Mechanisms for Increased Levels of Phosphorylation of Elongation Factor-2 during Hibernation in Ground Squirrels†

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ABSTRACT: Previously, eEF-2 phosphorylation has been identified as a reversible mechanism involved in the inhibition of the elongation phase of translation. In this study, an increased level of phosphorylation of eukaryotic elongation factor-2 (eEF-2) was observed in the brains and livers of hibernating ground squirrels. In brain and liver from hibernators, eEF-2 kinase activity was increased relative to that of active animals. The activity of protein phosphatase 2A (PP2A), a phosphatase that dephosphorylates eEF-2, was also decreased in brain and liver from hibernators. This was associated with an increase in the level of inhibitor 2 of PP2A (I_2^{PP2A}), although there was an increase in the level of the catalytic subunit of PP2A (PP2A/C) in hibernating brains and livers. These results indicate that eEF-2 phosphorylation represents a specific and previously uncharacterized mechanism for inhibition of the elongation phase of protein synthesis during hibernation. Increased levels of eEF-2 phosphorylation in hibernators appear to be a component of the regulated shutdown of cellular functions that permits hibernating animals to tolerate severe reductions in cerebral blood flow and oxygen delivery capacity.

Recent progress has defined a number of molecular and cellular changes that occur during the preparation, entrance, and torpor stages of hibernation (*19*, *25*). The process of mRNA translation is conventionally divided into three distinct phases termed (peptide-chain) initiation, elongation, and termination (*30*). Global rates of protein synthesis have been determined to be profoundly reduced in hibernating animals, the decreased rate of protein synthesis resulting from inhibition of both initiation and elongation (*10*).

One cellular mechanism that contributes to the regulated suppression of metabolism and thermogenesis during hibernation is reversible phosphorylation of enzymes and proteins that limits rates of flux through metabolic pathways (*42*). The acute regulation of mRNA translation depends on changes in the activity of initiation factors or elongation factors, which is also commonly mediated by alteration in their states of phosphorylation (*14*). Phosphorylation of eIF2α contributes to a substantial decrease in the rate of protein synthesis initation in hibernators (*10*), but the mechanism(s) involved in the regulation of elongation in hibernators is not known.

mRNA translocation on the ribosome involves elongation factor-2 (eEF-2),[1] and eEF-2 is phosphorylated and inhibited by a specific kinase (eEF-2 kinase), with phosphorylation reducing the ability of the factor to promote translocation possibly by decreasing its affinity for pretranslocation ribosomes (*1*, *24*, *32*, *36*, *37*). Increased levels of phosphorylation of eEF-2 occur in response to cellular stimulation by mitogens, growth factors, and neurotransmitters that influence intracellular Ca^{2+} levels (*31*, *35*, *37*). However, the precise physiological role of eEF-2 phosphorylation has not yet been elucidated.

eEF-2 kinase is a Ca^{2+}/calmodulin-dependent enzyme which appears to have eEF-2 as its only substrate (*22*). eEF-2 is dephosphorylated by protein phosphatase 2A (PP2A), and regulation of dephosphorylation by PP2A also appears to be important in determining the level of phosphorylation of eEF-2 (*24*, *32*, *33*). Mutation of Tyr307 and Leu309 in the PP2A catalytic subunit favors association with the α4 subunit (PP2A/α4) which promotes dephosphorylation of elongation factor-2 (*3*).

In this study, we have investigated the potential role of eEF-2 phosphorylation in reducing the rate of elongation in hibernators (*10*). The results obtained suggest that the level of eEF-2 phosphorylation is increased in hibernators through both an increase in eEF-2 kinase activity and a decrease in PP2A activity.

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[1] Abbreviations: eEF-2, eukaryotic elongation factor-2; eEF-2 kinase, eukaryotic elongation factor-2 kinase; PP2A, protein phosphatase 2A; I_2^{PP2A}, inhibitor 2 of protein phosphatase 2A; MBP, myelin basic protein.

