Endoplasmic Reticulum Stress Increases the Expression of Methyleneitetrahydrofolate Reductase through the IRE1 Transducer*

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Methylenetetrahydrofolate reductase (MTHFR), an enzyme in folate and homocysteine metabolism, influences many cellular processes including methionine and nucleotide synthesis, methylation reactions, and maintenance of homocysteine at nontoxic levels. Mild deficiency of MTHFR is common in many populations and modifies risk for several complex traits including vascular diseases, birth defects, and cancer. We recently demonstrated that MTHFR can be up-regulated by NF-κB, an important mediator of cell survival that is activated by endoplasmic reticulum (ER) stress. This observation, coupled with the reports that homocysteine can induce ER stress, prompted us to examine the possible regulation of MTHFR by ER stress. We found that several well characterized stress inducers (tunicamycin, thapsigargin, and A23187) as well as homocysteine could increase Mthfr mRNA and protein in Neuro-2a cells. The induction of MTHFR was also observed after overexpression of inositol-requiring enzyme-1 (IRE1) and was inhibited by a dominant-negative mutant of IRE1. Because IRE1 triggers c-Jun signaling, we examined the possible involvement of c-Jun in up-regulation of MTHFR. Transfection of c-Jun and two activators of c-Jun (LiCl and sodium valproate) increased MTHFR expression, whereas a reported inhibitor of c-Jun (SP600125) and a dominant-negative derivative of c-Jun N-terminal kinase-1 reduced MTHFR activation. We conclude that ER stress increases MTHFR expression and that IRE1 and c-Jun mediate this activation. These findings provide a novel mechanism by which the ER can regulate homeostasis and allude to an important role for MTHFR in cell survival.

The endoplasmic reticulum (ER)\(^2\) is a dynamic membranous organelle that plays a critical role in the folding, transport, and processing of newly synthesized proteins. Numerous xenotoxic agents and adverse metabolic conditions interfere with protein folding in the ER leading to cellular stresses, known collectively as ER stress. For example, tunicamycin blocks N-glycosylation and leads to the accumulation of misfolded proteins in the ER. Thapsigargin inhibits the ER Ca\(^{2+}\)-ATPase and is also a very potent ER stress inducer. The Ca\(^{2+}\) ionophore A23187 influences stress through depletion of Ca\(^{2+}\) stores in the ER. These chemicals, the most widely used agents to experimentally induce ER stress, activate a complex signaling pathway known as the unfolded protein response (UPR). The UPR represents a set of signaling cascades by which conditions within the ER are communicated to the protein translation machinery (to decrease ribosome activity and promote degradation of mRNAs for ER proteins) and to the nucleus (through transcription factors) to balance the folding capacity of the ER with the protein processing demand (1, 2). Three ER-resident transmembrane proteins, inositol-requiring enzyme-1 (IRE1), pancreatic ER stress kinase (PERK), and activating transcription factor 6 (ATF6) have been identified as proximal sensors of ER stress (1). Activation of these transducers/transcription factors results in the up-regulation of genes encoding ER chaperone proteins such as GRP78 that facilitate protein folding and reduce protein aggregation. The UPR also affects proteins that are not directly involved in ER function but have a role in cell survival after exposure to ER stress. Failure to counteract induced ER stress can result in activation of apoptosis.

Homocysteine (Hcy) is a thiol-containing amino acid generated by demethylation of methionine. Hcy can inhibit the biosynthesis of proteins normally secreted by some cells; this has been attributed to ER retention of proteins (3). The molecular targeting hypothesis for Hcy-induced damage (4) suggests that Hcy can form stable disulfide bonds with cysteine residues in proteins, thereby altering specific cellular processes and pathways. In vitro studies have provided experimental evidence for the effect of Hcy on expression of ER stress response genes (5, 6).

