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Localisation and regulation of the eIF4E-binding protein 4E-BP3

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Abstract The cap-binding protein eIF4E-binding protein 3 (4E-BP3) was identified some years ago, but its properties have not been investigated in detail. In this report, we investigated the regulation and localisation of 4E-BP3. We show that 4E-BP3 is present in the nucleus as well as in the cytoplasm in primary T cells, HEK293 cells and HeLa cells. 4E-BP3 was associated with eIF4E in both cell compartments. Furthermore, 4E-BP3/eIF4E association in the cytoplasm was regulated by serum or interleukin-2 starvation in the different cell types. Rapamycin did not affect the association of eIF4E with 4E-BP3 in the cytoplasm or in the nucleus.
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Key words: eIF4E-binding protein 3; eIF4E-binding protein 1; Primary T cell; Regulation; Nucleus

1. Introduction

Translation initiation is a regulatory stage in the modulation of gene expression. One important regulatory step in translation initiation is the availability of the cap-binding protein, eukaryotic initiation factor (eIF) 4E, to bind to the cap structure (m^7GpppN, where N is any nucleotide) of the mRNA. Binding of eIF4E to the cap structure recruits the eIF4F complex to the mRNA facilitating translation initiation [1].

Binding of eIF4E to the cap structure can be regulated in at least two ways, via phosphorylation of eIF4E [2] and via its availability through association with eIF4E-binding proteins (4E-BPs). Binding of 4E-BPs to eIF4E prevents formation of eIF4F complexes and therefore prevents translation initiation.

Three related 4E-BPs have been reported so far, of which 4E-BP1 is the best studied. Phosphorylation of 4E-BP1 leads to its dissociation from eIF4E, leaving eIF4E free to bind eIF4G and form eIF4F complexes [3,4]. Phosphorylation of 4E-BP1 occurs after numerous growth-stimulating treatments of cells and is regulated via the PI 3-K [5,6], mTOR and ERK signalling pathways [1,7].

4E-BP2 is also a phospho-protein, but is phosphorylated on fewer residues than 4E-BP1. Phosphorylation of 4E-BP2 is sensitive to LY294002 and rapamycin treatment [8]. Regulation of 4E-BP2/eIF4E association has not been described. However, it has been shown that levels of 4E-BP2 protein are upregulated during granulocyte differentiation [9].

4E-BP3 was the last of the eIF4E-binding proteins to be identified [10]. Like the other 4E-BPs, 4E-BP3 is able to associate with eIF4E and inhibits eIF4E-dependent translation. It has been reported that 4E-BP3 is a phospho-protein [10]. However, no data are available about its regulation.

In this report, we investigated the localisation and regulation of 4E-BP3 in different cell types.

2. Materials and methods

2.1. Primary T cell isolation and cell treatment

Buffy coats used for the isolation of T cells were prepared from freshly drawn blood from healthy donors and were obtained from the Scottish National Blood Transfusion Service (Edinburgh, UK). Mononuclear leukocytes were isolated as described [11]. T cells were suspended in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), 1 mM glutamine, 3 U/ml interleukin-2 (IL-2, Sigma), 1 μg/ml phytohaemagglutinin (PHA, Sigma), 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulphate and 0.25 μg/ml amphotericin B. The cells were kept in 75 cm^2 tissue culture flasks at a density of 4×10^6 cells/ml at 37°C and 5% CO_2. All tissue culture reagents were obtained from Gibco BRL unless stated otherwise. The cells were grown for 7 days before the experiments were performed.

2.2. Cell culture

Human embryonic kidney 293 (HEK293) and HeLa cells were grown on plates in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% foetal bovine serum (Gibco BRL).

2.3. Transfection and plasmids

HEK293 cells were transiently transfected with a his/myc-tagged 4E-BP3 construct [10] (kindly provided by F. Poulin and N. Sonenberg, Montreal, QC, Canada) by calcium phosphate precipitation of the DNA in HEPES-buffered saline [12]. Cells were treated as described in the figure legends and harvested in a buffer containing 20 mM HEPES–KOH pH 7.5, 50 mM β-glycerophosphate, 0.2 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM dithiothreitol, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml antipain, and 1 μg/ml pepstatin.

