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Repair of Isoaspartate Formation Modulates the Interaction of Deamidated 4E-BP2 with mTORC1 in Brain*[S]

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In eukaryotes, a rate-limiting step of translation initiation is recognition of the mRNA 5′ m^7GpppN cap structure by the eukaryotic initiation factor 4F (eIF4F), a heterotrimeric complex consisting of the cap-binding protein, eIF4E, along with eIF4G, and eIF4A. The eIF4E-binding proteins (4E-BPs) repress translation by disrupting eIF4F formation, thereby preventing ribosome recruitment to the mRNA. Of the three 4E-BPs, 4E-BP2 is the predominant paralog expressed in the mammalian brain and plays an important role in synaptic plasticity and learning and memory. 4E-BP2 undergoes asparagine deamidation, solely in the brain, during early postnatal development. Deamidation spontaneously converts asparagines into a mixture of aspartates or isoaspartates, the latter of which may be destabilizing to proteins. The enzyme protein L-isoaspartyl methyltransferase (PIMT) prevents isoaspartate accumulation by catalyzing the conversion of isoaspartates to aspartates. PIMT exhibits high activity in the brain, relative to other tissues. We report here that 4E-BP2 is a substrate for PIMT. *In vitro* deamidated 4E-BP2 accrues isoapartyl residues and is methylated by recombinant PIMT. Using an antibody that recognizes 4E-BP2, which harbors isoaspartates at the deamidation sites, Asn^{99} and Asn^{102}, we demonstrate that 4E-BP2 in PIMT−/− brain lysates contains isoaspartate residues. Further, we show that 4E-BP2 containing isoaspartates lacks the augmented association with raptor that is a feature of deamidated 4E-BP2.

Eukaryotic mRNA translation is regulated primarily at the initiation phase, during which the ribosome is recruited to the mRNA. The association of the cap-binding protein, eukaryotic initiation factor (eIF)[5] 4E, with the mRNA 5′ m^7GpppN cap structure, initiates an ordered series of reactions culminating in 40 S ribosome placement at the AUG (or its cognate) initiation codon. eIF4E binds the mRNA cap as part of a complex, termed eIF4F, which includes the initiation factors eIF4G and eIF4A. eIF4F recruits the ribosomal 43 S complex (40 S ribosome/Met-$tRNA_1^{Met}$-eIF2-GTP/eIF3) to the mRNA 5′ untranslated region (1–3). In mammals, three eIF4E-binding proteins (4E-BPs) (4) impair translation initiation by preventing eIF4F formation through competition with eIF4G for a common binding site on eIF4E (5–7). Under conditions favoring growth and proliferation, the 4E-BPs are hyperphosphorylated by the mammalian target of rapamycin complex 1 (mTORC1), which causes their release from eIF4E, eIF4F formation, and promotion of translation initiation (8–10).

mTORC1 is composed of the kinase mTOR, G protein β-subunit-like protein, and regulatory associated protein of mTOR (raptor), a large adaptor protein that recruits substrates to the mTOR kinase domain (11–14). A TOR signaling (TOS) motif common to mTORC1 substrates, including 4E-BPs, is necessary for the interaction with raptor (15–18). mTORC1 kinase activity is regulated by several signaling pathways, including phosphoinositide 3-kinase-protein kinase B, and serine/threonine kinase 11-AMP-activated protein kinase, which converge on the tuberous sclerosis complex to suppress its GTPase activity toward the small GTP-binding protein ras homolog enriched in brain, an activator of mTORC1 (19, 20).

Asparagine deamidation is a posttranslational protein modification comprising the nonenzymatic conversion of asparagines to aspartates. Although this process is spontaneous, the propensity of asparagines to undergo deamidation is dramatically disparate and is largely determined by the identity of the immediate C-terminal residue and the flexibility of the peptide backbone (21–23). Environmental factors, including pH, temperature, and ion concentration, also significantly influence deamidation rates (24). *In vivo* deamidation has been reported for >200 proteins (25). Although this modification has often been viewed as undesirable degradation of aged proteins, important molecular and cellular functions of deamidated B cell lymphoma-xL, fibronectin, histone $H1^o$, cytochrome *c*, protein kinase A, and rabbit muscle aldolase have been described (25, 26). The apparent evolutionary selection of sequences favoring asparagine deamidation (27, 28) and the coincidence of *in vivo* deamidation events with cellular processes suggest that deamidation may act as a "molecular clock" to regulate the onset of biologically significant events (28, 29). Indeed, protein deamidation may regulate diverse cellular processes, which include protein turnover (28, 30, 31), apoptosis (32, 33), chromatin remodeling (34), and regulation of cellular-matrix interactions (35).