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EXPERIMENTAL PROCEDURES

Animal Procedures. Induction of hibernation in ground squirrels was carried out as previously described (*8*). Thirteen lined ground squirrels, *Spermophilus tridecemlineatus*, were captured by a trapper licensed by the U.S. Department of Agriculture (TLS Research, Bartlett, IL). All experiments were approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke. The squirrels were housed individually in a room with an ambient temperature of 21 °C and a 12 h:12 h light:dark cycle, and were fed standard rodent diet and water ad libitum. To induce hibernation, the squirrels were placed separately in cages containing wood shavings in a cold chamber (hibernaculum) that was kept at 4 °C and 60% humidity. The hibernaculum was kept in constant darkness, except for a photographic red safe light (3−5 lux), and could be entered only through a darkened anteroom. Noise within the chamber was kept to a minimum. After hibernation for 2−6 days (a hibernation state will be sustained for 2−7 days after ground squirrels begin hibernating), animals were killed by decapitation. The brains and livers were removed quickly, frozen immediately on dry ice or homogenized immediately in the specified buffers, and then stored at −70 °C. Animals that had not yet entered hibernation in the cold chamber or that were aroused from hibernation were used as active controls.

Materials. ATP, benzamidine, Brij 35, glycerol, PMSF, β-mercaptoethanol, dithiothreitol, EDTA, EGTA, Tris, sodium dodecyl sulfate, sodium azide (NaN_3), aprotinin, pepstatin A, HEPES, myelin basic protein (MBP), leupeptin, antipain, histamine, diphtheria toxin (DT), ethylene glycol, sodium orthovanadate, sodium pyrophosphate, and trichloroacetic acid were obtained from Sigma (St. Louis, MO). Calmodulin and anti-PP2A/C antibody were from Calbiochem (La Jolla, CA). Inhibitor 2 of protein phosphatase-1 (PP1) was from New England Biolabs, Inc. (Beverly, MA). [γ-^{32}P]ATP was from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Anti-total eEF-2 antibody (G118) and anti-phospho-eEF-2 antibody (CC81) were prepared as previously described (*20*). Anti-eEF-2 kinase antibody (CC371) was prepared against a C-terminal domain of eEF-2 kinase fused to GST. Anti-inhibitor 2 of PP2A antibody was prepared as described previously (*6*, *17*, *18*).

Preparation of Tissue Extracts. Extracts were prepared from the brains and livers of active and hibernating ground squirrels by homogenization performed on ice using a glass/glass hand homogenizer for 15 strokes. For the eEF-2 assay, tissue samples were homogenized in 4 volumes of homogenization buffer containing 20 mM HEPES (pH 7.5), 50 mM NaCl, 25 mM KCl, 10 mM dithiothreitol, 3 mM benzamidine, 1% SDS, 1 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 0.2 mM PMSF, 1.25 μg/mL pepstatin A, and 1 mM protease inhibitor cocktail. Homogenates were centrifuged at 20000*g* for 30 min at 4 °C. For analysis of eEF-2 kinase protein, brain and liver samples from active and hibernating squirrels were homogenized in 4 volumes (w/v) of a buffer containing 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM EGTA, 15 mM β-mercaptoethanol, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.02% NaN_3, 100 mM NaCl, 10 μg/mL leupeptin, 10 μg/mL antipain, 10 μg/mL Trasylol, and 100 μM PMSF. Homogenates were centrifuged at 100000*g* for 30 min at 4 °C. For measurement of the amounts of PP2A and I_2^{PP2A}, tissue was homogenized (1:4, w/v) in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 1 mM PMSF, and 20 μg/mL leupeptin. Homogenates were centrifuged at 12500*g* for 10 min at 4 °C. The protein concentrations of the supernatants were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as a standard. Tissue extracts were adjusted for equal protein concentration and stored at −70 °C.

Determination of the Amounts of Phosphorylated eEF-2, Total eEF-2, eEF-2 Kinase, PP2A/C, and I_2^{PP2A} by Immunoblotting. Immunoblotting was performed according to the method of Frerichs et al. (*10*). In brief, tissue extracts containing 20 μg of protein were subjected to SDS−polyacrylamide gel electrophoresis (SDS−PAGE) (1.5 h at 125 V) on 8 or 14% Tris-glycine gels (NOVEX, San Diego, CA) followed by transfer to PVDF membranes (NOVEX) by electroblotting for 1.5 h at 25 V. Blots were incubated with a 1:1000 dilution of rabbit polyclonal antibodies specific for the phosphorylated form of eEF-2, total eEF-2, eEF-2 kinase, PP2A/C, or I_2^{PP2A}, followed by peroxidase-labeled anti-rabbit secondary antibody (1:4000 dilution). Immunoreactive bands were developed using an ECL kit (Western Blotting Analysis System, Amersham Pharmacia Biotech, Buckinghamshire, England). The optical density of the bands was measured using an EAGLE EYE II computer-controlled Image system (Stratagene, La Jolla, CA). All immunoblots were exposed to Hyperfilm (high-performance chemiluminescence film), and the optical densities measured for the immunoreactive bands were in the linear range. Results from immunoblots for samples obtained from up to seven active or seven hibernating animals were averaged. Optical density data from different experiments were normalized with the results for active liver being assigned the value of 1.