2.4. 7-Methylguanosine triphosphate (m^7GTP) Sepharose pull down, gel electrophoresis and Western blotting

The cells were treated as indicated in the figure legends, and then

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Abbreviations: eIF, eukaryotic initiation factor; FCS, foetal calf serum; m^7GTP, 7-methylguanosine triphosphate; 4E-BP, eIF4E-binding protein; HEK293 cells, human embryonic kidney 293 cells; IL-2, interleukin-2; LMB, leptomycin B; Mnk, mitogen-activated protein kinase-interacting kinase; PHA, phytohaemagglutinin

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harvested in EZ lysis buffer (Sigma) to obtain cytoplasmic and nuclear fractions. Laemmli sample buffer was added to part of each fraction and the extracts were analysed by SDS–PAGE and Western blotting. The rest of the sample (approximately 2 mg of extract) was used for isolation of eIF4E on m^7GTP Sepharose 4B (Amersham-Biosciences) (15 μl of slurry diluted with 15 μl of Sepharose CL-4B). Laemmli sample buffer was added to the beads and the samples were heated at 95°C for 10 min. The samples were run on a 17.5% SDS–polyacrylamide gel, transferred to Immobilon-P (Millipore), and detected by Western analysis. 4E-BP3 was detected with a polyclonal antibody raised in rabbit against the peptide PPTAPLSKLEELKC, eIF4E was detected with a monoclonal antibody raised against the whole protein, and eIF4GI with a polyclonal antibody raised in sheep against the peptide CKKEAVGDLLDAFKEAN. 4E-BP1 (SC-6025), lamin B (SC-6216), and α-tubulin (SC-8035) antibodies were obtained from Santa Cruz, myc antibody (9E10) from Sigma, and phospho-4E-BP1 T36/45 from Cell Signaling. The mitogen-activated protein kinase-interacting kinase 1 (Mnk1) antibody was raised in sheep and kindly provided by the Division of Signal Transduction Therapy (University of Dundee, Dundee, UK).

3. Results

3.1. Characterisation of the 4E-BP3 antibody

The 4E-BP3 antibody was raised in rabbit against a peptide (PPTAPLSKLEELKC) and subsequently purified by affinity chromatography. The antibody was tested on different sources of 4E-BP3 in the absence and presence of the BP3 peptide (Fig. 1). Firstly, the 4E-BP3 antibody recognised his/myc4E-BP3 expressed in HEK293 cells (Fig. 1A). Secondly, a clear signal was obtained after m^7GTP Sepharose chromatography. 4E-BP3 associated with eIF4E was detected in three different cell types (Fig. 1B). Lastly, the antibody was also able to detect 4E-BP3 in total cell extracts (Fig. 1C) as shown for primary T cells and HEK293 cells. In all three cases 4E-BP3 was detected as a clear band with the expected molecular weight (12 kDa), and in every case the signal was competed away in the presence of the peptide.