Deamidation occurs through nucleophilic attack by the C-terminal flanking peptide bond nitrogen toward an aspara-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[5] The abbreviations used are: eIF, eukaryotic initiation factor; 4E-BP, eIF4E-binding protein; mTORC1, mammalian target of rapamycin complex 1; raptor, regulatory associated protein of mTOR; TOS, TOR signaling; PIMT, protein L-isoaspartyl methyltransferase; AdoMet, *S*-adenosyl-L-methionine; GST, glutathione *S*-transferase; MES, 4-morpholineethanesulfonic acid; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

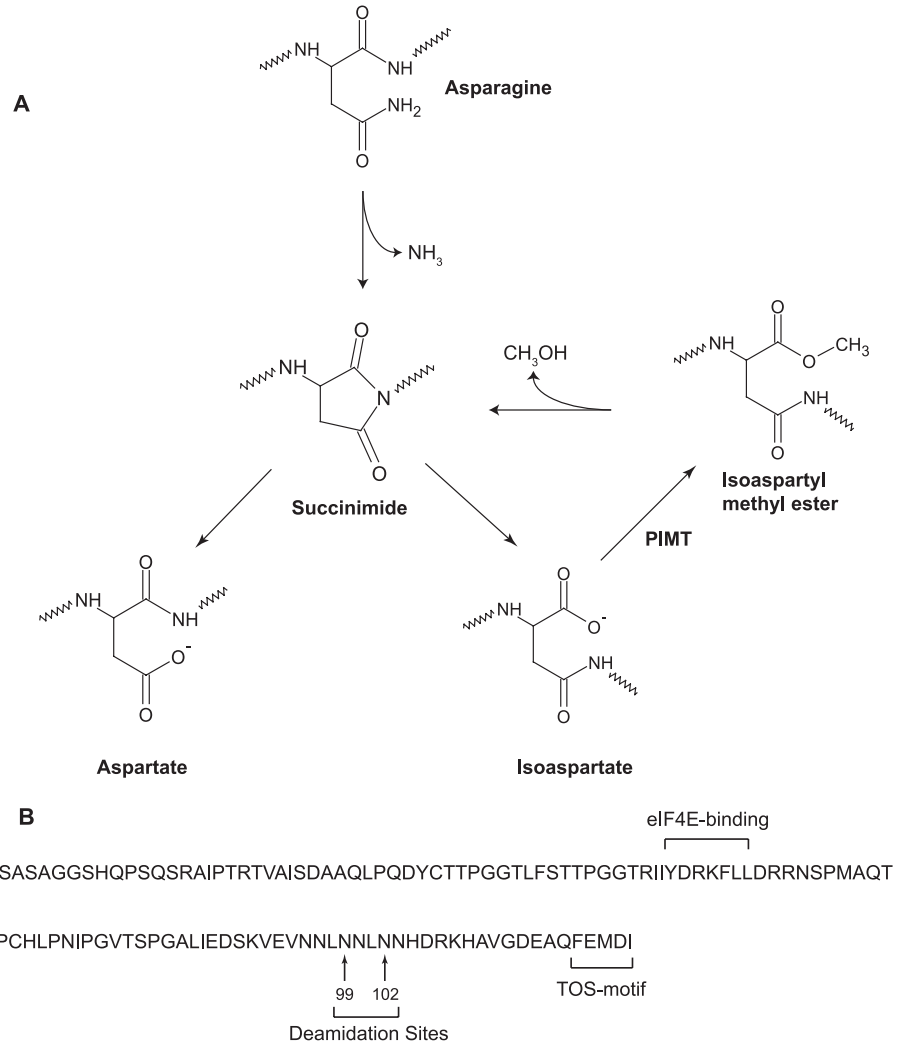


FIGURE 1. **PIMT repairs isoaspartates formed during spontaneous asparagine deamidation in proteins.** *A*, mechanism of aspartate or isoaspartate formation and repair of abnormal isoaspartyls by PIMT. Spontaneous asparagine deamidation yields a mix of aspartyls and isoaspartyls, of which the formation of the latter is favored by ~3:1. The deamidation reaction proceeds through the metastable succinimide intermediate. PIMT catalyzes the transfer of a methyl group to isoaspartyls thereby forming the corresponding carboxyl-methyl esters. These rapidly revert to the original succinimide intermediate, consequently leading to *de novo* formation of aspartyls and isoaspartyls. Net accumulation of repaired aspartyls is accomplished iteratively. *B*, amino acid sequence of murine 4E-BP2. Deamidation sites, eIF4E-binding site, and TOS motif are indicated.

