Regulation of the Unfolded Protein Response by eIF2B Isoforms

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Cells respond to a variety of stresses, including unfolded proteins in the endoplasmic reticulum (ER), by phosphorylating a subunit of translation initiation factor eIF2, eIF2α. eIF2α phosphorylation inactivates the eIF2B complex. The inactivation of eIF2B not only suppresses the initiation of protein translation but paradoxically up-regulates the translation and expression of transcription factor ATF-4. Both of these processes are important for the cellular response to ER stress, also termed the unfolded protein response. Here we demonstrate that cellular response resulting from eIF2α phosphorylation is attenuated in several cancer cell lines. The deficiency of the unfolded protein response in these cells correlates with the expression of a specific isoform of a regulatory eIF2B subunit, eIF2Bδ variant 1 (V1). Replacement of total eIF2Bδ with V1 renders cells insensitive to eIF2α phosphorylation; specifically, they neither up-regulate ATF-4 and ATF-4 targets nor suppress protein translation. Expression of variant 2 eIF2Bδ in ER stress response-deficient cells restores the stress response. Our data suggest that V1 does not interact with the eIF2 complex, a requisite for eIF2B inhibition by eIF2α phosphorylation. Together, these data delineate a novel physiological mechanism to regulate the ER stress response with a large potential impact on a variety of diseases that result in ER stress.

In eukaryotic cells, secreted and transmembrane proteins are folded in the endoplasmic reticulum (ER) prior to cellular transport. When unfolded proteins accumulate in the ER, as in periods of intense protein production (e.g. secretory myeloma), or when the ER milieu does not favor faithful folding (e.g. hypoxic tumors), a signaling pathway is activated to respond to this ER stress. This response, termed the unfolded protein response (UPR), permits the cell to adapt to ER stress via several mechanisms, including ER-associated protein degradation and generation of the XBP transcription factor (reviewed in Ref. 1). However, the central consequence of UPR activation is the attenuation of protein synthesis and the paradoxical concomitant increased translation of transcription factors, specifically ATF-4. Multiple studies have demonstrated that the UPR plays an important role in promoting cell survival in response to ER stress and also affects a variety of cellular phenotypes, including differentiation, tumor growth, and the inhibition of nonsense-mediated RNA decay (2–6).

The coordinated attenuation of protein synthesis and up-regulation of ATF-4 translation in response to ER stress is due to phosphorylation of the eukaryotic initiation factor eIF2α via the PKR-like ER-localized eIF2α kinase (PERK) (7–9). eIF2α can be also be phosphorylated by several cytoplasmic kinases that respond to a variety of stresses, including amino acid deprivation and double-stranded RNAs in an essential homeostatic process (10). The mechanism by which eIF2α phosphorylation attenuates protein translation has been extensively studied. eIF2α is one subunit of the trimeric eIF2 complex, which also contains eIF2β and eIF2γ. During the initiation of translation, the GTP-activated eIF2 complex forms a ternary complex with methionine-tRNA and binds to the 40 S ribosome. After this complex initiates translation, GTP is hydrolyzed, and eIF2 is released from the ribosome as an inactive eIF2-GDP complex. GDP must be exchanged for GTP in order for the ternary complex to reform and again initiate translation. This GDP-GTP exchange is carried out by the eIF2B complex. Phosphorylation of eIF2α on serine 51 by stress-activated kinases promotes the sequestration and functional inactivation of eIF2B.

Much of what is known about the eIF2B complex is derived from yeast studies (11–14). The eIF2B pentameric complex consists of both catalytic (γ, ε) and regulatory (α, β, and δ) subunits. In yeast, mutations in the regulatory subunits do not affect eIF2B GTP exchange activity directly but do reduce the ability of eIF2B activity to be inhibited by phosphorylated eIF2α. Overexpression of these subunits also reduces the inhibition of protein synthesis with eIF2α phosphorylation (15, 16). In mammalian cells, the eIF2B subunits that directly interact with eIF2α are not well delineated, although data suggest that recombinant human eIF2B interaction with eIF2α may be enhanced when eIF2α is phosphorylated (12, 14).