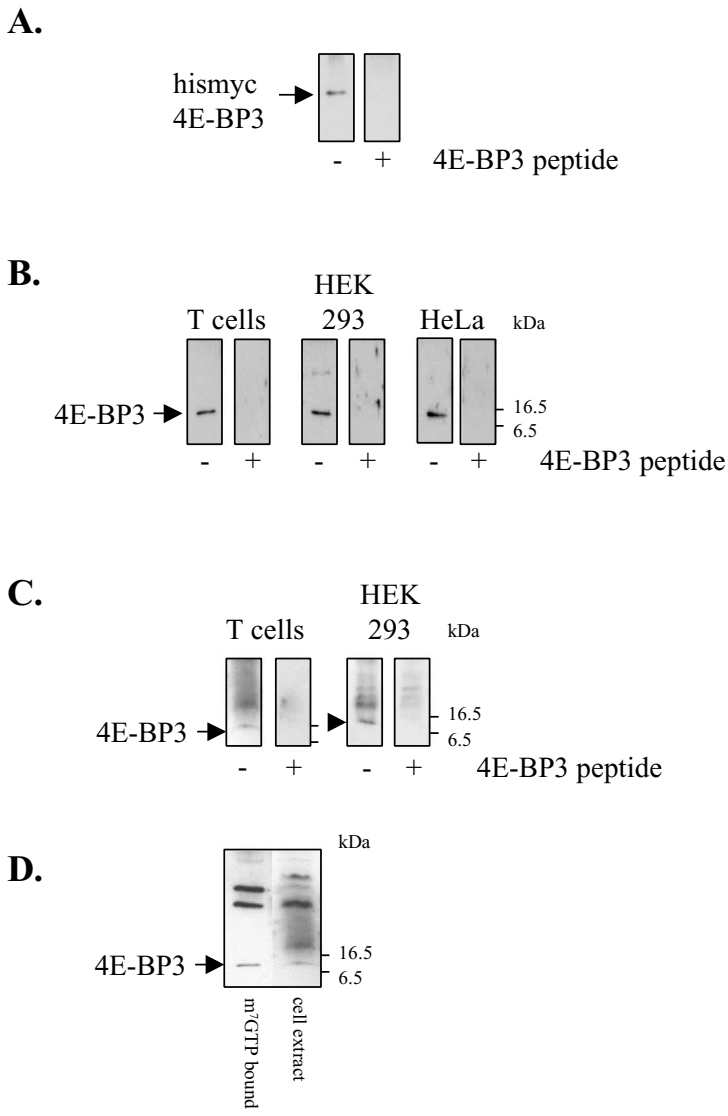

Fig. 1. Characterisation of the 4E-BP3 antibody. Different samples were analysed by SDS–PAGE and Western blotting to test the 4E-BP3 antibody. The samples were run in duplicate and Western blotting was performed using the 4E-BP3 antibody in the absence or presence of the 4E-BP3 peptide used to make the antibody. A: HEK293 cells transfected with a vector encoding his- and myc-tagged 4E-BP3. B: m^7GTP Sepharose pull downs from the indicated cell types. C: Whole cell lysates of the indicated cell types. D: An m^7GTP Sepharose pull down and a cell extract from T cells were aligned to confirm the position of 4E-BP3 in cell extracts. New England Biolabs prestained markers were run to assess the molecular weight. The antibody test was performed in duplicate.

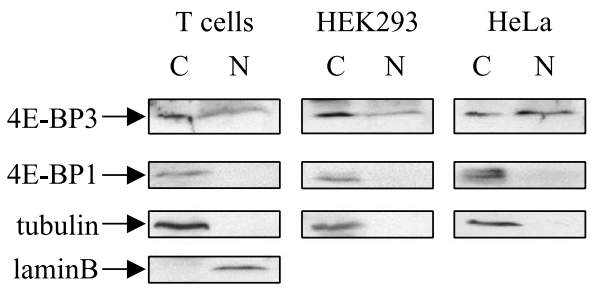

Fig. 2. 4E-BP3 is found in cytoplasmic and nuclear compartments. Cytoplasmic and nuclear fractions of primary T, HEK293 and HeLa cells were analysed by SDS–PAGE and Western blotting. 4E-BP3, 4E-BP1, tubulin (as a cytoplasmic marker) and lamin B (as a nuclear marker) were detected with the appropriate antibodies. Similar results were obtained in three experiments.