gine side chain amide group. This leads to the formation of a circular succinimide intermediate (36) (Fig. 1*A*). The hydrolysis of this short lived intermediate (physiological half-life of a few hours) yields both aspartate and isoaspartate residues, the latter of which comprise 70–85% of the reaction product (37). The atypical peptide bond (β-linkage) of the isoaspartyl residue prevents proper protein folding and is therefore thought to represent a form of damage to proteins aged *in vitro* or that accumulate in some tissues (36, 38, 39). To alleviate the potentially deleterious consequences of isoaspartate accumulation, the enzyme protein L-isoaspartyl methyltransferase (PIMT) promotes the conversion of these residues into the aspartate form. This occurs through the PIMT-catalyzed transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to the isoaspartyl carboxyl side-chain leading to reformation of the succinimide group (40, 41) (Fig. 1*A*). Iterative PIMT-catalyzed succinimide formation and decay thereby leads to replacement of isoaspartates with aspartates (42–44). PIMT is ubiquitous, although it exhibits high expression and has the greatest number of substrates in brain (45, 46). The critical requirement for this enzyme in neuronal function is underscored by its genetic deletion in mice. Most strikingly, of a host of reported maladies, PIMT−/− mice display enlarged brains, suffer severe epileptic seizures, and die on average 6 weeks postnatally (45, 47). Transgenic flies overexpressing PIMT exhibit increased lifespan (48), possibly through suppression of isoaspartate content. Because of the importance of PIMT in the brain, two recent studies were independently undertaken to globally identify its substrates in this tissue (46, 49). The considerable discrepancy in the identified PIMT substrates from these reports suggests that still other targets of this enzyme remain unidentified.

We recently discovered that the translation repressor 4E-BP2, the predominant 4E-BP paralog expressed in the mammalian brain, undergoes brain-specific deamidation during postnatal development at a unique asparagine-rich sequence (50). Considering this, we investigated whether 4E-BP2 is a PIMT substrate.

EXPERIMENTAL PROCEDURES

Materials—AdoMet (81.9 Ci/mmol) and unlabeled AdoMet were purchased from PerkinElmer Life Sciences and Sigma, respectively. EN^{3}HANCE^{TM} liquid autoradiography enhancer was from PerkinElmer Life Sciences. 4E-BP2 (no. 2845) antibody was from Cell Signaling Technology, and raptor antibody was from Millipore.

Vector Construction and Recombinant Protein Expression and Purification—Human PIMT cDNA was amplified by PCR with the addition of EcoRI and XhoI restriction sites at the 5′ and 3′ ends, respectively. The PCR product was digested and ligated into the pGEX-6P-1 vector at the above sites in-frame with the upstream glutathione *S*-transferase (GST) sequence. Recombinant GST-PIMT fusion protein was expressed in *Escherichia coli* BL21 (DE3) by induction with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside, followed by 3-h growth at 30 °C. Protein was purified on glutathione-Sepharose 4B resin according to the manufacturer's recommendations (GE Healthcare) and eluted in fractions with 10 mM glutathione in 50 mM Tris-HCl, pH 8.5. In the case of recombinant GST-4E-BP2 fusion

proteins, the 4E-BP moieties were released, following binding to glutathione-Sepharose 4B, by on-resin cleavage for 20 h at 4 °C with PreScission Protease (GE Healthcare). All purified proteins were stored at −80 °C in 10% glycerol.

In Vitro Methylation of Isoaspartates by Recombinant PIMT—Methylation of brain lysates was performed as described previously (49). Briefly, 40 μg of lysate was incubated with 2.5 μM GST-PIMT, 100 μM AdoMet (4 μCi of ^{3}H-labeled; concentration and specific activity were adjusted with unlabeled AdoMet), and 75 mM Na-MES, pH 6.2, in a total reaction volume of 27.5 μl. For methylation of recombinant 4E-BP2, the wild type protein was *in vitro* deamidated in 0.15 M Tris-HCl, pH 10, for 24 h at 37 °C. The protein solution was then neutralized with 0.5 M Na-MES and treated with 1 mM dithiothreitol for 10 min. Control wild type and 4E-BP2 N99D/N102D recombinant proteins were similarly treated with 0.5 M Na-MES and 1 mM dithiothreitol immediately before use. Methylation reactions were then carried out in 25-μl volumes containing 1 μg of recombinant protein, 2.5 μM GST-PIMT, 50 μM AdoMet (4 μCi), and 75 mM Na-MES (pH 6.2). In both cases, reactions were for 15 min at 30 °C and stopped by the addition of 5 × Laemmli sample buffer. Samples were resolved on 4–12% Criterion XT BisTris gels, pH 6.4 (Bio-Rad). Gels were then treated with EN^{3}HANCE^{TM}, dried, and exposed to autoradiographic film for tritium detection.