Measurement of eEF-2 Kinase Activity. eEF-2 kinase activity in active and hibernating brains and livers was measured as previously described (*24*). Briefly, eEF-2 kinase activity in cytosolic extracts was assayed in a reaction mixture (final volume of 100 μL) containing 50 mM HEPES (pH 7.4), 10 mM magnesium acetate, 5 mM dithiothreitol, 20 μg/mL calmodulin, 0.2 μg/mL purified eEF-2, 1 mM EGTA, 1.5 mM $CaCl_2$, 1 μg/mL leupeptin, and tissue extract containing 30 μg of protein. The reaction mixtures were preincubated at either 4 or 30 °C for 1 min, and reactions were initiated by the addition of 50 μM nonradioactive ATP, continued for 5 min at either 4 or 30 °C, and terminated by the addition of 20 μL of Tris-glycine SDS sample buffer. Assays were linear with respect to time of incubation and protein concentration. For the SDS−PAGE assay, the terminated mixtures were boiled for 5 min and proteins were subjected to electrophoresis and transferred to PVDF membrane. Blots were incubated with the antiphosphorylated form of eEF-2 antibody (CC81) (1:1000 dilution) followed by peroxidase-labeled anti-rabbit secondary antibody. Immunoblots were developed using ECL reagents. All immunoblots were exposed to Hyperfilm, and the optical densities measured for the immunoreactive bands were in the linear range.

Assay of PP2A Activity. PP2A activity in active and hibernating brains and livers was determined using the method of Damuni (*12*). Briefly, tissue extracts from brains

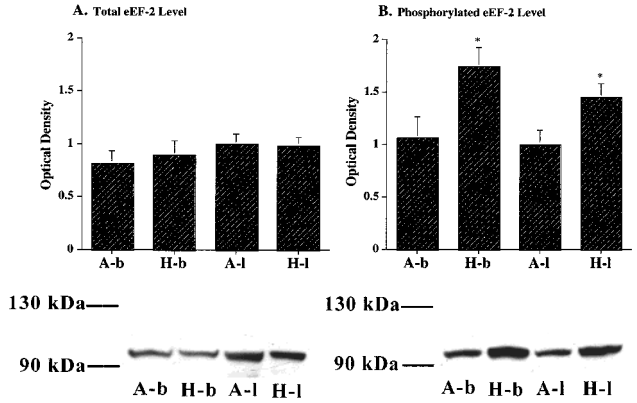

FIGURE 1: Hibernation-induced eEF-2 phosphorylation in brain and liver from ground squirrels. The total amounts of eEF-2 (A, $n = 7$) and phospho-eEF-2 (B, $n = 7$) were measured by immunoblotting in extracts from brains and livers of hibernating and active squirrels. Tissue extracts containing 20 μg of protein were subjected to SDS−PAGE (8% Tris-glycine gels) followed by transfer to PVDF membranes. Immunoblotting ECL data were analyzed using an EAGLE EYE II computer-controlled Image system. A representative blot is shown in each of the lower panels, and the data in the upper panels are a summary of seven experiments. The optical densities were normalized to that of samples obtained from active livers (A-l) (set to an arbitrary value of 1): A-b, active brains; H-b, hibernating brains; A-l, active livers; and H-l, hibernating livers. Asterisks indicate that $P < 0.05$, as compared to an active control.