Methylenetetrahydrofolate reductase (MTHFR) is a critical enzyme in Hcy metabolism. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. This reaction is the only source of 5-methyltetrahydrofolate, which serves as the methyl donor in the remethylation of Hcy to methionine. MTHFR first received medical recognition in reports of patients with homocystinuria (OMIM number 236250), an inborn error of metabolism that can be caused by...
deleterious mutations in the MTHFR gene (7). However, considerably greater interest has been generated in MTHFR through our identification of a common variant, $677^{Cys} \rightarrow Thr$ (A222V) (8), which increases risk for vascular disease, neural tube defects, and possibly other birth defects (9). This variant may also increase risk for certain neoplasias, although it lowers the risk of colorectal cancer when folate status is adequate. MTHFR may influence disease through elevation of plasma homocysteine, disruption in methionine or S-adenosylmethionine synthesis or altered distribution of folate metabolites with consequent effects on nucleotide synthesis (9). Mthfr knock-out mice have an elevation of plasma Hcy, a decrease of S-adenosylmethionine and DNA methylation, and altered folate distributions (10, 11). The cerebellar pathology in Mthfr$^{-/-}$ mice is associated with increased apoptosis (12).

Studies on MTHFR regulation are few in number. S-Adenosylmethionine is an allosteric regulator of MTHFR and the post-translational modification of MTHFR by phosphorylation leads to decreased activity and increased sensitivity of the enzyme to S-adenosylmethionine (13). Phosphorylation of MTHFR has been demonstrated for the smaller MTHFR isoform (70 kDa); phosphorylation of the larger isoform (77 kDa) has not been examined (13). We recently characterized 2 promoters for MTHFR, each of which may direct the synthesis of one of the two isoforms through different transcriptional start site clusters and alternative splicing (14). We also demonstrated that MTHFR expression was enhanced by NF-$\kappa$B through an effect on the downstream promoter (14). Because NF-$\kappa$B is an important modulator of cell survival that is activated by ER stress and because MTHFR is involved in several critical cellular pathways, we examined the possibility that MTHFR could also participate in the response to ER stress. Our findings suggest that MTHFR is up-regulated by ER stress and that this effect is mediated by IRE1 and c-Jun. This link between the ER stress pathway and a new regulatory mechanism for MTHFR supports an important role for this enzyme in cellular homeostasis or survival.

**Experimental Procedures**

**Cell Culture Conditions and Transfection**—Neuro-2a neuroblastoma cells and RAW264.7 macrophages were maintained in high glucose Dulbecco’s modified Eagle’s medium containing 10% serum at 37 °C. For ER stress induction, the cells were grown to 70% confluence and treated with tunicamycin, thapsigargin, A23187, or DL-Hcy (all from Sigma) for the time and growth conditions specified under “Results.” LiCl was from Fisher Scientific (Nepean, Ontario) and valproic acid, pyrrolidinethiocarbamate (PTC), Bay11-7082, lipopolysaccharides from *Escherichia coli* K-235, SP600125, DL-cysteine, and DL-methionine were from Sigma. Transfections were performed in Opti-MEM (Invitrogen) using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer’s protocol. All transfections were performed three times, in independent experiments, using different preparations of every tested plasmid.

**Overexpression of MTHFR Isoforms**—Overexpression of DNA constructs harboring the short or long isoforms of MTHFR was performed in *E. coli*, as previously described (15).

The inserts were transferred into the pCMV vector and transiently expressed in Neuro-2a cells after transfection.

**Other Plasmids**—Various plasmids were kindly provided by investigators as indicated: mPERKWT9E10, mIRE1B, mIRE1BdelC9E10 by Dr. David Ron, New York University School of Medicine, New York; pCGN-ATF6-1 (373) by Dr. Ron Prywes, Columbia University, New York; pCDNA3-Flag-JNK1 (APF) by Dr. Roger J. Davis, University of Massachusetts Medical School, Worcester, MA; pJRBC/JUN by Dr. Jawed Alam, Health Sciences Center, New Orleans, LA; and pCMV-JUN by Dr. Michael J. Birrer, National Cancer Institute, Bethesda, MD.