Raptor Immunoprecipitation and in Vitro Binding Assays—Raptor antibody was absorbed onto protein G-Sepharose beads for 30 min in homogenization buffer (10 mM K_3PO_4/1 mM EDTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 5 mM EGTA, 0.5% Nonidet P-40, 0.1% Brij 35, 0.1% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin). Brain lysate from 4E-BP2−/− mice was prepared in homogenization buffer, clarified by centrifugation, and 500 μg was incubated with 10 μl of prepared beads for 30 min to immunoprecipitate raptor. The indicated recombinant 4E-BP2 proteins (150 ng) were then mixed with immune complexes and incubated for 2 h at 4 °C with agitation. Beads were washed four times with 500 μl of homogenization buffer, resuspended in sample buffer, and subjected to SDS-PAGE (5–20%).

SDS-PAGE and Western Blotting—Protein samples were resolved by SDS-PAGE (15%) and electroblotted onto nitrocellulose membranes. Membranes were blocked in 5% powdered milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h followed by incubation with primary antibodies for 2 h at room temperature or overnight at 4 °C. Membranes were then washed with TBST three times and incubated with horseradish peroxidase-coupled secondary antibody for 1 h at room temperature, washed again, and treated with enhanced chemiluminescence prior to detection on x-ray film (Denville Scientific).

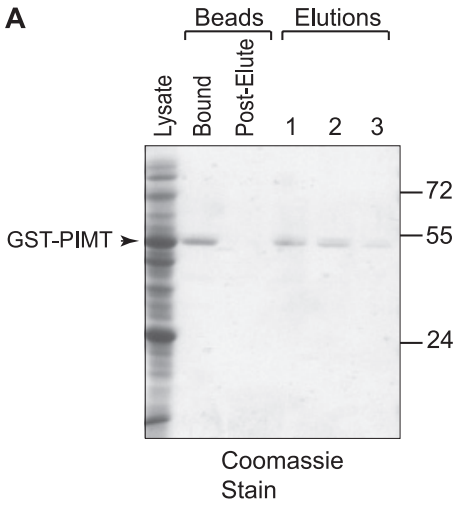
Iso4E-BP2 (Asp^{99}/Asp^{102}) Antibody Production and Purification—A peptide of amino acid sequence identical to that encompassing the deamidation susceptible region of 4E-BP2, with aspartates in β-linkages (isoaspartates) replacing asparagines 99 and 102, was purchased from American Peptide. The peptide was conjugated to immunogenic keyhole limpet hemocyanin via an ectopic N-terminal cysteine using the Imject Maleimide Conjugation kit (Pierce). Rabbits were immunized with the conjugated peptide, using Freund's Complete Adjuvant for first injection and Freund's Incomplete Adjuvant for subsequent injections, according to the McGill Animal Resources Centre protocol. For antibody purification, rabbit serum was incubated with the above peptide that had been previously conjugated to resin using the SulfoLink kit (Pierce). The antibody was eluted at low pH, dialyzed against 50% glycerol, and stored at −20 °C.

RESULTS

GST-PIMT Exhibits Functional Methyltransferase Activity in Vitro—Two 4E-BP2 asparagine residues (Asn^{99} and Asn^{102}; Fig. 1*B*) are deamidated during postnatal development in the mammalian brain (50). Considering the preponderance for isoaspartate accumulation and the critical requirement for PIMT in the brain (45, 47), we hypothesized that 4E-BP2 would be a PIMT substrate. To address this, we first adopted an *in vitro* assay system based on the use of recombinant PIMT, similar to that used in previous studies (46, 49, 51). To produce recombinant PIMT, the human PIMT isozyme II cDNA was subcloned into the pGEX-6P-1 vector for bacterial expression as a fusion protein to GST at the PIMT N terminus. Recombinant GST-PIMT was expressed in *E. coli* BL21 cells and purified to homogeneity (Fig. 2*A*). The activity of GST-PIMT was assessed by incubating it with wild type or PIMT−/− brain lysates and the tritiated methyl donor, AdoMet. As anticipated, GST-PIMT efficiently labeled the isoaspartates that accumulate in PIMT−/− brains, relative to wild type, as was evidenced by the pronounced tritium signals across the entire molecular weight range of the PIMT−/− PAGE lane (Fig. 2*B*). Similar results were previously obtained with different preparations of recombinant PIMT protein (46, 49).