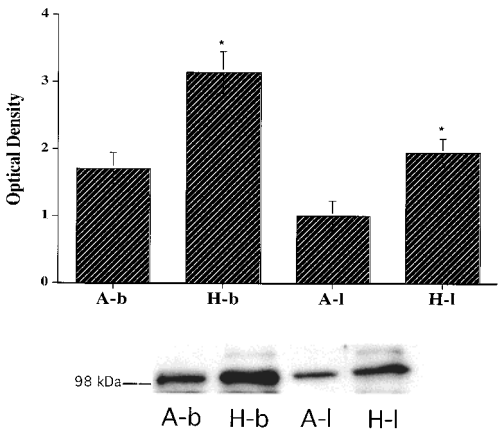

FIGURE 2: Increased eEF-2 kinase levels in brain and liver from hibernating ground squirrels. The total amount of eEF-2 kinase protein was measured by immunoblotting in extracts ($n = 6$) from brains and livers of hibernating and active ground squirrels. Samples were subjected to SDS−PAGE and analyzed as described above. A representative blot is shown in each of the lower panels, and the data in the upper panels are a summary of six experiments. The optical densities were normalized to that of samples obtained from active livers (A-l) (set to an arbitrary value of 1): A-b, active brains; H-b, hibernating brains; A-l, active livers; and H-l, hibernating livers. Asterisks indicate that $P < 0.05$, as compared to an active control.

and livers (protein concentration of 1 μg/μL) were diluted 1:800 with the assay buffer containing 50 mM Tris-HCl (pH 7.0), 10% glycerol, 1 mM benzamidine, 0.1 mM PMSF, 14 mM β-mercaptoethanol, 1 mM EDTA, 0.2 mg/mL bovine serum albumin, and 1 mM Brij 35. Diluted tissue extracts (5 μL) were added to a reaction mixture containing 35 μL of assay buffer and 5 μL of inhibitor 2 of PP1 (1:40 dilution). Reactions were initiated at 30 °C by the addition of 5 μL of ^{32}P-labeled MBP. After incubation for 5 min, reactions were terminated by addition of 0.1 mL of 12% TCA. The mixtures were centrifuged at 10 000 rpm in a Fisher microcentrifuge. An aliquot of the supernatant (0.12 mL) was mixed with 1 mL of scintillant, and the amount of radioactivity was determined in a liquid scintillation spectrometer. One unit of PP2A activity was defined as the amount of phosphatase that released 1 nmol of $^{32}P_i$ per minute from a ^{32}P-labeled substrate. Preparation of ^{32}P-labeled MBP has been described previously in detail (*12*, *17*).

Statistical Analysis. Results are expressed as means ± standard error of the mean (SEM). Comparison between different groups was performed using ANOVA and a Student's *t* test. $P < 0.05$ was taken as the level of significance.

RESULTS

Hibernation-Induced eEF-2 Phosphorylation. The total eEF-2 content and the amount of phosphorylated eEF-2 were measured by immunoblotting in the brain and liver extracts from the same animals. There were no differences in the total amount of eEF-2 in extracts from brains and livers of hibernating or active squirrels (Figure 1A). However, a significant increase in the levels of phospho-eEF-2 was observed in hibernating brains and livers as compared to that in active controls (a 65% increase in hibernating brains and a 45% increase in hibernating livers in phospho-eEF-2 content) (Figure 1B). It can be concluded that entry into hibernation is accompanied by an increased level of eEF-2 phosphorylation in both brains and livers, especially in brains.

Increased eEF-2 Kinase Protein Activity during Hibernation. eEF-2 is specifically phosphorylated by eEF-2 kinase and dephosphorylated by PP2A. An increased level of eEF-2 phosphorylation during hibernation may therefore result from an increased eEF-2 kinase content and/or activity or from a decreased PP2A content and/or activity. eEF-2 kinase protein levels, measured using an antibody specific for eEF-2 kinase, increased significantly in both hibernating brains (increased by 54.3%) and hibernating livers (increased by 51.5%) as compared to that in active controls (Figure 2). However, the level of eEF-2 kinase in livers was lower than that in brains (only 56.7% of the brain level in active animals and 61.5% in hibernating animals).

A significant increase in eEF-2 kinase activity was also observed in hibernating brains (increased by 110.8%) and livers (increased by 77.5%) as compared to those in corresponding tissues from active controls, when incubations were carried out at 4 °C (Figure 3). When eEF-2 kinase assays were carried out at 30 °C, a similar significant increase in the activity of eEF-2 kinase was observed in brains and livers of hibernating animals as compared to those of active animals (increased by 99.6% in hibernating brains and by 66.3% in hibernating livers), indicating that cold is not required for the activity differences.