**RNA Purification and Real Time RT-PCR**—Total RNA extraction from cultured cells and real-time RT-PCR were performed as previously described (14). Platinum SYBR Green qPCR Supermix-UDG was from Invitrogen. Generation of single amplicons of the expected sizes was confirmed by polyacrylamide gel electrophoresis and denaturation curves also confirmed amplification of unique products. Specificity of amplifications was verified by cloning and sequencing representative products. No significant amplification was observed with the use of “minus RT controls” (reverse transcriptase omitted during RT) as well as in “no template controls” (omission of cDNA). The amplicon signal for each target cDNA strongly correlated with serial dilution of template (r > 0.95). Oligonucleotides for quantitation of Mthfr, Nos2, and Gapdh were described in Pickell et al. (14). For detection of Grp78, the PCR primers 5'-GGTTCTCTCACTAAAATGAAGGAGA-3' (sense) and 5'-GTACGTAACAACTGATGGGTAA-3' (antisense) were employed for amplification of a 74-bp specific segment. Data analysis and calculations were performed according to the Relative Quantitative Analysis method, using Gapdh as the normalizer target. The internal reference dye was ROX. All analyses were standard procedures of the MX4000 QPCR System (Stratagene, La Jolla, CA).

**Analysis of Xbp1 mRNA Processing**—An RT-PCR assay was designed for visualization of Xbp1 splicing, using primers 5'-AGTAAGGCTGTGCGCGGCTCT-3' (sense) and 5'-GAAGATGTCTGGGAGAGTTAA-3' (antisense), that span the relevant Xbp1 splice site. Products were resolved on 8% polyacrylamide gels. The unsliced form generated an amplicon of 100 bp and a fragment of 74 bp was observed for the processed mRNA. Heteroduplexes were also observed, as seen in a similar experimental design (16).

**Western Blotting**—Cells were collected by scraping in phosphate-buffered saline. Cell lysis was performed in a lysis buffer (1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline) in the presence of Complete Mini (protease inhibitor mixture from Roche Applied Science, Laval, Quebec). After incubation on ice for 30 min, lysates were centrifuged (16,000 × g, 15 min, 4 °C). Supernatants were collected and protein concentrations assessed. Unless otherwise indicated, 50 μg of protein from each lystate was diluted with loading buffer, boiled, and loaded onto SDS-polyacrylamide gels. Samples were electrophoresed and proteins were transferred onto nitrocellulose. Relevant antigens were visualized after incubation with rabbit polyclonal primary antibodies followed by incubation with horseradish peroxidase-conjugated second-
ary antibody and detection as previously described (15). To ensure reproducibility of observations, all Western blot experiments were performed at least twice, using extracts from independent experiments. Antibodies against GRP78, FLAG, and β-actin were from Sigma. Antibodies against eIF2α and phospho-eIF2α were kindly provided by Dr. Nahum Sonenberg, McGill University, Montreal, Quebec. Polyclonal antibody against MTHFR was previously described (15). Other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

MTHFR Enzyme Assay—Cytosolic extracts and enzymatic assays were performed as in Tran et al. (15). Activities were measured in duplicate and less than 6% variation was observed between repeats.

RESULTS

Up-regulation of Mthfr mRNA by ER Stress—To determine whether several well documented ER stress inducers could alter expression of MTHFR, we treated Neuro-2a cells with tunicamycin, thapsigargin, and A23187 (Fig. 1A). As positive controls, we assessed Grp78 (Fig. 1B) and Nos2 (Fig. 1C) mRNA levels. GRP78 is a chaperone protein that is a standard marker of the ER stress response, whereas Nos2 activation can be attributed to activation of NF-κB signaling, because it is abolished when BAY11-7082 (Fig. 1C) or PDTC (data not shown) are administered with tunicamycin. Mthfr, Grp78, and Nos2 mRNA levels were all increased following treatment by the 3 stressors. Bay11-7082 (or PDTC, not shown) did not inhibit the up-regulation of Grp78 and Mthfr mRNAs by tunicamycin (Fig. 1, A and B), indicating that these 2 genes are activated by ER stress independently of NF-κB. Similar results were obtained when NF-κB inhibitors were combined with thapsigargin or A23187 instead of tunicamycin (data not shown). We performed the same types of experiments with RAW264.7 cells and reached the same conclusions (data not shown). In addition to Grp78 induction, Xbp1 mRNA processing confirmed the effectiveness of the ER stressors in our experimental system (Fig. 1D).

