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TOS Motif-Mediated Raptor Binding Regulates 4E-BP1 Multisite Phosphorylation and Function

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Summary

Background: The mammalian target of rapamycin, mTOR, is a serine/threonine kinase that controls cell growth and proliferation via the translation regulators eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1). We recently identified a TOR signaling (TOS) motif in the N terminus of S6K1 and the C terminus of 4E-BP1 and demonstrated that in S6K1, the TOS motif is necessary to facilitate mTOR signaling to phosphorylate and activate S6K1. However, it is unclear how the TOS motif in S6K1 and 4E-BP1 mediates mTOR signaling.
Results: Here, we show that a functional TOS motif is required for 4E-BP1 to bind to raptor (a recently identified mTOR-interacting protein), for 4E-BP1 to be efficiently phosphorylated in vitro by the mTOR/raptor complex, and for 4E-BP1 to be phosphorylated in vivo at all identified mTOR-regulated sites. mTOR/raptor-regulated phosphorylation is necessary for 4E-BP's efficient release from the translational initiation factor eIF4E. Consistently, overexpression of a mutant of 4E-BP1 containing a single amino acid change in the TOS motif (F114A) reduces cell size, demonstrating that mTOR-dependent regulation of cell growth by 4E-BP1 is dependent on a functional TOS motif.
Conclusions: Our data demonstrate that the TOS motif functions as a docking site for the mTOR/raptor complex, which is required for multisite phosphorylation of 4E-BP1, eIF4E release from 4E-BP1, and cell growth.

Introduction

The coordination of cell growth (an increase in cell mass/size) and cell division (an increase in cell number) is required for sustained cellular proliferation. The target of rapamycin (TOR) is an evolutionarily conserved protein serine/threonine kinase that integrates signals from nutrients and mitogens to supply a required input for cell growth and proliferation [1, 2]. Indeed, TOR deletion in *Drosophila* or specific inhibition of mammalian TOR (mTOR) by the immunosuppressant rapamycin results in reduced cell size and cell proliferation [2–4]. The best-characterized downstream targets of mTOR (also referred to as FRAP, RAFT, and RAPT) [5–8] are the ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) [9, 10].

S6 kinases phosphorylate the 40S ribosomal protein S6, which is thought to enhance translation of mRNAs containing repressive 5′-terminal oligopolypyrimidine (5′ TOP) tracts [11]. Recently, however, the role of S6K1 and ribosomal protein S6 phosphorylation in regulating 5′ TOP mRNAs' translation has been questioned [12]. 4E-BP1 functions as a repressor of translation; when hypophosphorylated in the absence of nutrients or growth factors, 4E-BP1 associates with eIF4E, the mRNA cap binding protein, to inhibit cap-dependent translation [13, 14]. Binding of 4E-BPs to eIF4E is regulated by ordered phosphorylation of critical proline-directed Ser/Thr residues on 4E-BP1 [15, 16]. 4E-BP1 contains at least six phosphorylation sites (Thr37, Thr46, Ser65, Thr70, Ser83, and Ser112) [17, 18], four of which (Thr37, Thr46, Ser65, and Thr70) are known to be regulated by mTOR signaling. Phosphorylation of Thr37 and Thr46 is thought to prime 4E-BP1 for sequential phosphorylation of Ser65 and Thr70, which results in the dissociation of 4E-BP1 from eIF4E [19, 20]. Free eIF4E then forms an active translation initiation complex [21]. eIF4E function is particularly required for the efficient translation of transcripts containing highly structured 5′ UTRs, a subset of which encode proteins involved in cell growth and proliferation [22]. Indeed, overexpression of eIF4E transforms rodent fibroblasts [23], while coexpression with 4E-BP1 blocks eIF4E-driven transformation [24].