PP2A Activity Is Decreased through Changes in the Activity of I_2^{PP2A} during Hibernation. PP2A activity in the brains and livers of active and hibernating squirrels was measured using [^{32}P]MBP as a substrate. ^{32}P-labeled MPB is used as a substrate to assay PP2A activity because PP2A is most sensitive to this substrate. Although PP2B and PP2C can dephosphorylate MPB in vitro, the activities of the phosphatases with this substrate are an order of magnitude lower relative to that of PP2A (*15*). The activity of PP2A

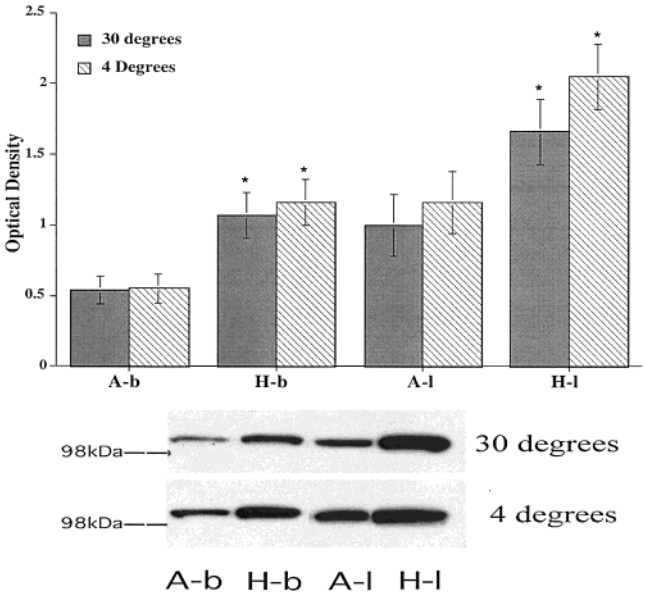

FIGURE 3: Increased eEF-2 kinase activity in hibernating ground squirrels. eEF-2 kinase activity in cytosolic extracts ($n = 8$) was assayed as described in Experimental Procedures at either 30 or 4 °C. Samples were subjected to SDS−PAGE, and proteins were transferred to PVDF membrane. Phospho-eEF-2 was detected with an anti-phospho-eEF-2 antibody. A representative blot is shown in each of the lower panels, and the data in the upper panels are a summary of eight experiments. The optical densities were normalized to that of samples obtained from active livers (A-l) (set to an arbitrary value of 1): A-b, active brains; H-b, hibernating brains; A-1, active livers; and H-1, hibernating livers. Asterisks indicate that $P < 0.05$, as compared to active controls at the corresponding temperature of 4 or 30 °C.

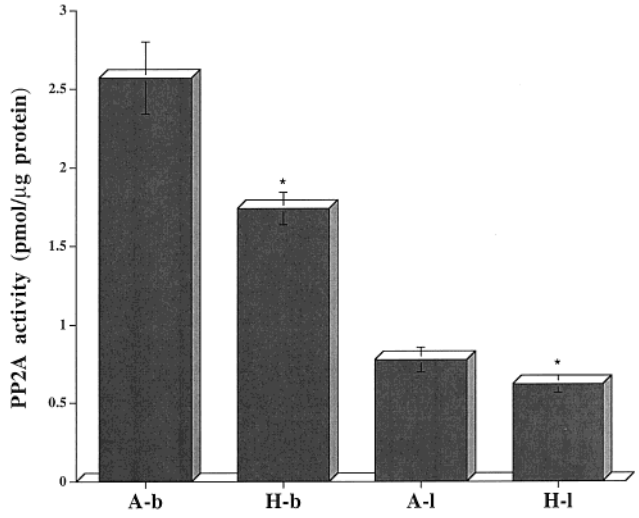

FIGURE 4: Decreased PP2A activity in brain and liver from hibernating ground squirrels. PP2A activity in cytosolic extracts ($n = 8$) was determined as described in Experimental Procedures using ^{32}P-labeled MBP as a substrate. Phosphatase activity was measured as the amount of ^{32}P released and is expressed in picomoles per microgram of extract protein: A-b, active brains; H-b, hibernating brains; A-l, active livers; and H-l, hibernating livers. Asterisks indicate that $P < 0.05$, as compared to an active control.

