u in the cell-free extract and its modification by ubiquitin indicate that Xbp-1u degradation is a robust process, and may well be constitutive.

The N and C Terminus Domains of Xbp-1 Determine Its Overall Intracellular Localization and Its Turnover—Xbp-1u can be divided into two parts (Fig. 2A). Amino acids 1–161, which are shared between Xbp-1u and Xbp-1s, were defined as the Xbp-1N terminus. The Xbp-1C terminus starts at the end of the intron (amino acid 168) and extends to the stop codon of Xbp-1u (position 267). Similarly, the segment referred to as Xbp-1C terminus is the domain unique to Xbp-1s, created by removal of the short intron. To examine the contribution of the different domains to intracellular localization and the turnover of Xbp-1, we generated GFP C-terminal fusions for each. For these experiments, Xbp-1u was used in a form that prevents its splicing (referred to as unsplicable), as described (12). This manipulation was necessary to prevent the splicing of GFP-Xbp-1u mRNA by endogenous Ire1. We also generated an unsplicable mutant in which the two lysines, located
at positions 235 and 252 of the Xbp-1u, were replaced by arginines. This mutation, referred to as K2R reportedly enhances the stability of Xbp-1u, probably by inhibiting its ubiquitination (10).

HeLa cells were transfected with the individual GFP constructs and imaged by fluorescence microscopy. The nucleus was visualized by staining with Hoechst 33342 (blue fluorescence). In parallel, turnover of the GFP fusion proteins was measured by pulse-chase analysis. Xbp-1u showed no preferential localization to either the nucleus or the cytoplasm and was evenly distributed in the cell (Fig. 2B). The Xbp-1N terminus was localized exclusively to the nucleus, most likely because of the basic leucine zipper domain and the three bipartite nuclear targeting sequences (residues 53–69, 68–84, and 69–85) (Fig. 2B). Conversely, Xbp-1C terminus was partially excluded from the nucleus (Fig. 2B, third panel). Our analysis for nuclear export sequences did not reveal any consensus nuclear export signals located in the Xbp-1C terminus. However, it should be borne in mind that nuclear export signals are less conserved, and for their function usually require post-translational modifications, such as phosphorylation, which are difficult to predict. We conclude that in the context of the full-length molecule, the Xbp-1C terminus neutralizes the nuclear localization signals in Xbp-1N terminus to ensure dynamic trafficking of Xbp-1u in and out of the nucleus.

Examination of the turnover of the GFP fusion proteins by pulse-chase analysis showed rapid turnover for GFP-Xbp-1u and GFP-C-Xbp-1C terminus. Cells were pulse-labeled with [35S]methionine for 30 min. Within 30 min of chase, both GFP-Xbp-1u and GFP-C-Xbp-1C terminus were degraded completely (Fig. 2B). GFP-Xbp-1N terminus showed much better stability and was detectable at the 60-min chase point. In contrast to what was observed for the cell-free products, in live cells GFP-Xbp-1N terminus was not completely stable and did decay...
FIGURE 2. Xbp-1C terminus(u) imposes cytoplasmic localization and rapid turnover. A, schematic depiction of the constructs fused to the N terminus of GFP. Light blue, Xbp-1N terminus; red, splicing region; white, Xbp-1C terminus(u); gray, Xbp-1C terminus(s); black, unspliceable splicing region. B and C, HeLa cells were transfected with the indicated construct. 24 h after transfection nuclei were stained with Hoechst and fluorescent images were taken using a ×40 objective. In parallel, cells were pulse-labeled with [35S]methionine for 30 min. Cells were lysed in 1% SDS, and lysate was then diluted to 0.07% SDS with Nonidet P-40 lysis mixture followed by immunoprecipitation with anti-GFP antibodies and analyzed by SDS-PAGE (12%). D, HeLa cells stably expressing the indicated constructs were pulse-labeled as before in the presence or absence of the proteasome inhibitor ZL3VS. Cell lysates and immunoprecipitation were carried out as in B.
over time. Because Xbp-1 C termi

nus(u) is sufficient to direct rapid de

gradation of an otherwise stable protein like GFP, and because it con

fers localization to the cytoplasm, we conclude that the degra
don of Xbp-1u occurs mostly, but not exclusively in the cytoplasm.