In Vitro Deamidated 4E-BP2 Accrues Isoaspartates—To determine whether 4E-BP2 deamidation produces isoaspartates, in addition to aspartyl residues, the PIMT assay was adapted for methylation of *in vitro* deamidated 4E-BP2. Recombinant 4E-BP2 was subjected to alkaline-induced deamidation, pH 10, by incubation for 24 h at 37 °C. This treatment produced similar quantities of unmodified, singly, and doubly deamidated 4E-BP2 (Fig. 3, *upper panel*, *middle lane*). PIMT methylation was then performed on alkaline-treated 4E-BP2, untreated wild type 4E-BP2, and the deamidation mimic 4E-BP2 N99D/N102D (described in Ref. 50). As expected, isoaspartyl groups were only detected in the wild type 4E-BP2 protein that was deamidated by alkaline treatment (*wt*; pH 10) (Fig. 3, *lower panel*). Thus, the formation of isoaspartyl residues during *in vitro* asparagine deamidation of 4E-BP2 renders it a PIMT substrate. Importantly, recombinant PIMT distinguishes between the isoaspartates of alkaline-treated 4E-BP2 and the aspartates at the same residues in 4E-BP2 N99D/N102D.

Antibody Specifically Recognizes 4E-BP2 Containing Isoaspartyls at Positions 99 and 102—To characterize further the isoaspartate formation in 4E-BP2, an antibody was produced that recognizes 4E-BP2 harboring isoaspartyl residues at both positions 99 and 102. The specificity of the immunopurified antibody was confirmed by Western blotting against recombinant 4E-BP2 proteins. The antibody, herein referred to as anti-



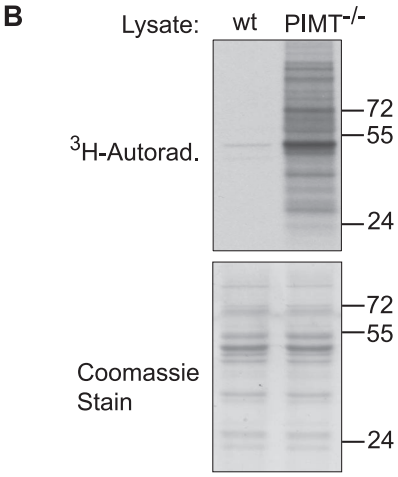


FIGURE 2. **Purified GST-tagged PIMT exhibits methyltransferase activity toward isoaspartyl residues in PIMT−/− brain lysates.** *A*, expression and purification of N-terminal GST-tagged PIMT is depicted. Human PIMT was expressed in *E. coli* as a fusion to N-terminal GST. Protein was purified on glutathione-Sepharose 4B resin and eluted in three fractions with 10 mM glutathione. The crude lysate, glutathione resin before and after elution, and three elutions were resolved by 10% SDS-PAGE, and proteins were visualized with Coomassie Brilliant Blue stain. The first two elutions were pooled and stored until use. *B*, GST-PIMT catalyzes the transfer of methyl groups from ^{3}H-labeled AdoMet to isoaspartyl residues accumulated in PIMT−/− brain lysates. 40 μg of wild type (*wt*) or PIMT−/− lysates were incubated with 2.5 μM GST-PIMT and 100 μM AdoMet (4 μCi ^{3}H-labeled) in 75 mM Na-MES, pH 6.2, for 15 min at 30 °C. Lysates were separated by 4–12% BisTris PAGE, and gels were processed for tritium detection.

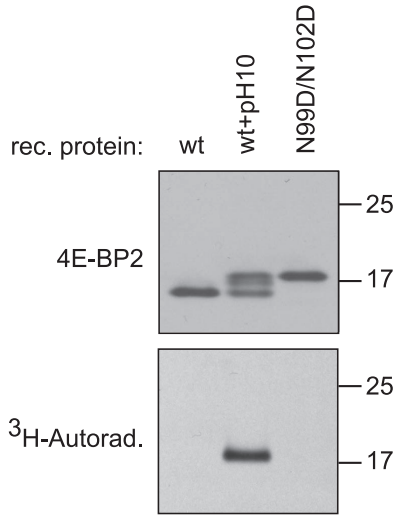


FIGURE 3. ***In vitro* deamidated 4E-BP2 accrues isoaspartyl residues and is a substrate for PIMT.** Recombinant 4E-BP2 was alkaline-deamidated in 0.15 M Tris-HCl, pH 10, for 24 h at 37 °C. Wild type (*wt*), alkaline-treated (*wt+pH10*), and the deamidated mimic (*N99D/N102D*) 4E-BP2 proteins were assayed for isoaspartyl content as in Fig. 2*B*. Recombinant protein (1 μg) was incubated with 2.5 μM GST-PIMT and 50 μM AdoMet in 75 mM Na-MES, pH 6.2, for 10 min at 30 °C. Samples were divided and resolved by SDS-PAGE (15%) (Western blotting; *upper panel*) or by BisTris PAGE (4–12%) (^{3}H detection; *lower panel*).

iso4E-BP2 (Asp^{99}/Asp^{102}), predominantly recognized 4E-BP2 that had been deamidated by alkaline treatment (Fig. 4*A*), thereby confirming its specificity for 4E-BP2 containing isoaspartates at these two positions. Very faint cross-reactivity was noticed with the nonisoaspartate 4E-BP2 N99D/N102D variant. This antibody also weakly detects 4E-BP2 containing a single isoaspartate at position 102, particularly when position 99 is concomitantly occupied by an aspartate, as shown in supplemental Fig. 1. Increasing amounts of isoaspartates were also detected with this antibody in recombinant wild type 4E-BP2 that had been "aged" *in vitro* by incubation for several days at pH 7 (Fig. 4*B*).