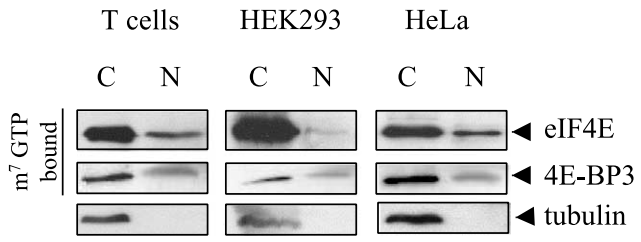

Fig. 3. 4E-BP3 is associated with eIF4E in the nucleus. eIF4E was purified from cytoplasmic and nuclear fractions of primary T (50×10^6 cells), HEK293 (10×10^6 cells) and HeLa cells (10×10^6 cells) using m^7 GTP Sepharose and was analysed by SDS–PAGE and Western blotting. 4E-BP3, eIF4E, and tubulin were detected with the appropriate antibodies. Tubulin was detected in total extracts. Similar results were obtained in three experiments.

However, various non-specific cross-reacting bands were evident in total cell extracts, and we aligned an m^7GTP Sepharose pull down and a cell extract from T cells and showed that 4E-BP3 in the cell extract ran at the same position and molecular weight as 4E-BP3 in a m^7GTP Sepharose pull down.

3.2. 4E-BP3 is present in the nucleus and the cytoplasm

Next, we examined the subcellular localisation of 4E-BP3 (Fig. 2). Interestingly, 4E-BP3 was detected both in the nucleus and the cytoplasm of primary T cells, HEK293 and HeLa cells. This differs from the localisation of 4E-BP1, the best-studied eIF4E-binding protein. 4E-BP1 was only present in the cytoplasm, as described previously in HEK293 cells, human malignant cell lines, human fibroblasts, and murine myoblasts [13]. Western blotting for tubulin indicated that there was no cytoplasmic contamination of the nuclear fraction.

3.3. 4E-BP3 is associated with eIF4E in the nucleus and the cytoplasm

It has been reported that 4E-BP3 associates with eIF4E in total cell lysates [10], as described for 4E-BP1 and 4E-BP2. Therefore, the association of 4E-BP3 with nuclear or cytoplasmic eIF4E was investigated (Fig. 3). 4E-BP3 was detected in a m^7GTP Sepharose pull down from both cytoplasmic and nuclear fractions of primary T cells, HEK293 cells and HeLa cells. Although hardly any eIF4E was detectable in the nucleus of HEK293 cells, a 4E-BP3 signal was still detected in the m^7GTP Sepharose pull down. This is likely due to a difference in sensitivity of the eIF4E and 4E-BP3 antibodies. The absence of tubulin in the nuclear fraction again showed that the nuclear fraction was not contaminated with cytoplasmic proteins.

3.4. Localisation of 4E-BP3

The effects of various stimuli on the localisation of 4E-BP3 was examined in several cell types. Primary T cells were treated with TPA and ionomycin or starved for FCS or IL-2. HEK293 cells and HeLa cells were serum-starved or treated with rapamycin (data not shown). However, we were unable to detect any significant change in the localisation of 4E-BP3 under any of these conditions.

One mechanism of shuttling proteins from the nucleus to the cytoplasm is via a CRM-1-mediated pathway, and this mechanism can be blocked using the inhibitor leptomycin B (LMB) [14] (Fig. 4). Primary T cells were incubated with leptomycin B for 16 h and the localisation of 4E-BP3 was analysed. However, the localisation of 4E-BP3 did not change upon LMB treatment. As a positive control for LMB treatment, we showed accumulation of the endogenous eIF4E kinase Mnk1 in the nuclear fraction, which is in agreement with a previous report that nuclear export of Mnk1 is sensitive to LMB [15]. This indicated that LMB treatment was sufficient to block CRM-1-mediated export in primary T cells. Apparently, 4E-BP3 is not transported out of the nucleus via a CRM-1-dependent pathway.

3.5. Regulation of the association of 4E-BP3 with eIF4E

The effects of different treatments on the association of eIF4E with 4E-BP3 and 4E-BP1 were examined in primary T cells, HEK293 and HeLa cells.

Primary T cells were starved either for FCS or for IL-2 and PHA. Starvation of primary T cells for FCS did not affect the association of eIF4E with 4E-BP3 in the cytoplasm. However, starvation for IL-2 and PHA resulted in a 90% decrease in the amount of 4E-BP3 bound to eIF4E in the cytoplasm (Fig. 5A). The association of eIF4E with 4E-BP1 was not affected by starvation.