A variety of diseases are thought to lead to ER stress, including diabetes, where rapid synthesis of the secreted insulin peptide can overwhelm the UPR and induce pancreatic islet death; neurodegenerative diseases, such as Alzheimer and Parkinson diseases, which are associated with the accumulation of abnormal proteins in the ER; and cancer, where cellular hypoxia is thought to cause misfolding in the ER and activation of PERK (reviewed in Ref. 17). The importance of precise regulation of the eIF2-eIF2B complexes in protecting cells against ER stress is...
demonstrated by the cellular and tissue phenotypes apparent with perturbations of this system, both experimentally and naturally occurring. Mouse embryo fibroblasts deficient in PERK or with elf2α that cannot be phosphorylated (elf2α S51A) do not attenuate protein translation or increase ATF-4 production with ER stress (18, 19). These cells are more sensitive to ER stress. Animals with defects in PERK develop diabetes, and animals with defects in the elf2α phosphatases Gadd34 and CREP develop an anemia, as do animals deficient in HRI, the elf2α kinase most abundant in erythropoietic progenitors (20–23). Both experimental and human cancers exhibit up-regulation of ATF-4, and transformed cells deficient in ATF-4 and/or PERK cannot form tumors (2). Finally, the importance of the elf2α complex in the maintenance of homeostasis is best appreciated from data demonstrating that the genetic basis of central nervous system hypomyelination, also called vanishing white matter leukoencephalopathy, is mutations in any of the genes encoding the five subunits of elf2α and a decrease in elf2α GTP exchange (24–27).

Although the UPR can theoretically be augmented or attenuated, depending on the folding environment in the ER, the rate of protein synthesis, or the mutations described above, additional factors that might modulate the UPR have not been well explored. We previously demonstrated that a wide variety of primary, immortalized, and neoplastic cell lines robustly activated the UPR when stressed, which led to a down-regulation of Id-1, a protein that inhibits differentiation and proliferation (3). In these hypoxic cells, elf2α was phosphorylated in a PERK-dependent manner, ATF-4 was translationally up-regulated, and the ATF-4 targets ATF-3 and CHOP were transcriptionally up-regulated. The up-regulation of ATF-3 was responsible for the transcriptional repression of Id-1. However, previous reports suggested that Id-1 was not down-regulated in the neuroblastoma cell lines SK-N-BE(2) and SH-SYSY when rendered hypoxic (28, 29). We confirmed this observation and noted that, despite a high basal phosphorylation of elf2α, ATF-4, ATF-3, and CHOP were not up-regulated in response to thapsigargin (a calcium ionophore that permeabilizes the ER membrane) or tunicamycin (which leads to the glycosylation of ER proteins) or when rendered hypoxic. XBP-1 splicing still occurred in these cells, indicating that the ER stress was occurring and suggesting that these cell lines had a specific defect in their elf2α axis. In this report, we demonstrate that naturally occurring isoforms of a regulatory subunit of elf2B, elf2Bδ, are differentially expressed in several cell lines and that these isoforms can regulate the activity of the UPR.

MATERIALS AND METHODS

Cell Lines and Reagents—Neuroblastoma cell lines were either obtained from ATCC or derived from patients as described previously (SK-N-BE(2), BE(2)c, SK-N-BE(2)s, SK-N-ER, IMR6, IMR32, and SK-N-LP) (30). Additional cell lines have been described previously (3). Cells were grown in either DMEM having 4.5 g/liter glucose and 10% FCS or RPMI with 10% FCS. Prior to rendering cells hypoxic, medium was changed with prewarmed DMEM containing 4.5 g/liter glucose and 10% FCS or RPMI with 10% FCS. For tunicamycin treatment, medium was changed 1 h prior to experimentation. Cells were incubated at 37 °C, either in 5% CO₂ or in a Plas-Labs environmental chamber, and oxygen concentration in the chamber was maintained at ±0.5% as described previously.

Generation of elf2Bδ Knockdown and Overexpressed Stable U2OS Cells—C-terminal FLAG-tagged isoforms of elf2Bδ were amplified from human cDNA using Phusion HF Master Mix from Finnzymes and primers (variant 2 (V2) (short) upstream, 5′-AATCAGCCGCGCGCATGCTGCTGTGGCCGCTG-3′; V1 (long) upstream, 5′-AATCAGCCGGCCGCCCAATGCCAAGCCGACCAGCCGGC-3′; and common downstream, 5′-GCTGCTACCAGGTTTCTGACGTGCTGCTTGTGA-3′) and cloned into the NotI and Aegel restriction sites of the retroviral plasmid pQCXIN. Plasmids encoding lentiviruses with shRNA sequences directed against elf2Bδ were obtained from Sigma (SHCLNG-015636), and both retroviruses and lentiviruses were generated using standard techniques as described previously, with selection of U2OS cells with puromycin and Geneticin (G418), respectively, as described below. After determining the sequence for the most effective knockdown (GCCATCAAGTTCCCTAACA), the corresponding sequences in the two expression plasmids for elf2Bδ were mutated by performing PCR with a primer containing the mutated sequence 5′-CCC-TGTACCAGGACATGCACATGCGATTAAATCTCTTTAACAGGAAATCACAG-3′, so that, in contrast to endogenous elf2Bδ, they were unaffected by the shRNA. All plasmids were sequence-verified.