4E-BP2 Is an in Vivo PIMT Substrate in Brain—Our previous work demonstrated that 4E-BP2 undergoes deamidation, uniquely in the brain, during the first 3 murine postnatal weeks (50). Thus, anti-iso4E-BP2 (Asp^{99}/Asp^{102}) antibody was used to determine whether 4E-BP2, the predominant 4E-BP expressed in the mammalian brain, is an *in vivo* substrate for PIMT in this organ. 4E-BP2 was immunoprecipitated from wild type or PIMT−/− brains lysates using anti-4E-BP2 antibody, and proteins were resolved by SDS-PAGE. Immunoprecipitation from 4E-BP2−/− brain lysates served as a negative control for antibody specificity. Western blotting with anti-iso4E-BP2 (Asp^{99}/Asp^{102}) antibody indicated that only 4E-BP2 from PIMT−/− brains contained appreciable amounts of isoaspartyl residues (Fig. 5). Faint signals in the *wild type lane* are likely due to cross-reactivity with unrelated proteins accumulated during the immunoprecipitation procedure, rather than with 4E-BP2 itself, as similar signals are also present in the 4E-BP2−/− lane.

Isoaspartate Accumulation in 4E-BP2 Does Not Alter Its Stability but Renders It Defective for Enhanced Raptor Binding—Formation of isoaspartyl residues in some proteins has been associated with either increased proteasomal degradation (52, 53) or aggregation (54). We therefore asked whether the accumulation of isoaspartates in 4E-BP2 in the PIMT−/− brain affects the stability of the protein. Western blotting on wild type and PIMT−/− brain lysates from animals aged approximately between 6 and 14 weeks postnatally indicated no apparent change in steady-state levels of 4E-BP2, thereby suggesting that 4E-BP2 is not subject to aberrant degradation or aggregation in the absence of PIMT-mediated repair (Fig. 6*A*). We previously reported that deamidation of 4E-BP2 enhances its interaction with raptor (50). Thus, we questioned whether deamidated 4E-BP2 containing primarily isoaspartates at the deamidation sites maintained increased raptor association. To address this, recombinant 4E-BP2 was treated at high pH to induce deamidation and then assayed for *in vitro* association with raptor protein, which had been previously immunoprecipitated from

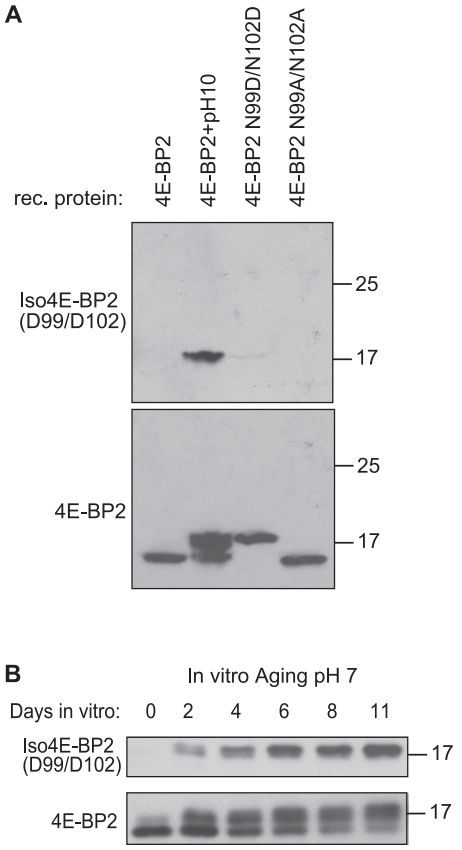


FIGURE 4. **Antibody raised in-house specifically recognizes 4E-BP2 harboring isoaspartyl residues at positions 99 and 102.** *A*, Western blot analysis using IgG purified from rabbit serum that was raised against a peptide containing the 4E-BP2 residues 99 and 102 in the isoaspartate conformation (iso4E-BP2 Asp^{99}/Asp^{102}). Wild type recombinant (*rec. protein*) 4E-BP2 protein was alkaline-deamidated as in Fig. 3 (*wt*+*pH10*). 0.5 ng of each recombinant protein was resolved by SDS-PAGE (15%) as indicated, and Western blotting was performed with anti-iso4E-BP2 (Asp^{99}/Asp^{102}) antibody (*uppper panel*). The nitrocellulose membrane was stripped of primary antibody and reprobed with a 4E-BP2-specific antibody (*lower panel*). *B,* Western blot detection of isoaspartyls accumulated in *in vitro* aged recombinant 4E-BP2. Recombinant protein was incubated at 37 °C in 0.15 M Tris-HCl, pH 7, and sampled on the indicated days. Samples were resolved by SDS-PAGE (15%) for Western blotting.