Hcy Induces ER Stress and Increases Mthfr mRNA Levels—Because Hcy adversely affects ER function (5, 17), we examined the effects of Hcy on ER stress and on Mthfr mRNA levels. Treatment of Neuro-2a cells with Hcy rapidly increased Grp78
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FIGURE 2. Mthfr mRNA is up-regulated by Hcy. Neuro-2a cells were incubated with 0.2 ( ), 1 ( ), or 5 mM (A) Hcy for the indicated times. Mthfr (A), Grp78 (B), or Nos2 (C) transcripts were measured by real-time RT-PCR and normalized to Gapdh. Measurements were performed in triplicate in three independent experiments. Results are presented as mean ± S.E. Significant differences with mock (two-tailed t test) are denoted by asterisks (*, p < 0.05; **, p < 0.01).

not mediated through NF-κB. We verified that Nos2 mRNA levels were increased after lipopolysaccharide treatment of RAW264.7 cells, and that this increase was abolished by PDTC or Bay11-7082, two inhibitors of NF-κB (data not shown, see also Ref. 14). However, because the up-regulation of Mthfr and Grp78 by Hcy was not affected by the NF-κB inhibitors (data not shown) and Hcy did not alter expression of Nos2, we conclude that the Hcy-induced increase of ER stress and Mthfr is not mediated by NF-κB.

Up-regulation of MTHFR Protein by ER Stress—Because Mthfr mRNA levels were increased by ER stress, we questioned whether MTHFR protein levels were also affected. We used tunicamycin or thapsigargin to study the effect of ER stress on MTHFR protein levels, in Neuro-2a and RAW264.7 cells (Fig. 3A). ER stress is significantly induced in these 2 cell lines in the presence of tunicamycin and thapsigargin, as verified by increased levels of GRP78 (Fig. 3A) and processing of Xbp1 (Fig. 3B). As expected, we obtained complex Western blot patterns for MTHFR because there are two possible protein isoforms (with apparent mass of 70 or 77 kDa (15)) and the shorter MTHFR isoform has been shown to undergo phosphorylation (13). The stress inducers clearly increased the amount of GRP78 as well as the intensity of the MTHFR protein (short isoform of 70 kDa, phosphorylated (SP) and non-phosphorylated forms). The identity in Fig. 3A of the short MTHFR subunit, with or without phosphorylation, was deduced by...
overexpression of the short and long isoforms in Neuro-2A cells, or by mixing bacterial extracts containing the overexpressed short and long isoforms with the Neuro-2A cells (Fig. 3C and supplemental Fig. 1A). We observed that overexpression of the short isoform after transfection into Neuro-2A cells generates mainly the phosphorylated form (Fig. 3C, Mock + S, and supplemental Fig. 1A), with smaller amounts of the non-phosphorylated protein. Transfection of Neuro-2A cells with a construct expressing the long MTHFR isoform (Fig. 3C, Mock + L) demonstrated that the long isoform of 77 kDa migrates slightly above the phosphorylated form of the short isoform (Fig. 3C, Mock + S) and that this long isoform does not appear to be efficiently expressed in Neuro-2A cells. We also confirmed, as initially reported (13), the identity of the phosphorylated and non-phosphorylated 70-kDa isoforms by treatment with alkaline phosphatase (Fig. 3D).

Mixing protein extracts from Neuro-2A cells with bacterial extracts containing the overexpressed short and long isoforms of MTHFR (supplemental Fig. 1B) supported our conclusion on the banding pattern of the 2 isoforms and confirmed that the long MTHFR isoform was not expressed in the cell lines used in the present study. Occasionally we observed a band that migrated slower than the long isoform (labeled with an asterisk in Fig. 3D). It is possible that this faint band represents the phosphorylated form of the long isoform but phosphorylation of the long isoform has not been examined by any group thus far. We also occasionally detected a band that migrated faster than the non-phosphorylated small isoform (labeled with 2 asterisks in Fig. 3D). This unidentified band is likely to be a degradation product of MTHFR, because a band of similar size was observed in Yamada et al. (13) and, in our studies, this protein appeared to increase in intensity under circumstances that increased the amount of the 70-kDa MTHFR protein (as shown in Fig. 3A).