To further investigate the intracellular location of Xbp-1u degra
dation, we fused the K2R mutant of unspliceable Xbp-1u to GFP. HeLa
cells were transfected with GFP-Xbp-1u or its K2R mutant. Under con
ditions of comparable transfection efficiency, the K2R mutant in its GFP
fused form was more stable than the wild type allele, as assessed by
pulse-chase analysis (the gel at Fig. 2C represents loading of equal
amounts of incorporated [35S]methionine). Imaging of the GFP-Xbp-1-
K2R mutant revealed accumulation of GFP signal in the cytoplasm.
Therefore, inhibition of Xbp-1u degradation results in accumulation in
the cytoplasm, and further shows that this is the major site of
degradation.

We applied a similar analysis to Xbp-1s. Xbp-1s was localized pre
dominantly to the nucleus (Fig. 2B). Analysis of Xbp-1 C termi

nus(s) did not show the preferential location to the cytoplasm, as was seen for Xbp-
1 C termi

nus(u). Instead, Xbp-1 C terminus(s) was evenly distributed between
the nucleus and the cytoplasm. Thus, the intracellular localization of
full-length Xbp-1s is governed by its N-terminal segment.

Pulse-chase analyses for GFP-Xbp-1s and GFP-Xbp-1 C termi

nus(s) indicated that they also decay quite rapidly, although at a slower rate
than their unspliced counterparts. We also noticed that Xbp-1s
appeared more diffuse at the later chase time points (Fig. 2B, fourth
panel), suggesting post-translational modification(s) in live cells, but
not in vitro (compare with Fig. 1A). Because Xbp-1s is localized pre
dominantly to the nucleus, its modification(s) and degradation probably
occur there.

To assess the rate at which Xbp-1u is degraded when expressed at
more physiological levels, we established stable HeLa cell lines derived
from single cell clones expressing either GFP-Xbp-1u in its unspliceable
form, or GFP-Xbp-1 C termi

nus(u). The expression of these constructs was
driven by a cytomegalovirus promoter. Remarkably, both fusion pro

teins were barely detectable by pulse-chase analysis, unless a protea
some inhibitor was included during the pulse and the chase. Regardless
of the pulse labeling time used, GFP-Xbp-1u or GFP-Xbp-1 C termi

nus(u) disappeared at a chase time equal to the duration of the pulse. We also
noticed that inclusion of the proteasome inhibitor did not completely
spare these proteins from degradation, suggesting that residual activi

ty of the proteasome, not blocked by ZL3VS, is sufficient to bring about
degradation of Xbp-1u (Fig. 2D). Alternatively, nonproteasomal pro
elysis may contribute as well. These results demonstrate that the rate of
Xbp-1u degradation, mediated by its C terminus, approaches its rate of
synthesis.

Xbp-1 C termi

nus(u) Does Not Require Ubiquitination for Its Degradation—

To investigate the mechanism by which GFP-Xbp-1 C termi

nus(u) is destroyed, we generated an HA-tagged version of Xbp-1 C termi

nus(u), a protein that only contains the two native lysine residues at the C termi

nus(u). We also generated the corresponding HA-Xbp-1 C termi

nus(u) K2R mutant, in which these lysines were replaced by arginines. These con

structs were transfected into 293T cells. Pulse-chase analysis was then
performed in the presence and absence of the proteasome inhibitor
ZL3VS, followed by anti-HA immunoprecipitation. Both polypeptides
were barely detected by pulse-chase analysis in the absence of protea
some inhibitor, again indicating a degradation rate close to that of their
rate of synthesis (Fig. 3A). For both constructs the inclusion of ZL3VS
markedly improved stability of the protein. Therefore, the presence of
primary amines in the sequence of the C terminus(u) is not required for
its proteasome-dependent degradation.