The mechanisms by which mTOR signals to its downstream targets, 4E-BP1 and S6K1, remain unclear. Direct phosphorylation of 4E-BP1 and S6K1 by mTOR, or indirect regulation of phosphorylation by an mTOR-regulated kinase or phosphatase, has been suggested [19, 20]. Recently, new insights have emerged regarding the biochemical mechanism by which mTOR signaling is regulated. The regulatory associated protein of mTOR, raptor, and its yeast homolog, the Kontroller of Growth-1 (KOG1), have been identified as novel TOR-interacting proteins and regulators [25–27]. Raptor association with mTOR is required for efficient S6K1 and 4E-BP1 phosphorylation [25, 26] and has been suggested to function as a scaffolding protein that brings mTOR in close proximity to its substrates [26]. In addition, raptor was suggested to function as a bidirectional regulator of mTOR, inhibiting mTOR under nutrient starvation conditions and activating it when adequate supplies of amino acids and carbohydrates are available [25]. Consistent with raptor's ability to regulate TOR signaling and cell growth, inhibition of raptor expression with siRNA reduces cell size [25].

We recently identified a TOR signaling (TOS) motif in the N terminus of all known S6 kinases and in the ex-

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treme C terminus of the 4E-BPs [28]. The TOS motif of S6K1 functions to receive mTOR signals, as mutagenic inactivation of this motif mimics the effect of rapamycin on S6K1 phosphorylation. S6K1 mutations that overcome the inhibitory effect of the nonfunctional TOS motif render S6K1 resistant to the inhibitory effect of amino acid withdrawal and to rapamycin. We have also presented preliminary data that supports the finding that mutation of Phe114 to Ala in the 4E-BP1 TOS motif (FEMDI) significantly inhibits insulin-stimulated phosphorylation of 4E-BP1 [28].

Here, we analyze in greater detail the mechanism by which the TOS motif mediates 4E-BP1 phosphorylation and function. We show that the TOS motif is required for phosphorylation of 4E-BP1 in vitro and in vivo at all the identified mTOR-regulated sites. The inability of mTOR to regulate 4E-BP1 phosphorylation in a TOS motif mutant (F114A) correlates with the inability of raptor to interact with 4E-BP1. Importantly, overexpression of 4E-BP1 (F114A) reduces cell size, similar to that observed when the dominant-inhibitory 4E-BP1 mutant (T37/46A) was overexpressed [2]. These results elucidate the mechanism by which mTOR controls the phosphorylation state of 4E-BP1 within mammalian cells: the TOS motif mediates 4E-BP1/raptor association to drive mTOR-dependent multisite phosphorylation of 4E-BP1, resulting in eIF4E release and cell growth.

Results

TOS Motif Is Required for 4E-BP1 Phosphorylation at All mTOR-Regulated Sites

mTOR regulates hierarchical 4E-BP1 phosphorylation (Thr37/46, Ser65, and Thr70), which is required for 4E-BP1's release from eIF4E [19]. To investigate which phosphorylation events on 4E-BP1 require the TOS motif (Figure 1A), we analyzed the phosphorylation of the nonfunctional TOS motif-containing mutant, 4E-BP1-F114A, by employing various 4E-BP1 phospho-specific antibodies. Basal and insulin-stimulated Thr37/46 phosphorylation was reduced in the 4E-BP1-F114A mutant protein when compared to wild-type 4E-BP1 in HEK293E cells (Figure 1B). The F114A mutation had a more pronounced inhibitory effect on phosphorylation of the later 4E-BP1 phosphorylation sites Thr70 and Ser65 (Figure 1B), of which residual phosphorylation was only detected on longer film exposures (data not shown). Therefore, the TOS motif is required for efficient 4E-BP1 phosphorylation at all mTOR-regulated sites. The residual phosphorylation of Thr37/46 (Figures 1B and 1C), Ser65, and Thr70 (data not shown) in 4E-BP1-F114A was still sensitive to the mTOR inhibitor, rapamycin, suggesting that either the single F114A point mutation of the TOS motif does not completely block the function of this TOS motif to mediate mTOR-dependent phosphorylation of 4E-BP1 or that the rapamycin-sensitive phosphorylation of Thr37/46, Ser65, and Thr70 can also occur independently of this motif. TOS motif-independent dephosphorylation of 4E-BP1 could be caused by a rapamycin-activated, and thus mTOR-inhibited, phosphatase. Regulation of 4E-BP1 phosphorylation by such a phosphatase has already been suggested [29, 30]. Similar regulation of these phosphorylation sites was also detected in U2OS cells (Figure 1C).