Preparation of Protein Lysate and Immunoblot—After scraping in phosphate-buffered saline, the cells were resuspended with lysis buffer containing protease inhibitors, briefly sonicated, and quantitated, and 40–50 μg of whole cell extract were electrophoresed and transferred to nitrocellulose as described previously. Membranes were then blocked and probed with antibodies for CHOP (Santa Cruz Biotechnology, Inc. (sc 7351), ATF4 (sc-200), ATF3 (sc-188), ID1 (sc-488), rabbit elf2Bδ (sc-28855), mouse elf2Bβ (31), elf2Bβ (sc-28852), elf2Bγ (sc-28853), FLAG, rabbit HA (TX2 29110, Gene Tex Inc.), elf2α (sc-133132), phospho-elf2α (119A11, Cell Signaling), and red anti-FLAG M2 Affinity Gel (F2426, Sigma). After incubation with a fluorescent secondary antibody (goat anti-rabbit from LI-COR Biosciences (92632211) and goat anti-mouse from LI-COR Biosciences (92632220)), the membranes were scanned and quantified in the Odyssey infrared imaging system (LI-COR Biosciences). Protein expression was quantitated by running a standard lysate to correct for gel, transfer, and hybridization variability. In all cases, expression was also normalized to an internal control (total elf2α), which is known to not vary with activation of the UPR.

XBP1 Splicing Assay—Cells were treated with tunicamycin (9 h), total RNA was collected, and total XBP1 and spliced XBP1 were assessed as described previously (3).

Assessment of Protein Synthesis—Cells were plated subconfluently in 6-well dishes. Before starting the experiment, the medium was changed with prewarmed DMEM containing 1 g/liter glucose and 10% FCS. The cells were then treated with tunicamycin (2.5 μg/ml) for 1 or 3 h, respectively. The cells were metabolically labeled for 30 min by changing to [35S]methionine (MP Biomedicals) containing medium (100 μCi/ml
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[35]S)methionine, 90% DMEM with 2 mM L-glutamine and without sodium pyruvate, L-Met and L-Cys, 10% regular DMEM, and 5% FCS). The cells were washed twice with ice-cold PBS and scraped, and the pellets were resuspended in the above mentioned lysis buffer. After freezing and thawing, cell lysates were further rotated at 4 °C for 1 h and spun down for 5 min at 13,000 rpm. Equal amounts of protein were loaded on 10% SDS gel and stained with Coomassie Blue. Autoradiograms were quantified by an Amersham Biosciences Storm 820 PhosphorImager and normalized to total protein loaded.

Real-time PCR—Total RNA were extracted using TRIzol reagents (Invitrogen). cDNA was prepared using the High Capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR was performed using SYBER Green qPCR Master Mix (Fermentas). Expression of 18 S rRNA was used for normalization of expression level of mRNA samples (3). Real-time primers included the following: eIF2β V2 (short), 5’-ATGGCTGTGCGCCCTGGCT-3’ (upstream) and 5’-CATCCCGGATCCCAGTCT-3’ (downstream); eIF2β V1 (long), 5’-CGGAGTCTCTCTGGCTACT-3’ (upstream) and 5’-GTCATTTCCCTCCACTG-3’ (downstream); 18 S, 5’-GGACAGGAGCAGGATGCA-3’ (upstream) and 5’-ACCACCCAGATCCGAAAGA-3’ (downstream).

Immunoprecipitations—The C-terminal HA-tagged eIF2β was amplified from human cDNA using primers 5’-AATGCCGGCCGCCGCACATGCTGGGGACAGATGATT-3’ (upstream) and 5’-TGCAACAGTTTTAAAGCTAGTCTGGGAGCTTGATATTAGCCTTTGGGACAGGACT-3’ (downstream) and then cloned into NotI and HindIII sites of retroviral vector pCDNA 3.1 (-). C-terminal FLAG-tagged isoforms of eIF2β were also cloned into NotI and HindIII sites of pCDNA 3.1 (-) by using previously mentioned primers. Both eIF2β and eIF2β plasmids were transfected into 293T cells. After 24 h, cells were divided and treated with tunicamycin or mock-treated for 7 h. To stop the treatment, cells were washed twice with ice-cold PBS, scraped in 500 μl of lysis buffer (150 mM NaCl, 20 mM HEPES, pH 7.5, 10% glycerol, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NAF, 17.5 mM glyceraldehyde, 1 mM PMSF, and protease inhibitors) and incubated for 5 min on ice. FLAG-tagged isoforms of eIF2β were pulled down by incubating EZview Red anti-FLAG M2 affinity gel (F2426, Sigma-Aldrich) with 600 μg of whole cell extracts for overnight at 4 °C. After extensive washing, eIF2β retained on the eIF2β beads was eluted with 4× Laemmi buffer and analyzed by immunoblot. Interactions of eIF2α and eIF2β were assessed with HA-tagged eIF2β and non-tagged eIF2α (provided by H. Harding), and interactions with eIF2β and eIF2β were done by first immunoprecipitating endogenous eIF2β with antibody (H300, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)) attached to fast flow protein A-Sepharose beads (Amersham Biosciences) and then immunoblotting with eIF2β antibody.

