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Signaling Pathways for Translation

Insulin and Nutrients

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Insulin Signaling and the Control of PHAS-I Phosphorylation

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1 Introduction

The rate of mRNA translation is determined by many factors including a family of proteins that control the availability of the mRNA cap-binding protein, eIF4E. The prototypic member of this family was originally described as a protein that became phosphorylated when rat adipocytes were exposed to insulin (Belsham and Denton 1980), a finding that presaged the important role of phosphorylation in controlling the protein's function. The phosphorylated protein had the unusual properties of remaining in solution when boiled or exposed to acid (Belsham and Denton 1980), and it was named PHAS-I (phosphorylated heat- and acid-stable) after its cDNA was found to lack homology with any others encoding known proteins (Hu et al. 1994). Subsequently, PHAS-I was found to be an eIF4E-binding protein, and named 4E-BP1 (Pause et al. 1994). We have retained the earlier nomenclature and will refer to the family as PHAS proteins. Mammals and many lower organisms including slime mold, insects, fish, and birds express one or more members of this family (Fig. 1A). The proteins have not been found in plants or yeast, although the latter contain an eIF4E-binding protein that possibly serves a homologous function but which has no amino acid sequence identity with the PHAS proteins, except in the eIF4E binding domain (Zanchin and McCarthy 1995). Thus, the distribution of PHAS proteins in nature appears less widespread than that of eIF4E, which is probably expressed in all eukaryotic organisms. Nevertheless, in mammals and many other species, PHAS proteins are important mediators of the actions of insulin, growth factors and nutrients on protein synthesis.

2 Mechanism of Translational Repression

PHAS proteins decrease the amount of eIF4E available for translation. When overexpressed in cells the proteins inhibit cap-dependent mRNA translation, but do not inhibit cap-independent translation driven by viral internal

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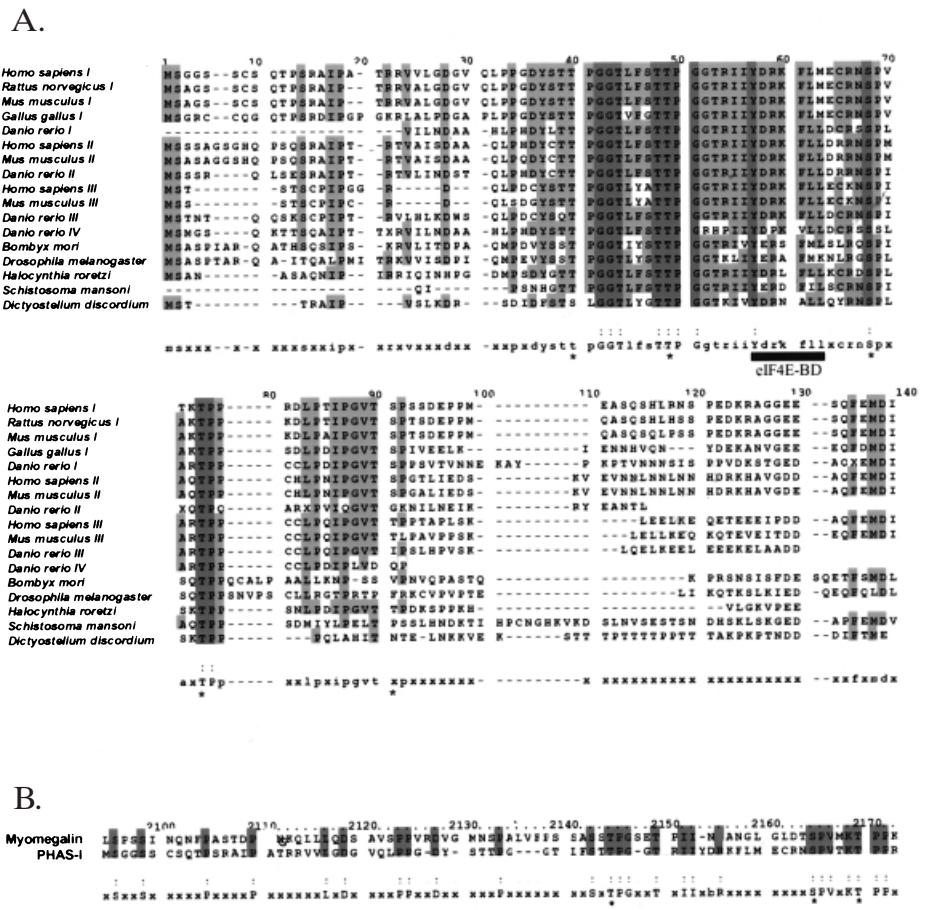