was found to be significantly reduced in both hibernating brain (~32% decrease in PP2A activity) and livers (~20% decrease in PP2A activity), as compared to that in active controls (Figure 4). These decreases in PP2A activity occurred despite an increase in the amount of catalytic

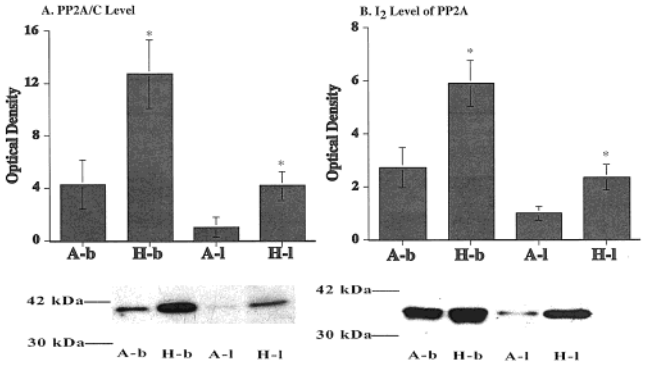

FIGURE 5: Increased levels of catalytic subunit and inhibitor 2 of PP2A in brain and liver of hibernating ground squirrels. The total amounts of $PP2A_C$ (A, $n = 6$) and I_2^{PP2A} (B, $n = 6$) were measured by immunoblotting in tissue extracts from brain and liver of active and hibernating squirrels. Tissue extracts containing 20 μg of protein were subjected to SDS−PAGE (14% acrylamide), and proteins were transferred to PVDF membrane. A representative blot is shown in each of the lower panels, and the data in the upper panels are a summary of six experiments. The optical densities were normalized to that of samples obtained from active livers (A-l) (set to an arbitrary value of 1): A-b, active brains; H-b, hibernating brains; A-l, active livers; and H-l, hibernating livers. Asterisks indicate that $P < 0.05$, as compared to an active control.

subunit of PP2A ($PP2A_C$) in hibernating brains (increased by 66%) and livers (increased by 76%) as compared to active controls (Figure 5A). There was no difference between hibernating and active animals in the amount of either the structural A subunit ($PP2A_A$) or the regulatory B subunit ($PP2A_{B\alpha}$) of PP2A (data not shown). However, the level of the inhibitor of PP2A, I_2^{PP2A}, detected by a specific antibody increased significantly in hibernating brains (increased by 54%) and livers (increased by 57%), as compared to levels in active controls (Figure 5B).

DISCUSSION

Entry into hibernation results from a regulated suppression of metabolic rates and physiological functions (such as the remarkable reductions in body temperature, respiratory rate, heart rate, blood pressure, cardiac output, and cerebral blood flow and metabolism) (*8*, *9*). During hibernation, the dramatic changes in body temperature and other physiological variables are associated with a slowing of many cellular processes. In hibernating squirrels, the initiation phase of protein synthesis is inhibited by phosphorylation of eIF2α, and the elongation phase, as measured by an increase in ribosomal transit time, is prolonged (*10*). In the study presented here, we have obtained results that suggest that the increase in transit time occurs as a result of an increase in the level of phosphorylation of the essential elongation factor, eEF-2. An increased level of eEF-2 phosphorylation results from both an increase in eEF-2 kinase activity and a decrease in PP2A activity. An increased level of phosphorylation of eEF-2 appears to be a hibernation state-specific and reversible mechanism for inhibition of translation elongation since no difference in eEF-2 phosphorylation was found in active squirrels that had been aroused from hibernation compared to those that had never hibernated (data not shown).

eEF-2 is a monomeric 100 kDa protein composed of 857 amino acid residues (*28*), which mediates the translocation

step in peptide-chain elongation by promoting transfer of peptidyl-tRNA from the A to the P site and the movement of mRNA relative to the ribosome by one codon (*24*, *31*, *35*). eEF-2 is phosphorylated by eEF-2 kinase, a Ca^{2+}/calmodulin-dependent protein kinase with a unique structure (*7*, *40*). Phosphorylation of eEF-2 at Thr56 by eEF-2 kinase inhibits protein synthesis (*23*) by a mechanism that likely involves a reduction in the affinity of phospho-eEF-2 for the pretranslocation ribosome (*1*, *35*), although eEF-2 phosphorylation may also influence the ability of the factor to catalyze mRNA translocation.

