PHOSPHORYLATION OF 4E-BP1 IS AN ORDERED, HIERARCHICAL PROCESS

Anne-Claude Gingras[1], Brian Raught[1], Steven P. Gygi[2], Wojciech Augustyniak[3], Joseph Marcotrigiano[4], Stephen K. Burley[4], Aleksandra Wyslouch-Cieszynska[3], Roberto D. Polakiewicz[5], Ruedi Aebersold[2], Nahum Sonenberg[1].
[1]Dept. of Biochemistry & McGill Cancer Centre, McGill U., Montréal, CANADA; [2]Dept. of Molecular Biotechnology, U. Washington, Seattle, WA; [3]Dept. of Biophysics, Institute of Biochemistry and Biophysics, Warszawa, POLAND; [4]Molecular Biophysics, The Rockefeller U., New York, NY; [5]Cell Signaling Technology, Beverly, MA.

The 4E-BPs are a family of translational inhibitory proteins which bind to the mRNA 5' cap-binding protein, eIF4E, and prevent its incorporation into an active initiation factor complex. The 4E-BP1/eIF4E interaction is regulated via phosphorylation: while hypophosphorylated 4E-BP1 interacts strongly with eIF4E, hyperphosphorylation of 4E-BP1 following treatment of cells with hormones, mitogens or growth factors induces dissociation from eIF4E. several sites are phosphorylated on 4E-BP1, via a PI3 kinase – Akt – mTOR signaling pathway. Using phosphopeptide mapping coupled to mass spectrometry, we have characterized the regulation of individual 4E-BP1 phosphorylation sites *in vivo*. Two threonine residues, Thr37 and Thr46, are phosphorylated *in vitro* by a FRAP/mTOR immunoprecipitate, but 4E-BP1 phosphorylated at these sites retains the ability to bind to eIF4E. recently, we also reported that phosphorylation of these sites is relatively insensitive to serum stimulation; however, phosphorylation of these residues is required for the subsequent phosphorylation of a group of serum-sensitive sites. Here, we identify Ser65 and Thr70 as the principal serum responsive 4E-BP1 phosphorylation sites. We have evaluated the impact of a variety of conditions (growth factor stimulation, amino acid deprivation, kinase inhibitor treatments, etc.) and observed significant differences in the regulation of the phosphorylation/dephosphorylation of Thr37 and Thr46, as compared to that of ser65 and Thr70. Furthermore, we have established, using two-dimensional IEF/SDS-PAGE and phosphospecific antibodies, the order of phosphate addition onto 4E-BP1: Phosphorylation first occurs simultaneously on Thr37 and Thr46. This event is followed by phosphorylation of Thr70. Finally, phosphorylation of 4E-BP1 culminates on Ser65, a site located near the region of 4E-BP1 responsible for interaction with eIF4E. The consequences of each of these phosphorylation events on 4E-BP1 binding to eIF4E are discussed.