Fig. 1A, B. PHAS proteins in different organisms. **A** The protein sequences deduced from the full-length cDNAs encoding the three mammalian PHAS proteins that have been cloned are presented. The proteins from the other species are based on ESTs detected in BLAST searches of GenBank performed in December 1999. Except for the mammalian proteins, the sequences have not been verified. The numbering (*I–IV*) of the predicted proteins in *Danio rerio* is arbitrary. The *asterisks* denote the positions of the five sites of phosphorylation that have been identified. The eIF4E-binding motif (eIF4E-BD) is *underlined*. **B** The deduced amino acids sequences of myomegalin (AF139185) and PHAS-I

ribosomal entry sites (Pause et al. 1994). Nonphosphorylated PHAS-I binds tightly to eIF4E, forming a complex that can be isolated from cell extracts by immunopurification with antibodies to either PHAS-I or eIF4E (Lin et al. 1994; Pause et al. 1994). Complexes containing PHAS-I and eIF4E may also be purified by using m^7GTP-Sepharose (Lin et al. 1994; Pause et al. 1994), indicating that binding of PHAS-I does not inhibit the association of eIF4E with the cap. Indeed, recent studies have demonstrated that PHAS-I actually increases the

affinity of eIF4E for the cap (Ptushkina et al. 1999). PHAS-I represses translation by binding to the dorsal surface of eIF4E and competitively inhibiting binding of eIF4G to eIF4E (Ptushkina et al. 1999). The recruitment of eIF4G, and its associated initiation factors, to the cap site is essential for the efficient scanning and/or binding of the 40 S ribosomal subunit (Gingras et al. 1999b; Rhoads 1999). eIF4G is a scaffold that organizes eIF4E, eIF4A, eIF3, and Mnk1. eIF4A is an mRNA helicase that facilitates translation of messages with structured 5′ UTRs, eIF3 provides a link to the 40 S ribosomal subunit, and Mnk1 is a protein kinase that phosphorylates eIF4E. The roles of the proteins that bind to eIF4G are described in more detail elsewhere in this volume.

The critical residues in PHAS-I that are required for binding to eIF4E have been mapped to the central region of the protein (Haghighat et al. 1995; Mader et al. 1995). The motif, YXXXXLΦ (where X is any amino acid and Φ is a hydrophobic amino acid residue), is found in PHAS proteins in all species (Fig. 1A) and it is also found in eIF4G, consistent with the competition between PHAS-I and eIF4G for eIF4E. Phosphorylation of PHAS-I, as occurs in response to insulin and certain growth factors, promotes dissociation of the PHAS-I/eIF4E complex (Lin et al. 1994; Pause et al. 1994). However, phosphorylation does not function as a simple on–off switch, and the mechanisms involved in the control of eIF4E binding are complicated. Nevertheless, dissociation of the PHAS-I/eIF4E complex allows eIF4E to bind to eIF4G, setting into motion the chain of events leading to increased protein synthesis. As would be expected, inverse relationships between the fraction of eIF4E bound to PHAS-I and the rate of mRNA translation (Mothe-Satney et al. 2000a), and between the amounts of PHAS-I and eIF4G bound to eIF4E (Kimball et al. 1997), have been observed. However, increased binding of PHAS-I to eIF4E is not always associated with a decrease in the amount of eIF4E bound to eIF4G (Marx and Marks 1999). The explanation for the apparent discrepancy is not clear, but might relate to the presence of other PHAS isoforms in the cell types investigated.

3 PHAS Isoforms

Three PHAS isoforms have been identified in mammalian cells. cDNA encoding 4E-BP2, which we will refer to as PHAS-II, was cloned by screening an expression library with a ^{32}P-labeled eIF4E probe (Pause et al. 1994). PHAS-III (4E-BP3) was initially detected as an expressed sequence tag (EST) by searching GenBank for species that were homologous to PHAS-I (Lawrence et al. 1995). The full-length PHAS-III cDNA was recently isolated and the recombinant protein has been expressed and shown to be a bona fide eIF4E-binding protein (Poulin et al. 1998). The general physical properties of the three mammalian PHAS isoforms are similar. All are heat- and acid-stable, and contain 100–118 amino acids with an overall sequence identity of approximately 60%. In solution, PHAS-I is found predominantly in a random coil conformation (Fletcher and Wagner 1998). The lack of ordered structure helps to explain the

stability of the protein at elevated temperatures or under acidic conditions. All isoforms exhibit anomalous mobility when subjected to SDS-PAGE, but the largest discrepancy between actual M_r and electrophoretic mobility occurs with PHAS-I, which has an M_r equal to 12,400 but which exhibits a mobility expected of a protein of M_r equal to 22,000. This behavior may be due to the relatively high proline content of the protein.