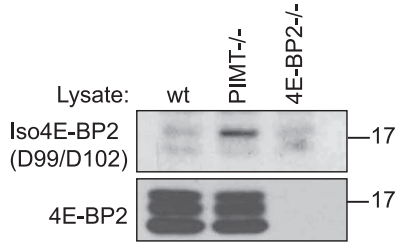


FIGURE 5. **4E-BP2 is an *in vivo* substrate for PIMT in the mammalian brain.** Western blot analysis using the iso4E-BP2 D99/D102 antibody (Fig. 4) detects isoaspartyl residues in 4E-BP2 from PIMT−/− brain lysates. 4E-BP2 was immnoprecipitated from 200 μg of whole brain extracts (7 weeks postnatal) and resolved by 15% SDS-PAGE. Immunoprecipitates from 4E-BP2−/− brain lysates controlled for antibody specificity. *wt*, wild type.

cell lysates and immobilized on protein G-Sepharose resin. It is noteworthy that the longer pH 10 treatments performed here on recombinant 4E-BP2 proteins (72 h) yielded a single isoapartyl-containing isoform derived from wild type protein (doubly deamidated) as opposed to the three forms found following a shorter treatment (Figs. 3 and 4*A*). This uniformity allows ease

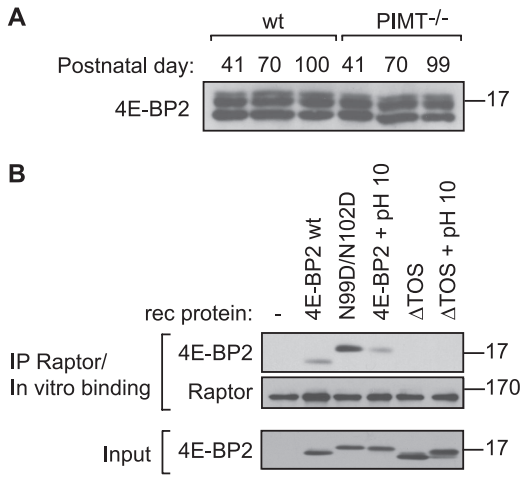


FIGURE 6. **Accumulation of isoaspartyl residues in 4E-BP2 in the brain does not alter protein stability but reduces its interaction with raptor relative to 4E-BP2 N99D/N102D.** *A*, steady-state level of 4E-BP2 is unaffected in PIMT−/− brains as assessed by Western blotting. Brain lysates (30 μg) from wild type (*wt*) or PIMT−/− mice of indicated ages were resolved by 15% SDS-PAGE. *B*, isoaspartyls in 4E-BP2 are detrimental to the enhanced association of deamidated 4E-BP2 with raptor. Raptor was immunoprecipitated (*IP*) from 4E-BP2−/− murine brain, and the resulting immune complex immobilized on Protein G-Sepharose resin was incubated with the indicated recombinant 4E-BP2 protein (*rec. protein*) variants. Bound complexes were separated by SDS-PAGE (5–20%). Here, pH 10 treatment of 4E-BP2 protein was for 72 h. This longer treatment induces a single, isoaspartyl-containing (double-deamidated) form for wild type 4E-BP2. The 4E-BP2 C-terminal TOS motif is required for raptor binding; 4E-BP2 variants lacking this sequence do not bind raptor thereby serving as negative controls for the binding assay.

of comparison in raptor binding relative to the other 4E-BP2 variants. In agreement with our earlier findings (50), the deamidation mimic, 4E-BP2 N99D/N102D, exhibited a dramatic enhancement in raptor binding, relative to the wild type form (Fig. 6*B*, compare *second* and *third lanes*). Strikingly, *in vitro* deamidated 4E-BP2 (4E-BP2; pH 10) which contains isoaspartates at the principal deamidation sites displayed raptor binding efficiency similar to that of wild type 4E-BP2 and thus was considerably reduced with respect to the deamidation mimic (Fig. 6*B*, compare *fourth lane* with *second* and *third lanes*). No binding was detected for 4E-BP2 variants lacking the C-terminal residues comprising the TOS motif, which is required for interaction with raptor, as we demonstrated previously (50). Thus, this result indicates that the accumulation of isoaspartates in 4E-BP2 during deamidation counteracts its gain of function in raptor-binding.