Because the HA epitope tag starts with alanine, it is often acetylated
when positioned at the N terminus (16). Therefore, the degradation of
HA-tagged Xbp-1 C termi

nus(u) K2R might represent a proteasome-de

pendent but ubiquitin-independent degradation pathway. To further
address this possibility, we constructed a fusion protein between ubiqui

itin and the HA-tagged Xbp-1 C termi

nus(u) separated by a proline resi

due (Ub-P-Xbp-1 C termi

nus(u)). Because ubiquitin C-hydrolases quickly
cleave the peptide bond between the ubiquitin Gly37 residue and a C-termi

nal extension, this method generates polypeptides with the predeter
mined N-terminal residue of choice (17). The Ub-P-Xbp-1 C termi

nus(u) K2R construct is designed to yield a C terminus(u) fragment that carries
no lysines and a proline residue at its N terminus, and therefore cannot
be ubiquitinated.

The Ub-P-Xbp-1 C termi

nus(u) and its K2R mutant were transfected into
293T cells. Pulse-chase analysis was conducted 24 h after transfection
in the presence or absence of the proteasome inhibitor MG132. We used
MG132 instead of ZL3VS, because MG132 is a reversible inhibitor that
can be removed by washing. As expected, in the absence of proteasome
inhibitor very little of the polypeptide was recovered by anti-HA immu
noprecipitation (Fig. 3B). This degradation is probably medi
ated by Xbp-1 C termi

nus(u). When chased in the presence of MG132,
Ub-P-Xbp-1 C termi

nus(u) was stabilized and gradually converted into
P-Xbp-1 C termi

nus(u) (Fig. 3B), showing that this construct is pro
cessed as expected in living cells. We repeated this experiment followed
by a chase period of 90 min in the presence of MG132 to allow maximal
conversion into P-Xbp-1 C termi

nus(u). Cells were then washed to remove
MG132 (Fig. 3C, diagram). We followed the fate of P-Xbp-1 C termi

nus(u) or its K2R mutant generated from the ubiquitin fusion protein precur
 sor in the presence of MG132, and saw similar degradation profiles for
both the P-Xbp-1 C termi

nus(u) and its K2R mutant. Removal of MG132
did not affect the rate of degradation either. Likewise, we did not observe
accelerated degradation of the remaining intact Ub-P-Xbp-1 C termi

nus(u) (Fig. 3C). Thus, we conclude that the removal of MG132 did not restore the
proteasomal function. Nonetheless, the fact that the P-Xbp-1 C termi

nus(u) K2R mutant, although not containing any acceptor sites for ubiquitination,
was unstable and decayed at comparable rates to P-Xbp-1 C termi

nus(u) supports the notion that ubiquitination in this degradation reaction is
dispensable (Fig. 3D).

The ts20 cells express a temperature-sensitive E1 activity. Within 2 h
at the restrictive temperature (42 °C), de novo ubiquitination is blocked
and the cells undergo a G2 cell cycle arrest (11). We used these cells to
explore a requirement for ubiquitination in the degradation of Xbp-1u.
Unspliceable Xbp-1u was expressed by transfection and the cells were
incubated for 12 h at 42 °C or kept at 32 °C, the permissive temperature.
The 12-h exposure to 42 °C eliminates completely the high M, ubiquiti
nated species observable on SDS-PAGE. As an internal control we fol
owed the degradation of Ikb, which is degraded by the proteasome in a
strictly ubiquitin-dependent manner (18).

As expected, Xbp-1u was rapidly degraded at 32 °C. At 42 °C we
observed a reduction in the rate of degradation, but degradation still
continued. In contrast, Ikb degradation was completely blocked at the
restrictive temperature. Note the different time scale used in the analy
sis of Xbp-1u and Ikb turnover (Fig. 3E). We conclude that an ubiquitin
independent degradation mechanism significantly contributes to the
overall elimination of Xbp-1u.