**Effect of Individual ER Stress Transducers/Transcription Factors**

To determine which branch of ER stress, PERK, ATF6, or IRE1, was involved in MTHFR activation, we used plasmids that overexpressed the relevant transducers/transcription factors. Cells overexpressing PERK or IRE1 accumulate large quantities of these proteins, which then dimerize and are activated, even in the absence of ER stress (22, 23). We used a N-terminal derivative of ATF6, which lacks the transmembrane and C-terminal regions of the original protein. This truncated and activated form of ATF6 (amino acids 1–373) results in a protein that localizes entirely in the nucleus, even in the absence of ER stress (24).

We did not observe an increase in MTHFR expression after transfection of PERK and ATF6 constructs (Figs. 4A and B, respectively); confirmation of overexpression of the plasmids was performed by immunoblotting for the c-Myc and hemagglutinin tags for PERK and ATF6, respectively. Furthermore, the induction of eIF2α phosphorylation (Fig. 4A) and the increased levels of GRP78 (Fig. 4B) confirmed the functionality of the expression plasmids for PERK (1) and ATF6 (25), respectively. In contrast, MTHFR protein levels increased when Neuro-2A cells were transfected with plasmids overexpressing IRE1 (Fig. 4C). Similar results were obtained when native IRE1 or a c-Myc-tagged IRE1 were transfected; overexpression of the latter plasmid was confirmed using a c-Myc antibody (Fig. 4C). The overexpression of IRE1 also increased levels of GRP78 and phosphorylated c-Jun (p-c-Jun, its active form; Fig. 4C), as previously reported (23, 26). The degree of increase in GRP78 and c-Jun expression varied depending on transfection conditions (Fig. 4D, +, +, +, or +++ lanes). The optimal transfection conditions for c-Jun were also associated with higher levels of MTHFR expression.

A vector expressing a dominant-negative form of IRE1 (IRE1delC) partially abolished the effect of tunicamycin (Fig. 4E), as well as the effect of Hcy (Fig. 4F) on MTHFR protein levels. IRE1delC also attenuated the basal expression of MTHFR (Fig. 4, E and F). Our assay for Xbp1 processing showed detectable levels of ER stress in our basal conditions (particularly visible in the top panels of Figs. 3B and 4G); this is
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likely to contribute to the decrease in basal MTHFR expression by IRE1delC. The dominant-negative IRE1 mutant efficiently eradicated the tunicamycin- and Hcy-induced increases of p-c-Jun (Fig. 4, E and F, respectively). Evaluation of Xpb1 processing after Hcy treatment (Fig. 4G) demonstrated increased splicing in the presence of Hcy; this finding is consistent with the proposed IRE1 activation by Hcy because Xpb1 is downstream of IRE1 in ER signaling (1).

Hcy appeared to inhibit the phosphorylation of the short MTHFR isoform (Fig. 4F) because the Hcy-induced increase in MTHFR protein was largely limited to an increase in the non-phosphorylated band; this pattern is distinct from that seen with the other stress inducers or IRE1, which did not restrict activation to the non-phosphorylated short isoform (as shown in Figs. 3A and 4, C and D). The Hcy-dependent decrease in MTHFR phosphorylation is consistent with the findings of Yamada et al. (13) who suggested that the phosphorylation of MTHFR is decreased by a low S-adenosylmethionine/S-adenosylhomocysteine ratio.

Effect of Other Regulators of p-c-Jun—To further address the involvement of c-Jun in the up-regulation of MTHFR, we investigated the effects of LiCl and valproate, which can increase the levels of active c-Jun (27, 28). We also examined SP600125, a synthetic, classic inhibitor of stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (which activates c-Jun by phosphorylation (29)). Treatment of cells with LiCl and valproate enhanced the levels of p-c-Jun and MTHFR without an increase in GRP78 (Fig. 5A), suggesting that p-c-Jun can affect MTHFR expression without an increase in ER stress. These compounds in combination with tunicamycin increased both p-c-Jun and GRP78, with a concomitant increase in MTHFR. LiCl increased the amount of the non-phosphorylated MTHFR protein, as mentioned above for Hcy, suggesting that it might also affect phosphorylation of MTHFR. The protein sequence encompassing MTHFR phosphorylation sites (13) resembles a glycogen synthase kinase-3 (GSK-3) target sequence (30). LiCl is commonly used for inhibition of GSK-3β (31) but may be a potent inhibitor of other protein kinases (32); therefore a LiCl-sensitive kinase may be involved in MTHFR phosphorylation.