RESULTS

Activation of the Unfolded Protein Response Varies among Cell Lines and Is Inactive in Some Neuroblastoma Cell Lines—We previously noted that Id-1 was down-regulated in most hypoxic cells due to activation of the UPR, phosphorylation of eIF2α, and subsequent induction of the transcriptional repressor ATF-3 (which is an ATF-4 target) (3). However, we also observed that in several hypoxic neuroblastoma cell lines, ATF-4 and ATF-3 were not induced, and indeed Id-1 was not down-regulated. These cells also did not induce ATF-3 or other ATF-4 targets when treated with a variety of chemicals known to induce ER stress and activate the UPR. XBP-1 splicing and ligation was normal in these cells, demonstrating that these cells were still able to sense ER stress and suggesting that there was a defect specifically in the eIF2α axis.

In order to identify cellular factors that might modulate the eIF2α axis of the UPR, we assessed eIF2α phosphorylation; ATF-4, CHOP, and ATF-3 protein induction; and Id-1 response in response to ER stress in multiple cell lines, including over 10 neuroblastoma cell lines. Although there was some variation in both the response to different stresses and in the induction of specific UPR-induced proteins, in general, we observed a robust activation of the UPR in multiple non-neuroblastoma human cell lines (including LN229, HaCAT, HeLa, and U2OS cells) when they were rendered hypoxic or treated with the chemical reagent tunicamycin over several time periods (chosen because they demonstrated, on average, the most significant activation of the UPR) (Fig. 1, A and B, group I cells and data not shown). UPR activation differed significantly among stressed neuroblastoma cell lines. In some of the tested cell lines (e.g. IMR6, SK-N-LP, SK-N-ER, SH-EP1, and IMR32), UPR activation in response to hypoxia and tunicamycin was similar to HeLa cells; these cells were defined as group IA cells (Fig. 1, A and B) (data not shown). However, several neuroblastoma cell lines (e.g. SK-N-BE(2), SK-N-BE(2)s, BE(2)c, and SH-5YSY) had a nearly absent response, and we defined these as group II cells (Fig. 1, A and B) (data not shown). When the signals from these immunoblots were quantified, there was a significant difference in UPR activation between group II cells and group I cells when they were rendered hypoxic (Fig. 1C) or treated with tunicamycin (Fig. 1D). Of note, although eIF2α phosphorylation was induced with ER stress in group I cells, for the most part, eIF2α phosphorylation did not dramatically increase with stress and was highly phosphorylated basally in group II cells, perhaps due to the inefficient up-regulation of the eIF2α phosphatase Gadd34 which is an ATF-4 target. Consistent with these results and our previous publication, we observed differences in hypoxia-induced Id-1 expression between these three groups of cell lines. Id-1 was significantly down-regulated in hypoxic group I cells. However, in general, Id-1 was up-regulated or minimally down-regulated in hypoxic group II cells (Fig. 1, A–D), confirming that in some neuroblastoma cells, an inactive unfolded protein response prevents the hypoxic down-regulation of Id-1 by ATF-3.

Inactivity of the Unfolded Protein Response Correlates with Expression of a Distinct Isoform of eIF2β—We next sought to identify the molecular basis underlying the altered UPR in group II cells by identifying genetic differences between these cells and group I/IA cells that have an intact UPR. Because there are no obvious gross genetic deletions or translocations that correlate with UPR activation in the these groups (e.g. SK-N-BE(2), SK-N-BE(2)s, and BE(2)c have mutated p53, whereas SK-N-ER, IMR6, IMR32 and SK-N-LP have wild type p53, and...
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The unfolded protein response is blunted in several neuroblastoma cell lines. A, HeLa, HaCat, SK-N-ER, SK-N-BE(2), SK-N-BE(2)c, SH-5YSY, and BE(2)c cell lines were rendered hypoxic for 16 or 24 h on tunicamycin for 16 or 24 h, and protein was collected and immunblotted for expression of components of the UPR. Cells were divided into those that induced (groups I and IA) and did not induce (group II) the UPR. Representative immunoblots are displayed. 