Modulation of Xbp-1u Stability Affects the UPR—To examine
whether the unusually rapid degradation of Xbp-1u affects the mamma
lian UPR, we designed two Xbp-1 mutants that selectively affect Xbp-
1u, but not Xbp-1s. The first mutant was engineered to contain a stop
codon immediately after the intron. This mutant, designated as "stop,"
FIGURE 3. Ubiquitination is not required for C terminus(u)-mediated degradation. A, 293T cells were transfected with the indicated vectors. 24 h after transfection cells were pulse-labeled with [35S]methionine for 30 min and chased up to 60 min in the presence or absence of ZL3VS. Cells were lysed in 1% SDS, and lysate was then diluted to 0.07% SDS with Nonidet P-40 lysis mixture followed by immunoprecipitation with anti-HA antibodies and analyzed by SDS-PAGE (12%). A background band is labeled by the asterisk. B, same as in A. MG132 was used to block the proteasome instead of ZL3VS. C, 293T cells were transfected with the indicated vectors and pulse-chased according to the diagram. Cell lysis and immunoprecipitation were carried out as in A. D, autoradiograms were quantified by phosphorimager. E, ts20 cells were transfected with pcDNA3.1 that encodes unspliceable Xbp-1u. 24 h later the cells were incubated for an additional 12 h at 42 °C or kept at 32 °C. Cells were pulse-labeled for 20 min and chased up to 1 h for Xbp-1 analysis or up to 3 h for Ikb analysis. Cells were lysed in 1% SDS, and lysate was then diluted to 0.07% SDS with Nonidet P-40 lysis mixture followed by immunoprecipitation. Immunoprecipitates were analyzed by SDS-PAGE (12%). F, autoradiograms were quantified by phosphorimager.

Role of Unspliced Xbp-1 Degradation in the UPR

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expresses the stable N-terminal fragment (amino acids 1–175) instead of the full-length Xbp-1u. Upon splicing, the stop mutant should yield the exact open reading frame of Xbp-1s (Fig. 4A).

Xbp-1C terminus(u) contains two tandem proline (PP) elements. We reasoned that the PP elements might impose conformational constraints on the secondary structure of the polypeptide, and might therefore be required for as yet to be determined protein-protein interactions. These interactions might play a role in degradation. We therefore replaced the two PP sequences with leucine-histidine (LH), and refer to this Xbp-1 mutant as PP2LH. Again, this mutation was introduced in a manner that does not alter the open reading frame of Xbp-1s (Fig. 4A).

Wild type Xbp-1 and the two mutants were cloned into the pMig retroviral vector, harboring an internal ribosomal entry site-GFP element. Xbp-1+/−/− MEFs were transduced with these vectors. An empty pMig retrovirus was used as a negative control. After transduction, GFP positive cells were sorted and propagated in culture. Levels of GFP were comparable for the pMig control and the different Xbp-1 constructs (not shown). We then assayed the expression and stabilities of Xbp-1 by pulse-chase analysis. Wild type Xbp-1u was not detectable unless proteasome inhibitor was included (Fig. 4B). These results again demonstrate that Xbp-1u is degraded at a rate comparable with its rate of synthesis, preventing it from reaching detectable levels. As expected, the