Incubation of Neuro-2a cells with SP600125, alone, or in combination with tunicamycin and/or LiCl, decreased the amount of p-c-Jun, as expected, and also decreased the global amount of MTHFR protein (Fig. 5B). SP600125 also reduced the levels of Mthfr mRNA, in the presence and absence of tunicamycin (Fig. 5C). However, because this inhibitor also increased the ratio of non-phosphorylated/phosphorylated MTHFR (Fig. 5B), as did LiCl, it is possible that SP600125 affects the unidentified kinase(s) responsible for MTHFR phosphorylation, because it does not exclusively inhibit SAPK/JNK (32).

Hcy Increases MTHFR Protein Levels and the Non-phosphorylated/Phosphorylated MTHFR Ratio—As shown in Figs. 2 and 4F, Hcy induced ER stress and MTHFR expression in Neuro-2a cells, possibly through a c-Jun-dependent mechanism. To further explore the effects of Hcy, we first confirmed that the effect was specific to this amino acid, by examining other sulfur amino acids, L-cysteine and L-methionine. Incubation with these other amino acids did not increase GRP78 levels (Fig. 6A); L-cysteine and L-methionine also had no effect (data not shown). The increase in Hcy-dependent ER stress was accompanied by an increase in the non-phosphorylated MTHFR isoform. Additional experiments (Fig. 6B), comparing the effects of Hcy and LiCl separately and in combination, demonstrated that both compounds increased the non-phosphorylated:phosphorylated MTHFR ratio. The proposed decrease in
MTHFR phosphorylation could be dissociated from an increase in ER stress, because LiCl affected MTHFR phosphorylation without an effect on GRP78.

The effects of Hcy and SP600125, alone or in combination, were also studied (Fig. 6, C and D). Hcy alone induced ER stress (increased GRP78 levels), increased p-c-Jun protein levels, and increased MTHFR mRNA and protein; the increase in the non-phosphorylated:phosphorylated MTHFR ratio was also observed. SP600125 alone had no effect on ER stress but also increased the non-phosphorylated:phosphorylated MTHFR ratio, presumably by modulating the kinase that phosphorylates MTHFR. The combination of Hcy and SP600125 limited the p-c-Jun increase, blocked MTHFR induction at the mRNA and protein levels, and also affected MTHFR phosphorylation. These experiments supported our hypothesis that Hcy mediates ER stress and increases MTHFR expression through c-Jun. They also confirmed that Hcy, LiCl, and SP600125 affect MTHFR phosphorylation although the nature of the kinase(s) remains to be determined.

The non-phosphorylated isosform of MTHFR was proposed to be more active than the phosphorylated isoform (13). Table 1 shows that MTHFR activity is increased by tunicamycin, LiCl, and Hcy under the same conditions in which MTHFR protein levels were measured; these results indicate that the modulation of MTHFR protein levels by these compounds is consistent with their modulation of enzyme activity.

**Regulation of p-c-Jun by Transfection**—To directly examine the regulation of MTHFR by c-Jun, Neuro-2a cells were transfected with 2 c-Jun plasmids, pRJB/CJUN or pCMV-CJUN (Fig. 7A). A marked increase in total c-Jun as well as in p-c-Jun (active form of the transcription factor) was observed. These findings suggest that Neuro-2a cells can phosphorylate large amounts of c-Jun protein. This is not surprising because the levels of activated SAPK/JNK enzyme are substantially higher in cells of neuronal origin or in the brain than in peripheral organs (27), even if total SAPK/JNK protein levels are similar (33). Transfection with the c-Jun plasmids resulted in an increase of MTHFR, more specifically in the phosphorylated isosform, because we did not add any of the previously mentioned compounds that can inhibit MTHFR phosphorylation (Hcy, LiCl, or SP600125). Transfection with these c-Jun constructs also increased Mthfr mRNA levels (Fig. 7B).

