Seasonal and state-dependent changes of eIF4E and 4E-BP1 during mammalian hibernation: implications for the control of translation during torpor

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van Breukelen, Frank, Nahum Sonenberg, and Sandra L. Martin. Seasonal and state-dependent changes of elf4E and 4E-BP1 during mammalian hibernation: implications for the control of translation during torpor. Am J Physiol Regul Integr Comp Physiol 287: R349–R353, 2004. First published April 1, 2004; 10.1152/ajpregu.00728.2003.—Mammalian hibernation involves cessation of energy expenditure and depression of protein synthesis. To further elucidate the mechanisms employed in depressing translation, we surveyed key eukaryotic initiation factors [eIF2, eIF4B, eIF4E, eIF4GI and -II, and 4E-binding protein-1 (4E-BP1), -2, and -3] for their availability and phosphorylation status in the livers of golden-mantled ground squirrels (Spermophilus lateralis) across the hibernation cycle. Western blot analyses indicated only one significant locus for regulation of translational initiation in ground squirrel liver: control of elf4E. We found seasonal variation in a potent regulator of elf4E activity, 4E-BP1. Summer squirrels lack 4E-BP1 and apparently control elf4E activity through direct phosphorylation. In winter, elf4E is regulated through binding with 4E-BP1. During the euthermic periods that separate bouts of torpor (interbout arousal), 4E-BP1 is hyperphosphorylated to promote initiation. However, during torpor, 4E-BP1 is hypophosphorylated and cap-dependent initiation of translation is restricted. The regulation of cap-dependent initiation of translation may allow for the differential expression of proteins directed toward enhancing survivorship. eukaryotic initiation factor 4E; 4E binding protein-1; protein synthesis

IN RESPONSE TO LIMITED FOOD availability and harsh environmental conditions, many mammals enter a state of depressed metabolism or torpor (see Refs. 2, 27 for review). Ground-dwelling sciurid rodents are the masters of this adaptive hypothermia because they can maintain body temperatures (Tb) below 0°C for up to 3 wk with metabolic rates as low as 1/100th of basal metabolic rate. These squirrels follow a strict circannual rhythm of reproduction, fattening, and hibernation. The winter hibernation season is comprised of a series of sequential bouts of torpor, wherein Tb approaches that of ambient temperature. These torpor bouts are interrupted by periodic rewarmings, or interbout arousals, to core temperatures near 37°C that usually last <24 h.

Concordant with limited energy availability and low Tb, hibernators depress protein synthesis during torpor but fully restore it during each interbout arousal (4, 8, 26). Translational initiation in the liver is acutely depressed during entrance into torpor at Tb ≤ 18°C, but the data are not fully explained by temperature effects alone and indicate that an active, rapidly reversible mechanism for inhibiting initiation is also required (26). The mechanisms that surround this depression and resumption of protein synthesis are incompletely understood. Translation initiation is mediated by the eukaryotic initiation factors (eIFs). These proteins are responsible for loading initiator methionyl-tRNA onto the translational start site of appropriate transcripts and for recruiting the ribosomal subunits for initiation of translation. An examination of the pathway for initiation of translation reveals two major loci for regulation: the functions of eIF2 and elf4E (reviewed in Ref. 15; Fig. 1). Several studies have implicated elf2f, elf4B, elf4E, and elf4FG in controlling translational initiation under a variety of conditions in nonhibernators (6, 11, 12, 15). Partial phosphorylation (from 2 to 13%) of elf2f has been implicated in the down-regulation of translational initiation in brain during torpor in ground squirrels (8), but this change may not be sufficient to fully explain the degree of translational depression (8) and may not occur in all tissues (16). elf2f is the only translation factor examined in hibernators to date. Therefore, to gain a broader perspective on translational control during hibernation, we surveyed the key eukaryotic initiation factors elf2f, -4B, -4E, -4GI, and -4GII, as well as the 4E binding proteins 4E-BP1, -2, and -3, for their availability and phosphorylation status in the liver across the hibernation cycle.

MATERIALS AND METHODS

Animals. Adult golden-mantled ground squirrels (Spermophilus lateralis) were captured at Sugarloaf, Boulder County, CO. Some squirrels were killed immediately for seasonal controls (summer active; SA; n = 3). Additional squirrels were implanted with temperature-sensitive radiotelemeters (Minimitter, Sun River, OR) that allowed for the precise determination of torpor state and then placed into an environmental chamber. Ambient temperature was maintained at ~5°C, and food was restricted to promote torpor. Torpid ground squirrels had Tb of approximately 5–7°C. Squirrels were killed after being torpid for 7 days or 80% of their torpor bout (late torpor, LT; n = 3) and when euthemic in between torpor bouts (interbout aroused, IBA; n = 3). Livers were removed, snap frozen in liquid N2, and stored at −80°C until use.