DISCUSSION

We report here that 4E-BP2 is a substrate of PIMT. Deamidation of endogenous 4E-BP2 in the PIMT−/− murine brain or of recombinant 4E-BP2 deamidated under alkaline pH or by *in vitro* aging caused the formation of isoaspartyl residues. Detection of isoaspartates in 4E-BP2 in the PIMT−/− brain indicates that 4E-BP2 is an accessible intracellular substrate to PIMT and that PIMT normally acts to suppress isoaspartate accumulation following deamidation in the wild type brain. 4E-BP2 deamidation occurs principally on two asparagines (Asn^{99} and Asn^{102}) near the C terminus (50), and this leads to slower migration of the deamidated species in SDS-PAGE. Thus, in the PIMT−/− brain the slow migrating forms of

4E-BP2 harbor 70–85% isoaspartyl content at each deamidation site, as this is the ratio favored by succinimide hydrolysis. Our antibody directed against 4E-BP2, containing isoaspartates at the deamidation sites (anti-iso4E-BP2), recognized the slowest migrating 4E-BP2 species formed by alkaline-induced deamidation (Fig. 4). In using this antibody to demonstrate, by Western blotting, the accumulation of isoaspartyls in 4E-BP2 from PIMT−/− brain lysates (Fig. 5), it was also shown that the 4E-BP2 signal from wild type brain was not above background levels (determined with the 4E-BP2−/− lysate). This indicates that PIMT-mediated repair of 4E-BP2 is sufficiently efficacious to maintain it in the aspartate-containing form, at least as given by the limit of detection of this assay.

The appearance and accumulation of 4E-BP2 deamidation in murine brain in the early postnatal weeks parallel approximately those of PIMT expression (50, 55). This suggests that, in addition to 4E-BP2, numerous PIMT substrates accrue isoaspartyl residues during the same time frame and that increased PIMT expression is required to catalyze the conversion to aspartates sufficiently. Indeed, the large number of isoaspartates formed in the brain, relative to other tissues, rapidly increases within the first 100 postnatal days in PIMT−/− mice (56).

Studies aimed at cataloguing the complement of PIMT substrates in the brain did not identify 4E-BP2 as a substrate (46, 49). A possible explanation for this is that 4E-BP2 from the PIMT−/− brain lysates yielded either a minor radiolabel signal from exogenous PIMT methylation or was not detected by protein staining in the two-dimensional isoelectric focusing SDS-PAGE analyses. Consequently, it would not have been identified.

Isoaspartyl groups tend to preclude several different protease activities (52, 57, 58). In contrast, other work suggests that isoaspartates predispose calmodulin to elevated rates of ubiquitin-independent proteasomal degradation (53). Similarly, total isoaspartate levels are maintained at a constant level in aged PIMT-deficient mice (greater than 100 days of age) by enhanced degradation and urinary excretion of damaged proteins (56). Thus, it is conceivable that isoasparate accumulation in 4E-BP2 in PIMT−/− mice might affect the stability or turnover of the protein. However, no changes were observed in 4E-BP2 levels upon comparing wild type and PIMT−/− brain lysates from mice aged between 40 and 100 days (Fig. 6*A*). Although no gross changes in 4E-BP2 expression arise from isoaspartate formation in PIMT−/− brains in the first 100 postnatal days, it is possible that 4E-BP2 harboring isoaspartyl residues would be degraded and excreted in older knock-out mice in which total brain isoaspartyl content has reached a plateau. We observed that 4E-BP2 deamidated *in vitro*, so as to accrue isoaspartates at positions 99 and 102, lost the proclivity for enhanced raptor association that is a function exhibited by deamidated 4E-BP2 (not harboring isoaspartyls), relative to the wild type protein (Fig. 6*B*). 4E-BP2 deamidation occurs in the brain during early postnatal development (50). Therefore, the PIMT-catalyzed methylation of 4E-BP2 to prevent isoaspartate accumulation may be necessary to preserve the enhanced association of deamidated 4E-BP2 with raptor in the nervous system.

Genetic ablation of 4E-BP2 in mice begets impairments in certain forms of learning and memory and alters synaptic plasticity, a cellular correlate of memory formation (59–61). Moreover, 4E-BP2 undergoes deamidation during early postnatal development, and expression of deamidated 4E-BP2 in hippocampal CA1 neurons slows the kinetics of excitatory synaptic transmission at the Schaffer collateral pathway (50). Considering this, it is conceivable that PIMT-mediated suppression of isoaspartate accumulation in deamidated 4E-BP2 in brain may be required for optimal synaptic function during memory formation. In summary, we have identified 4E-BP2 as an endogenous substrate for PIMT-mediated repair of isoaspartyl residues that accrue by asparagine deamidation in postnatal brain development.

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