A distinct eIF2Bδ isoform is preferentially recognized by a monoclonal antibody. A, protein from several cell lines with inactivating ATPase and active UPRs was immunblotted for eIF2Bδ using a monoclonal antibody as well as a polyclonal antibody directed against the C terminus. eIF2α is shown as a loading control. B, two isoforms, derived from alternative splicing of the eIF2Bδ mRNA, result in peptides with divergent N-terminal sequences. C, overexpression of V2 and V1 eIF2Bδ isoforms demonstrates that the V1 form is recognized by a monoclonal antibody, whereas both forms are recognized by a polyclonal antibody raised against the C-terminal portion of human eIF2Bδ.

only SK-N-LP has a p14ARF deletion, we focused on potential molecular events that could specifically block activation of the UPR despite eIF2α phosphorylation, as noted in group II cells (Fig. 1). When eIF2α is phosphorylated, it binds to and inactivates eIF2B, which is composed of several catalytic and regulatory subunits. Reconstituted rat eIF2B complexes containing mutations in a regulatory eIF2B subunit, eIF2Bδ, or missing eIF2Bδ entirely are known to be resistant to eIF2α phosphorylation (24–27, 32, 33). We thus compared the expression of eIF2Bδ with UPR activation in several cell lines. In all cells, we noted that eIF2Bβ was uniformly present, as was eIF2α. Although a polyclonal antibody, raised against the C terminus of human eIF2Bδ sequence, showed equivalent expression in all cell lines, a monoclonal antibody raised against full-length rat eIF2Bδ (31) showed higher expression in two neuroblastoma cell lines with an inactive UPR as opposed to several cells with an active UPR (Fig. 2A).

The rat and human eIF2Bδ proteins have high (87%) homology, particularly in the carboxyl region. Although the differences observed in immunoreactivity of the two antibodies could be due to several factors, including recognition of protein modifications, we hypothesized that the monoclonal antibody recognizes a sequence distinct from the carboxyl region of eIF2Bδ recognized by the polyclonal antibody. A review of genetic databases revealed three referenced transcripts for the eIF2Bδ gene (36). V1 encodes a 543-amino acid peptide, and two other variants encode almost identical 523- and 522-amino acid peptides. Intriguingly, the proteins predicted from V1 and the other two isoforms differ in their N-terminal sequences. Specifically, V1 and V2 are identical after amino acids 32 and 11, respectively, but have no similarity upstream to these regions (Fig. 2B). When these two isoforms were overexpressed in HeLa cells, the polyclonal antibody recognized proteins expressed from either construct, whereas the monoclonal antibody preferentially recognized V1, the longer isoform protein expression in the carboxyl region (Fig. 2C). Together these data suggest that the short variant of eIF2Bδ (V2) does not contain an epitope well recognized by the monoclonal antibody and that several neuroblastoma cell lines with absent UPRs highly express the long variant of eIF2Bδ (V1), which is recognized by the monoclonal eIF2Bδ antibody.

We therefore examined eIF2Bδ isoform protein expression in the cell panel we had previously assessed and classified for UPR activation, in order to determine whether the expression of eIF2Bδ isoforms correlates with UPR activity. Again we noted relatively consistent eIF2α and eIF2Bβ protein levels among this diverse panel of cell lines (Fig. 3A). In most cells, eIF2Bγ expression was also relatively constant. Although several cell lines did not appear to have a form recognized by our antibody, eIF2Bγ expression in these cells did not correlate with UPR activation in response to ER stress. Total eIF2Bδ protein,
as assessed by the polyclonal antibody, and the long variant derived from V1 mRNA, as assessed by the monoclonal antibody, varied dramatically among cell lines (Fig. 3A). Of the 13 cell lines tested, those cells with the inactive UPR (group I) had four of the top five highest ratios of the long eIF2Bβ expression to total eIF2Bβ expression (Fig. 3B, left). When these were normalized to total eIF2α and quantified (from three separate experiments), we noted that, on average, group II cells had a significant increase in expression of the long protein expressed from V1 as compared with group I cells (Fig. 3B).

In order to determine whether the protein expression of eIF2Bβ variants in these cell lines is also reflected at the mRNA level, and also because we noted that the specificity of the monoclonal antibody decreases with increasing overexpression of the eIF2Bβ isoforms (data not shown), we designed PCR primers to quantitatively assess individual eIF2Bβ isoform expression. One set of primers amplifies a region in the common 3′ area of the gene and thus assesses total eIF2Bβ mRNA expression; a second set amplifies only the V1 eIF2Bβ isoform, and a third set amplifies only V2. These primers selectively amplify these isoforms, as demonstrated with control PCRs with cDNAs for each of the isoforms (Fig. 3C). Expression of both isoforms was noted in all cell lines and quantified (Fig. 3D). There was no significant pattern of eIF2Bβ V2 mRNA expression among cell lines, but V1 eIF2Bβ mRNA was expressed highest in the group II cells (Fig. 3D, left). On average, there was a slight although non-statistically significant increase in V2 mRNA expression between group I and group II cells (Fig. 3D, right). There was also no statistical difference in V1 eIF2Bβ mRNA expression between group I and group IA cells (data not shown), both of which have active UPRs. However, there was a highly significant increase in V1 eIF2Bβ expression, responsible for generating the long eIF2Bβ protein, in group II cells compared with group I and IA cells (p < 0.05 versus group I cells). C. PCR primers were designed to amplify either both isoforms of eIF2Bβ (common), only V1, or only V2. Specificity is demonstrated by utilizing cDNAs for each isoform. D. PCR primers were used to assess the expression of eIF2Bβ mRNA isoforms of group I and group II cells (left), along with average expression in group I and group II cells (with S.E. and p values by Student’s t-test).