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Sample preparation and Western blot analyses. Livers were pulsed in liquid N$_2$ and homogenized in either sample buffer A or B. Sample buffer A consisted of 50 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM DTT, 1 mM EDTA, 50 mM glycerophosphate, 1 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate, 0.1 mM orthovanadate, and 50 mM okadaic acid. Samples were centrifuged for 10 min at 10,000 g, 4°C, to remove cellular debris. Sample buffer B consisted of 50 mM Tris-HCl, pH 8.3, 20% glycerol, 2% SDS, and 0.4 M β-mercaptoethanol. The homogenate was centrifuged at 10,000 g at 4°C for 30 min to remove cellular debris. Protein concentration of all samples was determined using a modified Lowry assay. Except as otherwise noted, the general protocol for Western blotting was as follows. Samples were electrophoresed by SDS-PAGE and transferred to an appropriate membrane. Nonspecific protein binding was blocked by incubation of the membrane in 3% milk in 10 mM Tris-HCl, pH 8, and 150 mM NaCl (Tris-buffered saline; TBS) with 0.5% Tween-20 (TTBS). Incubation conditions for use of the primary antibody were specific to the antibody as described later. Washing between incubation steps consisted of one 5-min wash in TBS, followed by two 5-min washes in TTBs, and a final 5-min wash in TBS. All visualization was performed using ECL (Amersham) with either film or a Bio-Rad Gel Doc imager.

eIF2α, eIF4B, and eIF4E blotting. Forty (eIF2α and eIF4B) or 80 μg (eIF4E) total protein samples made up in sample buffer A were electrophoresed on 15% (30:0.5 acrylamide:bisacrylamide) gels before transfer to polyvinylidene fluoride (PVDF) membrane. For primary antibody incubations the following conditions were used. A monoclonal antibody raised against eIF2α was diluted 1:5,000 in high-salt (500 mM NaCl) TTBS with 3% milk. A rabbit polyclonal antibody raised against eIF4B was diluted 1:1,000 in standard TTBS with 3% milk. A monoclonal antibody raised against rabbit eIF4E (Transduction Laboratories cat. no. E27620) was used at 1:1,000 in standard TTBS with 1% milk and 1% BSA.

Western blot analyses indicated only one significant locus for regulation of translational initiation in ground squirrel liver: control of eIF4E. eIF4E concentration did not change as a result of season or state (data not shown), but eIF4E activity was apparently regulated through reversible phosphorylation. Western blot analysis revealed three bands (Fig. 2). In winter animals (IBA and LT), the uppermost band (i.e., the hyperphosphorylated form of eIF4E) had ~40% greater intensity than the corresponding band for summer animals (P < 0.05; ANOVA). The middle band (less phosphorylated) was reduced reciprocally in winter compared with summer (P < 0.05; ANOVA), and no change in the hypophosphorylated lower band was detected.

Western blot analyses of the eIF4E binding protein 4E-BP1 demonstrated that 4E-BP1 is not detectable in the summer animals but is present in winter (Fig. 3A). During the interbout arousal, 4E-BP1 was hyperphosphorylated in contrast to torpor, where it was hypophosphorylated (Fig. 3B). No significant changes were observed for 4E-BP2 and no 4E-BP3 was detected (data not shown).

Phosphorylation of eIF2α was observed to accompany downregulation of the initiation of protein synthesis under a variety of conditions, including heat shock, viral infection, amino acid or glucose starvation, and Ca$_2^+$ mobilization (5). In ground squirrel livers, no significant difference in eIF2α phosphorylation or availability was detected (ANOVA; P > 0.05; data not shown).

Dephosphorylation of eIF4B is associated with the translational depression seen during heat shock, serum depletion, and...
mitosis, whereas phosphorylation of eIF4B is associated with the stimulatory effects of insulin on translation initiation (21). However, no changes in phosphorylation status were observed across the hibernation season for eIF4B (data not shown).