FIGURE 4. Generation of mutants of Xbp-1u with improved stability. A, schematic depiction of Xbp-1 mutants. Mutants were prepared in a manner that does not alter the sequence of Xbp-1s. The stop mutant expresses a truncated Xbp-1u (amino acids 1–175). In the PP2LH the two PP sequences were replaced by LH sequences. B, WT Xbp-1, stop, and PP2LH were cloned into the pMig retroviral vector and the corresponding viruses were made. Xbp-1+/−/− MEFs were transduced with the retroviruses and GFP positive cells were sorted to 100% purity. Cells were pulse-labeled with [35S]methionine for 30 min and chased up to 60 min in the presence of the proteasome inhibitor ZLVS where indicated. Cells were lysed in 1% SDS, and the lysate was then diluted to 0.07% SDS with Nonidet P-40 lysis mixture followed by immunoprecipitation with anti-Xbp-1 antibody and analyzed by SDS-PAGE (12%). C, cells were treated with tunicamycin (1 μg/ml) for 8 h. Cells were pulse-labeled with [35S]methionine for 30 min and chased up to 30 min. Cell lysis and immunoprecipitation were carried out as in B.
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 FIGURE 5. PP2LH mutant activates UPR target genes better than WT. Total poly(A)⁺ RNA was isolated from MEF cells either treated or not treated with tunicamycin (1 μg/ml, 8 h). The levels of mRNA of Erdj4, Bip, and Chop were quantified by real-time PCR and expressed relative to the levels of β-actin mRNA.

stop mutant was readily detected and showed improved stability (Fig. 4B). The PP2LH mutant was detected at time 0, but was rapidly degraded. Incorporation of ZL3VS delayed its degradation, but did not fully block it (data not shown). We conclude that whereas the mutation PP2LH indeed improves the stability of Xbp-1u, it does not rescue it from rapid degradation.

To verify that the different Xbp-1 constructs retain the ability of being spliced, we treated the cells for 8 h with tunicamycin. Under these ER stress conditions, Xbp-1s was generated (Fig. 4C). Following 30 min of chase, Xbp-1s appeared as a disperse band at lower intensity, consistent with post-translational modifications and subsequent degradation. Under these conditions, the synthesis of the stop mutant was still detected, suggesting that the synthesis of WT Xbp-1u and PP2LH also continues, but their expression does not reach readily detectable levels because of rapid degradation.

Finally we assayed whether expression of Xbp-1u analogs with enhanced stability would affect the induction of the UPR. To this end, we measured by real-time PCR the level of mRNA of three target genes of the UPR in response to tunicamycin treatment. We measured the mRNA levels of Erdj4, a specific target of the Xbp-1 pathway (8); Bip, a target shared by all UPR pathways (19, 20); and Chop, a target downstream of the Perk/Atf4 pathway (19). As expected, Erdj4 transcription was hardly induced in the Xbp-1⁺/⁻ GFP cells. Reconstitution with the WT Xbp-1 gene fully restored induction. The reconstitution of the Xbp-1 stop mutant attenuated the induction of Erdj4 in comparison to reconstitution with WT Xbp-1. This is probably because of the dominant negative activity of truncated Xbp-1. We consistently observed that cells expressing the PP2LH mutant were superior to WT Xbp-1 expressing control cells with respect to induction of the Xbp-1 specific target gene, Erdj4 (Fig. 5, left panel). Unexpectedly, similar results were obtained when we examined the induction of Bip and Chop (Fig. 5, middle and right panels). We conclude that Xbp-1u, if stabilized, enhances the overall induction of the UPR and may modulate expression not only of Xbp-1–specific but also of other genes not usually considered Xbp-1 targets.

DISCUSSION

Many proteins are short-lived, a property exploited to obtain careful control over protein levels for regulatory purposes. For instance, IκB when phosphorylated by the IKK signalosome, is degraded with a half-life of 5–15 min (21). Class I major histocompatibility complex products are diverted for degradation by the human cytomegalovirus-encoded proteins US2 and US11, also with a half-life of minutes (22, 23). Both of these degradation reactions are readily demonstrable by pulse-chase analysis. Here we characterized a much faster degradation reaction. Even when expressed in stable fashion under the control of strong promoters, such as the cytomegalovirus or long terminal repeat, Xbp-1u is not detectable by pulse labeling or immunoblots. We could also recapitulate the robust degradation of Xbp-1u in a cell-free environment, and detect ubiquitinated intermediates in reticulocyte lysate. We further demonstrate that this unprecedented rate of destruction is not achieved by coinfection. Rather, the C terminus of the unspliced product utilizes nuclear export and may override the need for ubiquitination. The net result is that no physiologically relevant levels of Xbp-1u are produced.