Expression of a Distinct Isoform of eIF2Bβ Attenuates the Unfolded Protein Response—Our data demonstrate that distinct eIF2Bβ isoform expression correlates with UPR activation in response to ER stress. To determine the causal role of each eIF2Bβ isoform in modulating the UPR, we sought to create a system in which we could study the effect of each eIF2Bβ isoform in isolation. We therefore generated several lentivirus shRNAs targeting distinct sequences in the 3′-region of the eIF2Bβ mRNA common in all isoforms, infected U2OS cells, and assessed total eIF2Bβ protein expression (Fig. 4A). After choosing the shRNA sequence with the most effective knockdown for further studies, we created cells expressing V1 or V2 eIF2Bβ isoforms that are impervious to this shRNA (due to mutations introduced into the nucleotide sequence that maintain fidelity in amino acid sequence). In this way, we were able to produce cells that selectively overexpressed each eIF2Bβ isoform ~3-fold, with minimal expression of endogenous eIF2Bβ; in parallel, we also constructed control cells infected with a scramble knockdown lentivirus and an empty retrovirus (Fig. 4B).

These cells were then assessed for UPR activity. Cells were treated with tunicamycin, and the induction of ATF-4 and its
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downstream targets CHOP and ATF-3 was determined at the protein level (Fig. 5A, quantitated in Fig. 5B), and the mRNA induction of two transcriptional targets of ATF-4, gadd34 and CHOP, was assessed by real time PCR (Fig. 5C). Although there was some variation in individual experiments, on average, we noted a severe blunting of UPR activation when V1 was over-expressed, as compared with the cells infected with V2 or with a scramble knockdown lentivirus and retroviral control. Of note, eIF2α phosphorylation was elevated basally and did not increase with ER stress in cells overexpressing V1 eIF2Bδ, similar to what was noted in group II neuroblastoma cells with an aberrant UPR (Fig. 1) (3). We used a similar strategy to express only V1 and V2 in 293 cells (Fig. 5D) and again noted that cells expressing V1 had a blunted UPR with tunicamycin treatment (Fig. 5E). To rule out the unlikely possibility that the impact of the eIF2 axis of the UPR with expression of eIF2Bδ V1 was due to an alteration in the formation of misfolded proteins in the ER, we assessed an eIF2-independent arm of the UPR, XBP-1 splicing, in manipulated U2OS cells in response to tunicamycin. As expected, splicing of XBP-1 occurred in all cell lines regardless of eIF2Bδ isoform expression (Fig. 5F).

In addition to inducing ATF-4 and ATF-4 targets, an important consequence of an activated UPR is the attenuation of protein synthesis. To assess rates of protein synthesis in the cell lines expressing distinct eIF2Bδ isoforms, U2OS and 293 cells were treated with tunicamycin and then pulsed with [35S]methionine. Cell lysates were collected, proteins were separated by acrylamide electrophoresis, and radioactive incorporation for each lane was assessed and normalized to total protein loaded. Basal levels of protein synthesis were similar between U2OS cell lines and between 293 cell lines, suggesting that expression of either eIF2Bδ isoform does not have a large impact on un-stressed cells. We reproducibly, in three independent experiments, noted a ∼30–40% decrease in protein synthesis after 1 and 3 h of tunicamycin treatment in all control and eIF2Bδ V2-expressing U2OS cell lines but not in U2OS cells expressing V1 of eIF2Bδ (Fig. 5G). Control and V2-expressing 293 cells had a more robust attenuation of protein synthesis in response to tunicamycin, but this repression of protein synthesis was again blunted in cells expressing eIF2Bδ V1 (Fig. 5H).

Our previous data demonstrated that SK-N-BE(2) cells have a blunted UPR and primarily express V1 eIF2Bδ (Fig. 1). We overexpressed V2 eIF2Bδ in these cells without knocking down endogenous eIF2Bδ to determine if this would rescue the UPR (Fig. 5I). In control cells (infected with an empty vector retrovirus), ATF-4 induction was again blunted as noted previously in unmanipulated cells (Fig. 5J). When treated with tunicamycin and then pulsed with [35S]methionine, these control SK-N-BE(2) cells did not demonstrate a suppression in protein synthesis, again demonstrating a defective UPR (Fig. 5K, quantitated on the right). However, with the expression of V2 eIF2Bδ, tunicamycin led to both an elevation in ATF-4 expression and a decrease in protein synthesis (Fig. 5, J and K, respectively). Together these data demonstrate that expression of distinct isoforms of eIF2Bδ can blunt the two downstream events of an activated UPR and eIF2α phosphorylation: the attenuation of protein synthesis and the up-regulation of ATF-4 and its downstream targets.