To rule out the possibility that the inhibitory effect of the TOS motif mutant (4E-BP1-F114A) on Ser65 and Thr70 phosphorylation could be a secondary consequence of reduced phosphorylation of the priming sites (Thr37/46), we compared the phosphorylation of Ser65 and Thr70 in wild-type and 4E-BP1-F114A, which contain acidic mutations in the priming sites Thr37/46 (4E-BP1-T37/46E) to mimic Thr37/46 phosphorylation. It has been reported that mutation of Thr37/46 to glutamic residues (4E-BP1-T37/46E) partially restores phosphorylation of Ser65 and Thr70, whereas mutation of Thr37/46 to alanines abolishes phosphorylation of Ser65 and Thr70 [15]. Phosphorylation of Ser65 and Thr70 in the TOS motif mutant containing acidic Thr37/46 mutations (4E-BP1-F114A) was significantly less than in a 4E-BP1 mutant with the acidic substitutions for Thr37/46 but with a functional TOS motif; this finding suggests that the TOS motif is required for efficient phosphorylation of Ser65 and Thr70 even when 4E-BP1 is primed by phosphorylation at Thr37/46. Therefore, the TOS motif is required for mTOR-dependent phosphorylation of 4E-BP1 at all known rapamycin-sensitive sites (Figures 1B–1D); this new role of the TOS motif is similar to its role in regulating the phosphorylation status of several sites in S6K1 [28].

A Functional TOS Motif Is Required for 4E-BP1/Raptor Complex Formation

How does the TOS motif mediate mTOR regulation of 4E-BP1? Recently, raptor and its yeast homolog KOG1 were identified as TOR-interacting proteins and regulators [25, 26]. Raptor forms a complex with 4E-BP1 and mTOR [26] and is thought to function as a scaffolding protein that recruits mTOR in close proximity to its downstream substrates [26]. To investigate whether the TOS motif is required for 4E-BP1 regulation by the mTOR/raptor complex, we cotransfected HA-tagged 4E-BP1 and myc-tagged raptor in HEK293E cells. The TOS motif mutant 4E-BP1 (F114A) failed to coimmunoprecipitate with raptor, whereas wild-type 4E-BP1 coimmunoprecipitated, as previously reported (Figures 2A, 2B, and 2D; [26]), indicating that the TOS motif in 4E-BP1 is required for its binding to raptor. The inability of 4E-BP1-F114A to bind to raptor is not a consequence of its hypophosphorylated status, as the similarly hypophosphorylated 4E-BP1-T37/46A mutant bound raptor as efficiently as wild-type 4E-BP1 (Figure 2A). We have also generated mutations in other residues within the TOS motif of 4E-BP1 and have tested their effect on 4E-BP1 phosphorylation (as assayed by enhanced mobility on SDS-PAGE) and 4E-BP1 coimmunoprecipitation with myc-tagged raptor. Paralleling their effect on 4E-BP1 phosphorylation, different mutations in the TOS motif inhibited the ability of 4E-BP1 to coimmunoprecipitate with raptor (Figures 2B and 2C). Importantly, the TOS motif was also required for 4E-BP1 to bind raptor under conditions [25] that preserve mTOR complexes (0.2% CHAPS as detergent and crosslinker DSP). Upon cotransfection of 4E-BP1-F114A and -WT with raptor, followed by lysis under these conditions, raptor and endogenous