To confirm the role of c-Jun in MTHFR expression, we also used a nonphosphorylatable mutant JNK1, in which the canonical TPY activation motif has been mutated to APF (34). Overexpression of this dominant-negative mutant of JNK1, confirmed by immunodetection of the FLAG epitope (Fig. 7, E and F), resulted in a reduction of Mthfr mRNA levels (Fig. 7, C and D) and limited Mthfr mRNA induction by tunicamycin (Fig. 7C) or Hcy (Fig. 7D). The kinase-inactive JNK also caused a marked decrease of MTHFR protein and limited the up-regulation of MTHFR protein by tunicamycin (Fig. 7E) or Hcy (Fig. 7F).

**DISCUSSION**

When mammalian cells are subjected to a variety of physiological stress conditions that target the ER, the cells respond by activating a defense mechanism referred to as the UPR. This response modulates several transcriptional and translational pathways, which include induction of stress-response genes, such as GRP78. In the present study, we identified ER stress as a novel inducer of Mthfr gene expression in Neuro-2a cells. ER stress induction causes an increase in Mthfr mRNA levels and in MTHFR protein levels. We propose that IRE1 is involved in the signal transduction after ER stress and that c-Jun acts downstream of IRE1 in the context of MTHFR regulation. This concept is based on several lines of evidence. We found that MTHFR was induced by diverse conditions that cause ER stress, such as exposure to Hcy, tunicamycin (which inhibits N-linked glycosylation), and thapsigargin or A23187 (which disrupt calcium homeostasis in the ER). Mthfr mRNA induction paralleled that of the ER stress gene Gtp78. Overexpression of a dominant-negative IRE1 abolished the MTHFR response to tunicamycin or Hcy treatment. Overexpression of IRE1 and c-Jun mimicked the effect of ER stress inducers on MTHFR.
levels. After treatment of cells with relevant agents (LiCl, tunicamycin, and valproate), the levels of active c-Jun correlated with levels of MTHFR. Inhibition of c-Jun signaling blocked the increase in MTHFR expression. Additional studies are required to determine whether c-Jun exerts its effect directly on the Mthfr promoter or through an indirect mechanism.

We cannot exclude the possibility that other branches of the UPR besides IRE1 may contribute to the MTHFR response to ER stress. Similarly, we cannot rule out intermediates other than c-Jun, downstream of IRE1, that might influence MTHFR expression.

Hcy has been shown to induce a pathway that can lead to apoptosis after activation of the UPR, through mediation by the transmembrane protein IRE1 (35). The cytoplasmic portion of IRE1 can interact with TRAF2 (26), an adaptor protein involved in activation of the SAPK/JNK. These results and the findings in Cai et al. (36) suggest that Hcy can increase the amount of p-c-Jun. Several studies have described the physiological relevance of these observations. For example, Grp78 mRNA levels are elevated in vivo in hyperhomocysteinemic mice (37). In vitro as well as in vivo studies have shown that Hcy-induced ER stress leads to transcriptional activation of genes involved in lipogenesis; this association contributes to hepatic steatosis (38). Robert et al. (39) reported that the SAPK/JNK transduction cascade is activated in vivo in the brains of hyperhomocysteinemic mice, resulting in a higher amount of p-c-Jun. Ramaswami et al. (40) observed that curcumin (an inhibitor of c-Jun) blocks the Hcy-induced impairment of endothelium-dependent vasorelaxation.

Results from microarray analysis of Mthfr<sup>-/-</sup> mice suggested that neuronal damage by severe hyperhomocysteinemia involves disruption of intracellular Ca<sup>2+</sup> (41). An elevation of Hcy can activate the SAPK/JNK cascade through over-stimulation of glutamate receptors and a disturbance of calcium homeostasis (references in Ref. 39). Our observations do not exclude the possibility that Hcy can activate the c-Jun pathway by mechanisms other than ER stress or that factors other than c-Jun (e.g., ATF6, PERK, or XBP1) may be required for optimal MTHFR response to ER stress.