Rapamycin or TPA/ionomycin treatment of T cells did not affect the eIF4E/4E-BP3 association in the cytoplasmic fractions (Fig. 5B). Rapamycin slightly increased the binding of 4E-BP1 to eIF4E, but TPA/ionomycin treatment did not affect this. The results obtained were not caused by changes in levels of eIF4E or 4E-BP3 (Fig. 5A,B).

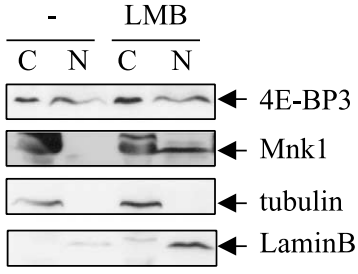

Fig. 4. Nuclear export of 4E-BP3 is not mediated by CRM-1. Primary T cells (50×10^6 cells) were treated with LMB (2 nM) for 16 h. Cytoplasmic and nuclear extracts were then prepared and analysed by SDS–PAGE and Western blotting. 4E-BP3, Mnk1, tubulin and lamin B were detected with the appropriate antibodies, as indicated. Similar results were obtained in four experiments.

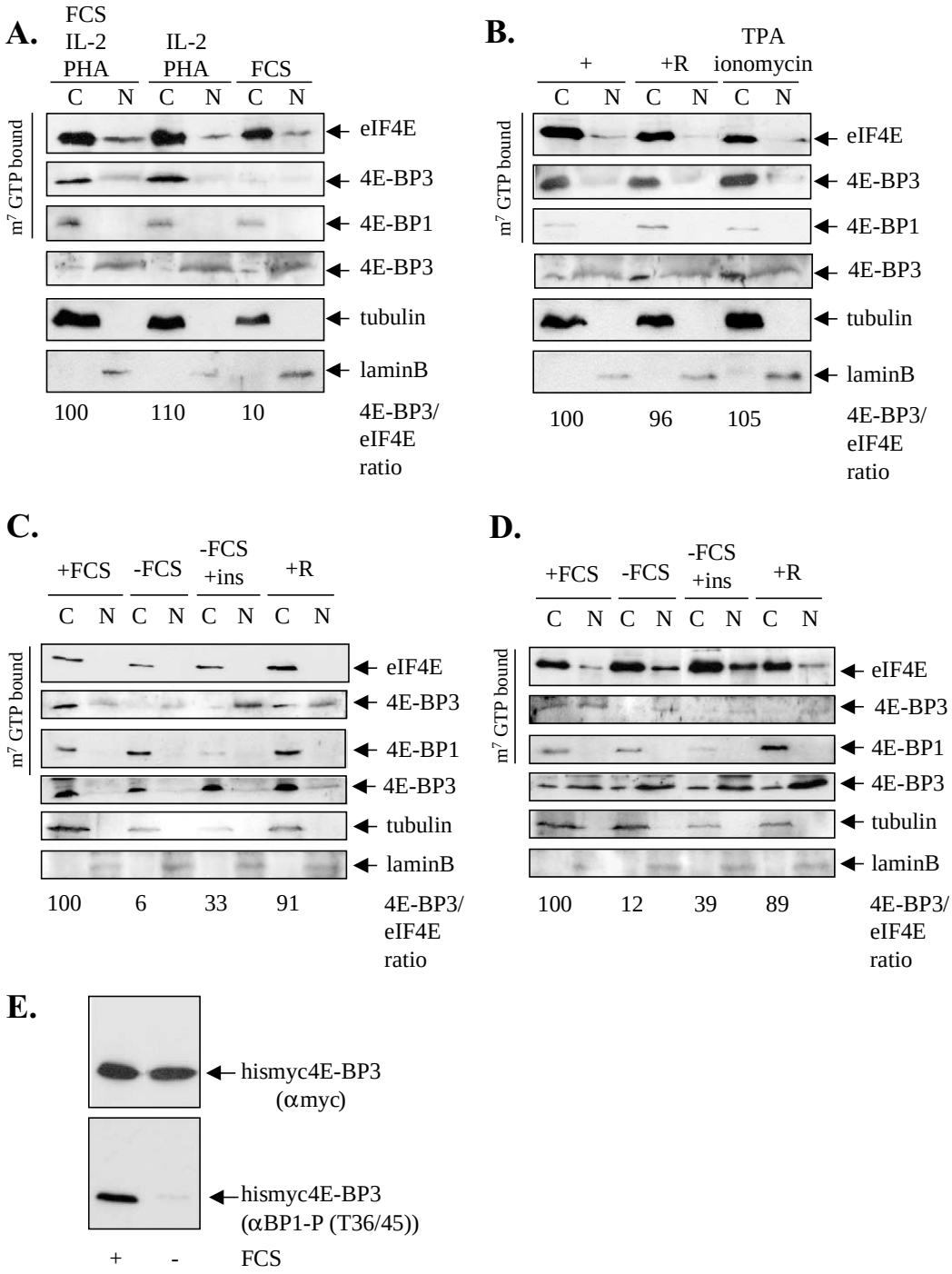

Fig. 5. Regulation of the association of 4E-BP3 with eIF4E in different cell types. A: Primary T cells (50×10^6 cells) were grown in complete medium containing FCS, IL-2, and PHA (FCS/IL-2/PHA) or were starved for either FCS in medium containing IL-2 and PHA (IL-2/PHA) or for IL-2 and PHA in medium containing FCS (FCS) for 20 h. B: Primary T cells (50×10^6 cells) were treated with rapamycin (100 nM) for 20 h or with TPA/ionomycin (1 mM/1 μg/ml) for 1 h. C: HEK293 cells (10×10^6 cells) were kept in growing medium, or serum-starved for 20 h, serum-starved for 20 h followed by insulin treatment (100 nM) for 1 h, or treated with rapamycin (100 nM) for 20 h. D: HeLa cells (10×10^6 cells) were kept growing in medium, were serum-starved for 20 h, were serum-starved for 20 h followed by insulin treatment (100 nM) for 1 h, or were treated with rapamycin (100 