There are two forms of eIF4G in mammals, and both undergo partial cleavage by poliovirus to downregulate translation of cellular proteins during infection (12). A similar partial cleavage of eIF4G is observed after ischemia in rats (6). Neither eIF4G I nor II showed any evidence for cleavage or other alterations by Western blot across the hibernation season in ground squirrel liver (data not shown).

**DISCUSSION**

This screen of translation factors for differences of expression or modification in the liver as a function of hibernation revealed only two proteins, eIF4E and 4E-BP1, with significant changes. eIF4 activity mediates recruitment of mRNA to ribosomes and is usually the rate-limiting step for initiation of translation (11). The specific role of eIF4E in this complex is to recognize the 5′-cap of the mRNA. Once eIF4E is bound to the capped mRNA, it can be recruited by eIF4G to the preinitiation complex, thereby forming the initiation complex. Regulation of eIF4E, and hence initiation of translation on capped mRNAs, is known to occur by two mechanisms: 1) direct phosphorylation; and 2) interaction with binding proteins, 4E-BPs. Hyperphosphorylation of eIF4E increases its affinity for the m′GTP cap structure of the mRNA approximately three- to fourfold relative to hypophosphorylated forms, enhancing translational initiation (23). Interaction of eIF4E with a binding protein, 4E-BP, competes with eIF4E’s binding of eIF4G, thereby preventing formation of the initiation complex. The affinity of 4E-BP for eIF4E is also known to be modulated by phosphorylation; when 4E-BP1 is hypophosphorylated, it is able to efficiently bind eIF4E to restrict cap-dependent initiation. In contrast, 4E-BP1 that is hyperphosphorylated on up to six known phosphorylation sites results in decreased binding of eIF4E and increased translation rates (10).

In ground squirrels, we have found that eIF4E is in a hyperphosphorylated and presumably more constitutively active form during winter than in summer (Fig. 2). This result initially appears counterintuitive: all studies to date indicate a severe depression of protein synthesis during hibernation, down to 0.13 to 0.5% of active rates (13, 28). Furthermore, because protein synthesis accounts for approximately 12–25% of a typical cellular energy budget (24), increased protein synthesis is at odds with a strategy of energy conservation like hibernation. It is worth noting that, although it is typical that enhanced phosphorylation of eIF4E increases translation, there are examples of stress responses that increase phosphorylation of eIF4E without concomitant increases in translation. For those situations, it has been proposed that negative effects occur on other components of the translational apparatus, such that eIF4E phosphorylation is a compensatory mechanism induced by stress as an attempt to stimulate translation (11).

In the case of hibernation, the rapid cycles between torpor and euthermia may benefit by a generally enhanced activity of eIF4E (hyperphosphorylated) that can be controlled by another component. Intriguingly, 4E-BP1 is not detected in squirrels sampled during the summer but is readily detected during winter (Fig. 3A). The winter steady-state levels of 4E-BP1 are altered by phosphorylation, with the hyperphosphorylated form with reduced affinity for eIF4E predominating during each interbout arousal and the hypophosphorylated forms with higher affinity for eIF4E predominating during torpor (Fig. 3B). The patterns of expression and phosphorylation of eIF4E and its binding protein 4E-BP1 suggest a novel model for regulation of translational activity across the circannual cycle of a hibernating mammal (Fig. 4). Because the steady-state level of the constitutively active, hyperphosphorylated form of
elf4E is lower in summer than in winter (Fig. 2), it appears that summer squirrels control cap-dependent initiation via direct phosphorylation and dephosphorylation of elf4E in the absence of 4E-BP1. In contrast, winter squirrels generally elevate the activity of elf4E and hence cap-dependent translation by hyperphosphorylation but then control it by binding and release of 4E-BP1. During torpor, when translational initiation is depressed, hypophosphorylated 4E-BP1 sequesters elf4E to restrict cap-dependent initiation. During interbout arousal, 4E-BP1 is hyperphosphorylated, releasing it from the binding site on elf4E that interacts with elf4G. Under these conditions, the hyperactivated elf4E can function maximally to recruit the preinitiation complex via binding of elf4G, thereby promoting cap-dependent initiation of translation. Hyperactivation of translation during interbout arousal has also been suggested based on the results of in vivo metabolic labeling experiments that used radioactive amino acid precursors to measure protein synthesis (28).

elf4E regulation specifically affects cap-dependent initiation of translation. In recent years, a cap-independent mechanism for initiation of protein synthesis has also been described; internal ribosome entry sites (IRES) allow bypass of the requirement for elf4E recognition of the cap structure (for review, see Ref. 14). While the exact in vivo function and extent of IRES-dependent initiation remain to be elucidated, recent work suggests IRES function may be particularly important in times of physiological stress, including mild hypothermia and oxygen stress (3, 7, 17, 18, 20). Thus the utilization of IRES-mediated translation at a time where the elf4E system is inactive may promote differential gene expression geared toward enhancing survivorship of torpor. Our laboratories are currently investigating the role of IRES-mediated initiation during torpor.