Expression of a Distinct Isoform of eIF2Bδ Interferes with the Interaction of the eIF2B and eIF2 Complexes—We next pursued the mechanism for a blunted UPR in the presence of the long variant of eIF2Bδ, focusing on the interactions of the eIF2 and eIF2B complexes in the presence of stress and eIF2α phosphorylation. When the α component of the eIF2 complex is phosphorylated, the eIF2 complex binds to the eIF2B complex and inhibits eIF2B exchange. Direct interactions between human endogenous eIF2 and eIF2B subunits have not been demonstrated (reviewed in Ref. 11). However, data primarily from assays using human recombinant proteins suggest that phosphorylation of eIF2α increases the interaction between eIF2β (not eIF2α) and eIF2Bδ and eIF2Be (14, 34).

We hypothesized that distinct isoforms of eIF2Bδ would display different interactions with the eIF2 complex, particularly in times of stress when eIF2α is phosphorylated. Specifically, we reasoned that the V1 eIF2Bδ would not bind, and thus inhibit, the eIF2 complex when eIF2α was phosphorylated. We first confirmed that a tagged eIF2β construct associated with other members of the eIF2 complex by immunoprecipitating tagged eIF2β and immunoblotting for endogenous eIF2α (Fig. 6A). A possible explanation for UPR attenuation in cells expressing V1 eIF2Bδ is that this expression renders the eIF2B complex unstable. We therefore also confirmed that both the V1 and V2 variants of eIF2Bα associate into an eIF2B complex by transfecting either construct into cells, immunoprecipitating eIF2Bβ, and immunoblotting for eIF2Bδ (Fig. 6B). These experiments demonstrated that both variants of eIF2Bδ associate with the catalytic eIF2B subunit eIF2Bβ.

FIGURE 4. Generation of cells that only express one eIF2Bδ isoform. A, U2OS cells were infected either with scramble (scr) or eIF2Bδ shRNAs, and protein expression for eIF2Bδ was determined. B, shRNA 1 (sh#1) from A (sequence defined under "Materials and Methods") was used to generate eIF2Bδ knockdown U2OS cells, which were then infected with retroviral empty vector (pQCXIN) or retrovirus expressing the V2 or V1 isoforms. Immunoblot (top) and quantitation (below) are displayed.
Consistent with multiple previous studies, we were unable to demonstrate interaction between endogenous components of the eIF2 and eIF2B complexes. We thus elected to transfected 293T cells first with HA tagged eIF2β and then with FLAG-tagged isoforms of eIF2Bδ or a plasmid control. After transfection, cells were treated with tunicamycin to induce eIF2α phosphorylation or mock-treated. Protein was collected after 3 h, a time point chosen because of maximal induction was assessed by immunoblot. OCTOBER 15, 2010 • VOLUME 285 • NUMBER 42

DISCUSSION

We have demonstrated that there is a large variation in UPR activation between cell lines in response to several provokers of ER stress (including hypoxia and tunicamycin). Although variations in the UPR could be due to differential rates of protein synthesis and/or differences in the ER capacity to reduce and fold proteins, our previous and current data do not demonstrate a difference in XBP-1 splicing in these cell lines, suggesting that misfolded proteins accumulate in the ER even in those cells with blunted UPRs. Thus, although we did not assess ATF-6 generation or ER-associated protein degradation, two other arms of the UPR, it appears that we have identified a specific defect in the eIF2 axis of the UPR. Theoretically, an aberrant eIF2 axis could be explained by alterations in the expression of the kinases or phosphatases responsible for regulating eIF2α phosphorylation. However, we did not note a deficiency in eIF2α phosphorylation in these cells but rather an
altered induction of downstream events with ER stress (i.e., induction of ATF-4 and suppression of protein synthesis). In fact, if anything, eIF2α phosphorylation was increased in cells that did not respond appropriately to ER stress, perhaps due to the poor inductions of the eIF2α phosphatase Gadd34 and ER chaperones that are responsible for protein folding (both of which are ATF-4 downstream targets) and/or a failure of these cells to appropriately suppress protein synthesis.

Our current work assessed eIF2α phosphorylation in hypoxic and tunicamycin-treated cells. These two stresses are known to work primarily though the ER-residing PERK kinase. Although we did not formally assess cellular phenotypes in response to amino acid starvation, double-stranded RNA, or activators of other eIF2α kinases, there is no reason to assume that our findings are unique to PERK-induced phosphorylation of eIF2α. Thus, we would expect that cell lines identified and generated with an aberrant UPR would also be defective in attenuating protein synthesis and up-regulating ATF-4 in response to other stresses that phosphorylate eIF2α.