nM) for 20 h. Cytoplasmic and nuclear fractions were prepared and an m^7GTP Sepharose pull down was performed followed by SDS–PAGE and western blotting. eIF4E, 4E-BP3 and 4E-BP1 were detected with the appropriate antibodies. Whole extracts were analysed by SDS–PAGE and western blotting to detect nuclear (lamin B) and cytoplasmic (tubulin) markers. Total amounts of cytoplasmic eIF4E and associated 4E-BP3 were quantitated using ImageQuant. The amount of 4E-BP3 associated with eIF4E in control cells was set at 100 ($n=3$). E: HEK293 cells were transfected with a vector encoding his/myc-tagged 4E-BP3. Total extracts were analysed by SDS–PAGE and Western blotting. Phosphorylation of 4E-BP3 was detected using phospho-4E-BP1 (Thr36/45) antibody and equal loading was verified using an antibody against the myc epitope. Similar results were obtained in three experiments.

We were unable to detect any significant effects of the different treatments of T cells on the association of eIF4E with 4E-BP3 in the nucleus (Fig. 5A,B).

HEK293 cells (Fig. 5C) or HeLa cells (Fig. 5D) were either grown in medium supplemented with FCS, serum-starved, serum-starved and subsequently treated with insulin, or treated with rapamycin. In the cytoplasmic fractions of both cell types, 4E-BP3 dissociated from eIF4E upon serum starvation. After insulin stimulation, partial recovery of the 4E-BP3/eIF4E association was observed. Rapamycin treatment did not affect the association of eIF4E with 4E-BP3.

In the nuclear fractions of both HEK293 and HeLa cells, association of eIF4E with 4E-BP3 did not significantly change upon any of the treatments studied (Fig. 5C,D).