The PHAS isoforms are widely distributed among different mammalian tissues, and more than one isoform may be found in the same cell type. However, the expression patterns differ significantly. At the mRNA level, PHAS-I expression is highest in adipose tissue, skeletal muscle, and pancreas (Hu et al. 1994; Tsukiyama-Kohara et al. 1996). PHAS-II mRNA is more uniformly expressed among tissues (Tsukiyama-Kohara et al. 1996). PHAS-III mRNA expression is highest in skeletal muscle, heart, kidney, and pancreas, and it is relatively low in brain and thymus (Poulin et al. 1998). Relative amounts of protein in different tissues estimated by immunoblotting do not always agree with the levels of mRNA, suggesting that expression of PHAS proteins themselves may be subject to control at the level of translation. For instance, PHAS-II expression is relatively high in liver and kidney, which express relatively low levels of PHAS-I (Lin and Lawrence 1996). The expression pattern of the PHAS-III protein in tissues has not been reported.

Most of the sequence divergence among the isoforms is in the regions preceding the highly conserved COOH termini (Fig. 1A). One possibility is that sequence differences allow targeting of the isoforms to different intracellular locations. Lack of information on the subcellular distribution represents a significant gap in our knowledge of these proteins, and the regulatory advantage achieved by expressing multiple isoforms of PHAS is not clear. Perhaps having redundant control of eIF4E availability could provide a selective advantage to multicellular organisms, as increasing eIF4E causes aberrant cell growth and may even promote malignant transformation (De Benedetti and Rhoads 1990; Lazaris-Karatzas et al. 1990). Functional overlap among PHAS isoforms may explain why knocking out PHAS-I in mice had little effect on the phenotype of the animals (Blackshear et al. 1997). Male PHAS-I knock-out mice weigh approximately 10% less than their wild-type littermates (Blackshear et al. 1997).

Have all the PHAS isoforms been identified? There are ESTs in the data bases that differ in sequence from those encoding the known PHAS proteins. However, these differences might reflect sequencing errors or cloning artifacts, and the sequences will need to be confirmed in independent clones to determine whether the species represent new gene products. Interestingly, in searching GenBank with TFASTA, we found a relatively large protein, myomegalin, that contains a region homologous to PHAS-I (Fig. 1B). Sequence identity between the two proteins in this region is 25% and the homology is 44%. Although myomegalin lacks the consensus eIF4E-binding motif, it does contain the equivalents of Thr45, Ser64, and Thr69, three of the Ser/Thr Pro (S/TP) phosphorylation sites that are conserved in all members of the PHAS

family (Fig. 1A, the middle three asterisks). Neither the significance of these sites in myomegalin nor the function of myomegalin is known.

4 Phosphorylation Sites in PHAS-I

Most of what is known regarding the phosphorylation of PHAS proteins is based on findings obtained with PHAS-I. PHAS-II appears to be regulated in the same manner as PHAS-I in 3T3-L1 adipocytes (Lin and Lawrence 1996), where both proteins are expressed, although there have been relatively few studies that have directly assessed the phosphorylation of PHAS-II or -III. Initially, the phosphorylation of PHAS-I was investigated by incubating cells in medium containing $^{32}P_i$. With the development of specific antibodies that allowed detection of the protein by immunoblotting (Hu et al. 1994; Lin et al. 1994), it became apparent that PHAS-I underwent a pronounced decrease in electrophoretic mobility when phosphorylated in the appropriate sites in vitro or when cells were incubated with insulin or certain growth factors. Three major electrophoretic forms of the protein can generally be detected after subjecting cell extracts to SDS-PAGE. For descriptive purposes these forms have been designated α, β, and γ, in order of decreasing electrophoretic mobility (Fig. 2). Agents that promote phosphorylation of PHAS-I in cells generally decrease α and increase γ, and this gel-shift forms the basis of a widely used method for detecting changes in the phosphorylation of PHAS-I. However, as will be discussed below, the phosphorylation of PHAS-I is much more complicated than suggested by the number of electrophoretic forms detected after SDS-PAGE, and the gel shift assay is not a reliable method for assessing changes in the phosphorylation of certain sites in the protein.

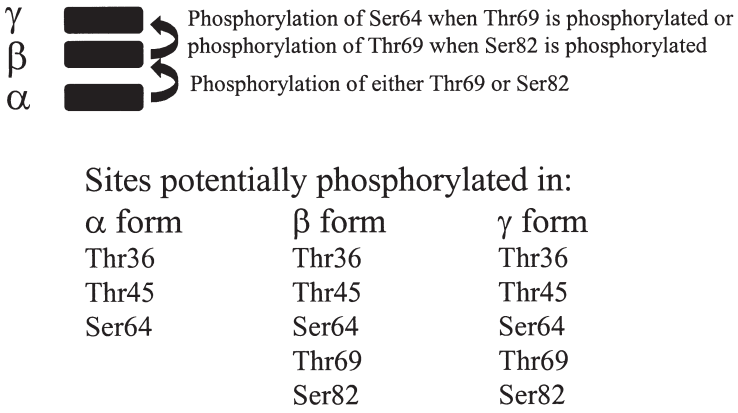

Fig. 2. The influence of phosphorylating different sites on the mobility of PHAS-I subjected to polyacrylamide gel electrophoresis in the presence of SDS