Other examples of ubiquitin–independent, 20 S proteasome-dependent degradation are on record. NAD(P)H quinone oxidoreductase 1 was recently demonstrated to bind and regulate the degradation of p53 and ornithine decarboxylase (24, 25). Addition of dicoumarol inhibited NAD(P)H quinone oxidoreductase 1 activity and promoted the degradation of both substrates by a ubiquitin-independent mechanism. At least for p53, this mechanism occurs independently of its mdm2 ubiquitin-dependent pathway. We think that similarly to p53, mechanisms involving both ubiquitin–dependent and –independent pathways synergize to promote the degradation of Xbp-1u.

Yeast cells have evolved a different mechanism to avoid expression of unspliced Hac1p, the counterpart of Xbp-1. This is caused by a block in translation to prevent the expression of unspliced Hac1p altogether. Why should the expression of the unspliced Xbp-1 and Hac1 proteins be avoided? We speculated that Xbp-1u, unless rapidly removed, may perturb UPR signaling. To address this hypothesis we generated two Xbp-1 mutants, in which only the unspliced open reading frame was affected in its expression. One mutant, termed stop, eliminates completely the C terminus(u) domain. This mutant generates a stable Xbp-1 fragment that localizes to the nucleus and most likely competes with Xbp-1s, and so acts in a dominant negative fashion. The second mutant, termed PP2LH, was engineered to perturb the secondary structure of Xbp-1u, consequently alter its interactions with the degradation machinery, and thus impede its degradation. This approach was only partially successful but did yield a slightly more stable Xbp-1u.

We analyzed the expression of these constructs in Xbp-1⁺/⁻ MEFs to assess their contribution to the UPR. We observed the synthesis of the Xbp-1 stop mutant even under extreme conditions of ER stress, induced by tunicamycin treatment. This indicates that under physiological conditions Xbp-1u is synthesized constitutively. Indeed, under physiological induction of Xbp-1 splicing, as in the case of B cell differentiation into plasma cells, or in the course of viral infection, the vast majority of the mRNA of Xbp-1, nonetheless, remains in its unspliced form. It suggests that if Xbp-1s and Xbp-1u would have had comparable stabilities, then at any given time Xbp-1u expression would dominate, which might have dire consequences for a stressed cell to respond by induction of the UPR.

The Xbp-1 mutants allowed us to address this possibility. When we analyzed the induction of UPR target genes in MEFs reconstituted with
the different alleles of Xbp-1, we were surprised to see stronger induction of Erdj4, Bip, and Chop in the PP2LH mutant as compared with WT controls. We cannot exclude the possibility that the PP2LH is a gain of function mutation, however, we did not see induction of Bip when PP2LH was overexpressed by itself in an unsplciseable version (not shown). We also analyzed the localization of a GFP-unsliceable PP2LH Xbp-1 fusion protein, and found it evenly distributed between the nucleus and cytoplasm, very much like the GFP-Xbp-1u. Therefore, PP2LH must interact with other components of the UPR signaling pathway, in the course of activation by tunicamycin block.

Expression of these constructs in the B cell line BCL-1 activated all UPR targets similarly upon tunicamycin treatment. Therefore, the contribution of Xbp-1u for maintaining basal levels of transcription of multiple genes was inferred by Shen et al. (26). In Caenorhabditis elegans, 324 genes require the UPR machinery for transcription constitutively. Of these genes, 72 required ire-1. Interestingly, 239 required xbp-1. Only 13 genes overlapped between the two C. elegans deletion mutants. The difference between ire-1 and xbp-1 deficiency can be attributed to the transcription capacity of xbp-1u. Therefore, in C. elegans Xbp-1u accounts for the transcription of the majority of the constitutive UPR targets (26).

We conclude that under active UPR conditions, the presence of small levels of Xbp-1u significantly modulates the UPR signaling pathway. The functional consequences of interference with Xbp-1u degradation in the context of a living organism still remain to be discovered.

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