As expected, the association of 4E-BP1 with eIF4E increased after serum starvation and rapamycin treatment, while dissociation of 4E-BP1 was detected upon insulin stimulation.

The effects of these treatments on the association of 4E-BP3 with eIF4E might be secondary to effects on the association of eIF4E with 4E-BP1. However, since these effects were not always reciprocally related, it seemed possible that 4E-BP3 was subject to direct regulation. Threonine 23 in 4E-BP3 is the phosphorylation site equivalent to threonine 36/45 (Thr37/46 in the human sequence) in 4E-BP1 and the phosphorylation state of Thr23 in 4E-BP3 can be detected with the phospho-4E-BP1 (Thr35/46) antibody [16]. HEK293 cells were transfected with his/myc-tagged 4E-BP3 (Fig. 5E), and subsequently left untreated or serum-starved. Upon serum starvation of HEK293 cells, a significant reduction in the phosphorylation of 4E-BP3 at this site was detected, suggesting a direct regulation of 4E-BP3.

4. Discussion

The regulation of 4E-BP1 has been widely studied. However, the regulation of the other two eIF4E-binding proteins, 4E-BP2 and 4E-BP3, has not been described in much detail. Until now, no information was available about the regulation of 4E-BP3.

Most interestingly, we found that 4E-BP3 is present in the nucleus as well as in the cytoplasm. The nuclear localisation of 4E-BP3 distinguishes 4E-BP3 from 4E-BP1, since 4E-BP1 is only present in the cytoplasm [13]. Furthermore, 4E-BP3 is associated with eIF4E in both the nucleus and the cytoplasm, and it will be important to assess the function of the nuclear 4E-BP3 and the cytoplasmic 4E-BP3 in future studies.

Subcellular localisation of 4E-BP3 was studied, however, no significant changes were found under a variety of conditions. Furthermore, the mechanism of nuclear export that can be experimentally tested, the CRM-1-mediated pathway, was not involved in the translocation of 4E-BP3 (Fig. 4). Therefore, the mechanism involved in the export and/or translocation of 4E-BP3 remains unclear. Considering the small size of the 4E-BP3 protein it is possible that 4E-BP3 is able to diffuse into and out of the nucleus, via the nuclear pore, without an active shuttling mechanism.

Regulation of the association between 4E-BP3 and eIF4E was shown in the cytoplasm. Dissociation of 4E-BP3 was detected upon starvation in the different cell types. In HEK293 cells this coincided with an increased association of 4E-BP1 and eIF4E, but in primary T cells no change was observed in the association of 4E-BP1 with eIF4E. Apparently, under some conditions, the dissociation of 4E-BP3 was not merely a consequence of increased binding of 4E-BP1 (Fig. 5).

A decrease in the phosphorylation of 4E-BP3 at Thr23 was seen in serum-starved HEK293 cells (Fig. 5E). Under these conditions, this decreased phosphorylation of 4E-BP3 at Thr23 coincided with dissociation of 4E-BP3 from eIF4E in HEK293 cells. This is an unexpected finding since dephosphorylation of the equivalent site (Thr36) in 4E-BP1 indirectly leads to association of 4E-BP1 with eIF4E [17]. However, this finding suggested that 4E-BP3 is itself regulated. Indeed, we have previously shown [16] that phosphorylation of his/myc4E-BP3 is relatively low in serum-starved HEK293 cells and increases somewhat in response to insulin. In particular, insulin increases the phosphorylation of Thr23 [16].

In this paper, we focused on the novel finding that 4E-BP3 is a nuclear as well as a cytoplasmic protein and on the finding that the association of cytoplasmic 4E-BP3 with eIF4E is regulated in several different cell types. However, it will be important to assess in future studies whether the association of eIF4E/4E-BP3 is an actively regulated process, i.e. via phosphorylation of 4E-BP3, or whether the association is regulated passively, i.e. via competition for binding with 4E-BP1, 4E-BP2, eIF4GI, eIF4GII, or 4E-T.

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