The lack of 4E-BP1 during summer could have broader implications for hibernation physiology than simply its probable role in translational control during the torpor-arousal cycles of hibernation; seasonal variation in 4E-BP1 expression may also contribute to the annual cycle of weight gain and fat deposition in ground squirrels. In preparation for hibernation, adult golden-mantled ground squirrels increase body fat threefold (19). The development of large depots of brown adipose tissue (BAT) is particularly critical for the successful employment of hibernation because this is the primary site of non-shivering thermogenesis during arousal from torpor (22). The absence of 4E-BP1 during summer may contribute to the accumulation of BAT per se. White adipose tissue (WAT) of knock-out mice for 4E-BP1 (Elf4ebp1/−/−) contains numerous multilocular adipocytes, which are normally associated with BAT (25). Indeed, expression of the mRNA for uncoupling protein 1 (UCP1) increases sixfold in the absence of 4E-BP1. UCP1, which is usually expressed in BAT, is responsible for thermogenesis via the uncoupling of oxidative phosphorylation from ATP synthesis (1). Thus it appears that 4E-BP1 may be involved in the emergence of BAT from WAT. Interestingly, knockout animals also experience 15% higher metabolic rates than controls and thus 4E-BP1 may play a role in overall energy expenditure (25).

We found no evidence of increased phosphorylation of elf2α in the liver during hibernation, in contrast to the results of an earlier study in brain where a sixfold increase in elf2α phosphorylation, from 2 to 13% of the total elf2α present in the extract, was observed during torpor (8). The earlier study also reported reduced translational capacity of brain (based on incorporation of a radiolabeled amino acid) in a single animal as it entered torpor, at temperatures above the extremely cold Tb of torpor. It was concluded that an active inhibition of initiation, likely the observed phosphorylation of elf2α, was responsible for the downregulation of protein synthesis in the torpor phase of hibernation (8). However, in light of the results of this study and other more recent data, this conclusion warrants further discussion. We subsequently showed that translational initiation in liver is depressed at Tb ≤ 18°C as squirrels enter torpor. Furthermore, initiation and elongation are fully coupled again only at Tb ≥ 18°C as the animals rewarmed during arousal (26). The lone entrance animal used by Frerichs et al. (8) had a core Tb of 19°C at the onset of the experiment, and then its Tb dropped to 7.5°C by the conclusion of the labeling period. Because the majority of the labeling period for their entrance animal was spent with a Tb below 18°C, where initiation slows dramatically if not ceases altogether, the protocol utilized by Frerichs et al. (8) would not distinguish passive from active mechanisms. Other investigators have also found variability in the degree of elf2α phosphorylation during torpor. In the kidney, elf2α becomes phosphorylated while no such changes were found in BAT (16). Such variability between tissue types and the limited degree of phosphorylation (87% of elf2α remained in the active form during torpor in the original study) may indicate that mechanisms in addition to elf2α phosphorylation are required to achieve the degree of suppression of protein synthesis that has been observed in torpid hibernators (8, 13, 16, 26). The control of elf4E activity during hibernation via seasonal expression of 4EB1, whose activity is in turn controlled by reversible phosphorylation in a manner consistent with suppressing translation during torpor but permitting it during interbout arousal, could complement or supplant the control of elf2α depending on the tissue type.

The tremendous energetic outlay required for protein synthesis conflicts with the energy-sparing strategy of hibernation. Yet, proteins are essential to maintain cellular integrity and function. It is noteworthy that eliminating the activity of elf4E would have an effect in addition to a general reduction in protein synthesis; blocking its activity could also be used to alter the population of mRNAs that are translated because capped, but not IRES-containing, mRNAs cease translation when elf4E is inactivated (11, 14). Although further investigation is required, the regulation of cap-dependent initiation of translation may facilitate differential gene expression by utilizing IRES-mediated translation on a global scale to produce proteins critical to the implementation and/or survival of torpor. The identification of such a strategy that conserves energy by directing limited biochemical efforts toward promoting survivorship would have significant impact on our understanding of hibernation.

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