Activation of eEF-2 kinase occurs in response to a variety of hormones, neurotransmitters, and growth factors that influence cellular Ca^{2+} levels (*30*, *36*). As a consequence, the phosphorylation of eEF-2 and the resulting inhibition of protein synthesis are often transient in nature. Such a transient process may act in several ways. For example, eEF-2 phosphorylation may conserve cellular energy during exocytosis in certain cell types. Transient eEF-2 phosphorylation would be expected to result in a decrease in the levels of short-lived protein repressors, and this in turn may influence other cellular processes (*27*). Recent studies have demonstrated that inhibition of protein synthesis by eEF-2 phosphorylation is associated with an alteration in the pattern of protein expression (*41*). Translational block is a common mechanism involved in the transition of cells from one physiological state into another (*38*). Once a self-maintained pattern of synthesized proteins is disrupted by translational block, new genes may be expressed and new programs of gene expression may enable the transition of cells into a new physiological state (*4*, *39*, *43*). eEF-2 phosphorylation may therefore influence the molecular coordination required for entrance into hibernation or arousal from hibernation in ground squirrels.

PP2A is a major protein serine/threonine phosphatase that has been implicated in the modulation of important cellular processes, including intermediary metabolism, signal transduction, cell cycle progression in DNA replication, transcription, and protein synthesis (*3*, *18*). The PP2A holoenzyme is a heterotrimeric complex consisting of an $M_r \sim 65$ kDa structural subunit (A), an $M_r \sim 52$ kDa regulatory subunit (B), and an $M_r \sim 36$ kDa catalytic subunit (C) (*16*). The deduced amino acid sequences of at least two forms of each of the A, B, and C subunits are 86, 85, and 97% identical (*6*). PP2A can be inactivated by phosphorylation of tyrosine or threonine residues (*2*, *12*). Recently, two potent heat-stable PP2A-specific inhibitor proteins, designated inhibitor 1 of PP2A (I_1^{PP2A}) and inhibitor 2 of PP2A (I_2^{PP2A}), have been characterized (*6*, *17*, *18*). I_1^{PP2A} and I_2^{PP2A} are the products of distinct genes. I_2^{PP2A} is specific for PP2A and has little or no activity toward PP1 or PP2C (*17*). The inhibitor proteins are noncompetitive and potent inhibitors of PP2A with apparent K_i values of ~30 and ~25 nM, respectively (*17*). I_1^{PP2A} and I_2^{PP2A} appear to act as inhibitors of PP2A, at least in part, by binding to its catalytic subunit. A reduction in PP2A activity during hibernation was found to be associated with an increase in the level of I_2^{PP2A}. $PP2A_C$ levels actually increased during hibernation.

PP2A is only one of the four major protein serine/threonine phosphatases present in the cytoplasm of mammalian cells (*5*). The other three are PP1, PP2B, and PP2C. Several studies have shown that PP2A specifically dephosphorylates phospho-eEF-2. In vitro, bisphosphorylated eEF-2 is dephosphorylated efficiently by PP2A, but hardly at all by the other major cellular protein phosphatase, PP1 (*11*, *33*). Studies in intact cells using PP2A inhibitors indicate that PP2A is the main phosphatase for eEF-2, although PP2C can dephosphorylate eEF-2 at some level in vitro, and recent results support the idea that regulation of eEF-2 dephosphorylation by PP2A is modulated by rapamycin-sensitive signaling pathways (*3*). This process involves a novel PP2A regulatory subunit called α4, and it appears that the PP2A/α4 complex may function downstream of the mammalian target for rapamyicin (mTOR) to specifically promote eEF-2 dephosphorylation. A reduction of PP2A/α4 activity in response to an increase in the levels of I_2^{PP2A}, coupled with an increase in eEF-2 kinase levels and activity, may be responsible for the increase in the level of eEF-2 phosphorylation and the reduced rate of elongation of translated proteins observed in tissue from hibernating ground squirrels.

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