Our hypothesis, that expression of distinct eIF2Bδ isoforms might regulate the cellular response to eIF2α phosphorylation, was based on the documentation of several distinct reference eIF2Bδ isoforms. In addition, we were able to amplify these isoforms by RT-PCR in multiple cell lines. Because of alternative splicing, the mRNAs of these isoforms differ in their 5′ sequence, which results in peptides with different N-terminal amino acid sequences. The advent of exon arrays, in which multiple probes can assess the expression of individual exons, and whole transcriptome sequencing has elevated the appreciation for the number of alternative splicing events in the human transcriptome (35). Although alternative spliced transcripts may display distinct expression patterns during differentiation and transformation, in most cases the events that regulate these alternative splicing events are unknown. It is unclear what mechanisms are responsible for the differential expression of eIF2Bδ isoforms and whether isoforms are dynamically regulated during transformation or differentiation or in different tissues. Although we have observed different UPR activities in neuroblastoma cell lines as they differentiate in culture (data not shown), we have not yet attempted to correlate these findings to eIF2Bδ isoform expression.

Our data suggest that the longer protein expressed from V1 eIF2Bδ does not interact with the eIF2 complex; this interaction is necessary to attenuate eIF2β activity, suppress protein translation, and induce ATF-4. We also studied an additional eIF2Bδ mRNA isoform found in the UCSC data base (36) and in our cell lines, which is identical to V2 except that it is missing the first 105 nucleotides and thus the first 35 amino acids. When cells had endogenous eIF2Bδ replaced with this variant, they did not behave differently than V2 or control cells in suppressing protein synthesis, up-regulating ATF-4 and ATF-4 targets when stressed, or interacting with phosphorylated eIF2α (data not shown). These data demonstrate that an N-terminal truncated eIF2Bδ can function normally and further suggest that it is the extended amino portion the V1 eIF2Bδ isoform that interferes with eIF2Bδ interaction with the eIF2 complex. Formal testing of this hypothesis will be aided by co-crystallization of the human eIF2-eIF2B complexes.

Because the integrated stress response plays such a fundamental role in health and disease, physiological and pathological factors that influence its activity can have a major impact on many aspects of biology. Thus, both experimentally provoked and naturally occurring mutations in components of the eIF2-eIF2B axis (including mutations in eIF2α kinases, eIF2α phosphatases, and eIF2B regulatory subunits) as well as deletions in downstream components of the integrated stress response (e.g., ATF-4 and its targets CHOP and ATF-3) are all associated with diverse disease states, including myelin degeneration, diabetes, and anemia. Several recent studies have also demonstrated the importance of protein translation in allowing cells to adapt to transformation and the tumor microenvironment and perhaps promoting the transformation process itself (37, 38). Specifically, the UPR is thought to allow cancer cells to adapt to hypoxia and grow as large tumors (2). Seemingly paradoxically, eIF2B activity has also been demonstrated to increase during in vitro transformation models, probably due to an increase in eIF2Be mRNA (39). Consistent with this finding, reduced eIF2B activity via knockdown of eIF2Be decreases the transformation capability of a breast cancer cell line, as demonstrated by soft agar growth and growth in nude mice, and eIF2Be mRNA is up-regulated in a variety of tumors (39, 40). However, the increase of eIF2B activity in malignancy does not necessarily suggest that the regulation of this activity, via the UPR, does not play an important role in transformation.

We elected to assess UPR activity in a panel predominantly consisting of neuroblastoma cells because the hypoxic down-regulation of Id-1, an event dependent on UPR activation, does not occur in several neuroblastoma cell lines (3, 28, 29). However, our assessment of UPR activity and eIF2Bδ isoform expression was limited to a relatively small number of cell lines. Id-1 is elevated in several cancers, including breast cancer, prostate cancer, and acute myelogenous leukemia, and may play an important role in neovascularization, metastasis, and endothelial cell biology (41–46). Future studies should explore the intactness of the UPR and expression of eIF2Bδ isoforms in these cancers. The induction of ATF-3 in hypoxic cells or exogenous expression of ATF-3 can decrease Id-1 expression and promote differentiation of neuroblastoma cell lines (3). A plethora of data suggests that hypoxia can either inhibit or promote differentiation, depending on the cell type (47, 48). It is tempting to speculate that at least part of the reason for the disparate effects of hypoxia on cell differentiation could be the disparate hypoxic activation of the UPR and down-regulation of the Id proteins. Modulation of the UPR may also help explain the variable penetrance of many of the diseases that activate the UPR (e.g., diabetes). The role of the novel mechanism, which we have demonstrated regulates the UPR, in cancer and other diseases affected by the UPR deserves further study.

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