In addition to transcriptional regulation, cells under ER stress control the translational capacity, protecting against further accumulation of unfolded proteins in the ER but allowing synthesis of proteins essential for cell survival. Translational inhibition occurs through phosphorylation of the α-subunit of eukaryotic initiation factor 2, catalyzed by PERK (22). GRP78 mRNA can still be translated when general, cap-dependent translation of mRNAs is inhibited, indicating that cap-independent translation initiated at an internal ribosome entry site is utilized (42). It would be interesting to address the molecular mechanisms that facilitate the translation of Mthfr. Its long, GC-rich 5′-untranslated region, containing multiple upstream ATGs (15), might possess structural features that permit its effective translation during ER stress. Translation of methionine synthase, the enzyme that functions immediately downstream of MTHFR, by transferring the methyl group from 5-methyltetrahydrofolate to homocysteine for the synthesis of methionine, is influenced by a functional internal ribosome entry site element (43). It has also previously been reported that methionine synthase mRNA levels are increased by treatment of cultured cells with Hcy (44).

MTHFR phosphorylation sites lie in a protein sequence segment similar to that of a putative GSK-3 consensus motif (13). There is no strict consensus motif for substrate phosphorylation by GSK-3, but many GSK-3 substrates require prior phosphorylation to form the motif, -S/TXXXS/T(P)-, before phosphorylation by GSK-3 is possible (45). The downstream phosphorylated residue Thr-34 in MTHFR plays an important role as the priming phosphorylation site (13). We observed that MTHFR phosphorylation is sensitive to the GSK-3 inhibitor lithium (30), supporting the concept that the kinase(s) regulating MTHFR shares some properties with GSK-3. Protein phosphorylation is one of the most important modifications for cel-
lular regulation. Greater understanding of the significance of MTHFR phosphorylation will require identification of the kinase(s) and phosphatase(s) responsible for its reversible modification. We observed that the ratio of non-phosphorylated/phosphorylated MTHFR is increased by Hcy, LiCl, and SP600125. It is interesting to note that differential display analysis, in addition to revealing the increased expression of GRP78, identified RTP (reducing agents and tunicamycin-responsive protein) as a novel up-regulated gene after Hcy treatment of cultured human umbilical vein endothelial cells (17). RTP, a soluble protein induced by ER stress, was shown to be phosphorylated at several sites, and Hcy treatment of cells resulted in dephosphorylation of RTP (46). Because many of the features displayed by RTP were observed for MTHFR, it would be interesting to determine whether both enzymes are modified by the same kinase(s) and phosphatase(s).

It is not surprising that Hcy might increase MTHFR expression in an effort to reduce hyperhomocysteinemia and therefore limit the Hcy-induced stress. The increase in MTHFR expression by the more classic ER stress inducers is more intriguing and suggests that MTHFR, a protein that has no known direct involvement in ER function, has a role in play in the UPR. Protein secretion irreversibly depletes intracellular amino acid pools and ER stress has already been noted to activate some genes involved in amino acid import and metabolism (Ref. 47 and references therein). Up-regulation of MTHFR by ER stress could contribute to amino acid sufficiency by supplying methionine for protein synthesis, to allow the cell to adapt to the metabolic consequences of high ER activity. Alternatively, increased MTHFR expression would increase methionine levels for production of S-adenosylmethionine and methylation reactions. This increase could contribute to the general increase in DNA methylation induced by homocysteine (48) or to specific methylation changes associated with ER stress, such as the methylation modification of nucleosomes at the GRP78 promoter (49). ER stress can induce the UPR, which can lead to cell death as well as to prosurvival mechanisms. The interplay between these processes has clinical implications in neurodegenerative diseases, atherosclerosis, and cancer (38, 50). MTHFR deficiency has been shown to modify the risk for atherosclerosis, cancer, and birth defects; methyl group metabolism also has a role to play in neurodegenerative diseases.

Our finding of MTHFR up-regulation by ER stress through IRE1 is compatible with a role for MTHFR in the recovery from stress and in the fine balance that exists between cell death and survival. IRE1 signaling enhances cell viability shortly after the onset of ER stress (51). Cells with a deficiency in MTHFR may not be able to recover optimally from ER stress or other forms of cellular injury; this could contribute to the wide spectrum of clinical consequences of MTHFR deficiency.

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