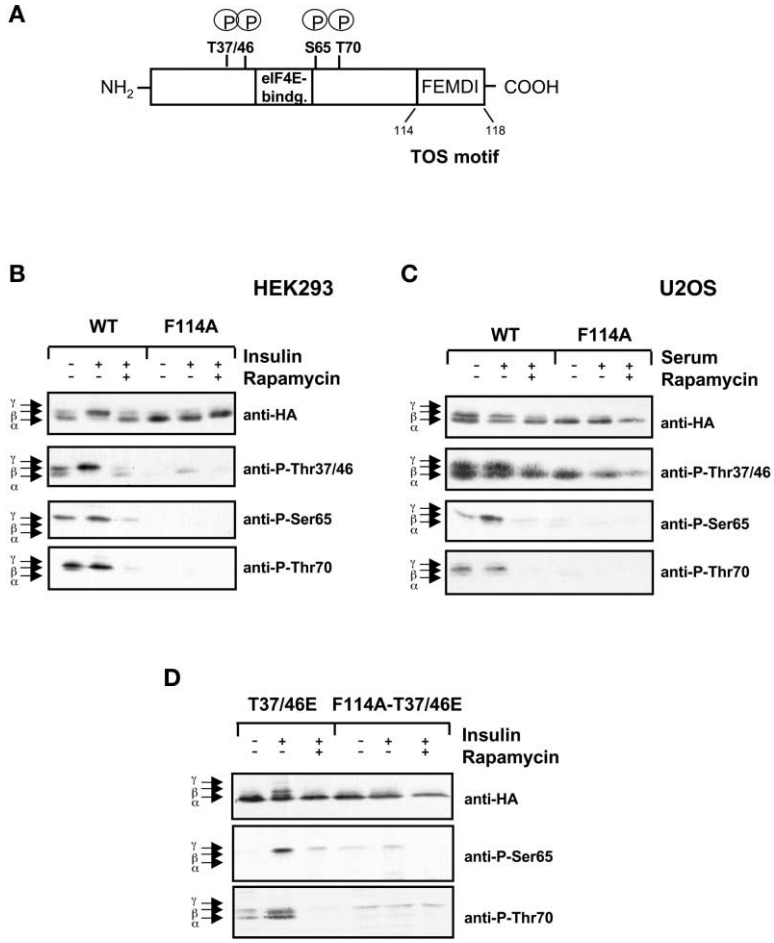

Figure 1. TOS Motif Is Required for Multisite 4E-BP1 Phosphorylation

(A) A schematic model of 4E-BP1 and the TOS motif, mTOR-regulated phosphorylation sites (Thr37/46, Ser65, and Thr70), and the eIF4E binding site (eIF4E-bindg.).

(B and C) (B) HEK293E or (C) U2OS cells were transfected with wild-type HA-4E-BP1 (WT) and HA-4E-BP1-F114A (F114A). Transfected cells were starved, rapamycin treated, stimulated, and lysed as described in the Experimental Procedures. Protein expression levels were assayed by immunoblotting with anti-HA antibody (upper panels). In vivo phosphorylation of 4E-BP1 at Thr37/46 (anti-P-Thr37/36), Ser65 (anti-P-Ser65), and Thr70 (anti-P-Thr70) was evaluated by immunoblotting with 4E-BP1 phosphopeptide-specific antibodies.

(D) HEK293E cells were transfected with HA-4E-BP1-T37/46E (T37/46E) and HA-4E-BP1-F114A-T37/46E (F114A-T37/46E). Transfected cells were starved, rapamycin treated, stimulated, and lysed as described in the Experimental Procedures. Protein expression levels were assayed by immunoblotting with anti-HA antibody (upper panel). In vivo Ser65 (anti-P-Ser65) and Thr70 (anti-P-Thr70) phosphorylation was evaluated by immunoblotting with phosphopeptide-specific antibodies.

mTOR coimmunoprecipitated with wild-type, but not with the TOS motif mutant of 4E-BP1 (Figure 2D). Our data indicate that a functional TOS motif is required for 4E-BP1/raptor complex formation.

TOS Motif Is Required for Efficient In Vitro Phosphorylation of 4E-BP1 by mTOR

Since the TOS motif is required for efficient 4E-BP1 phosphorylation at all mTOR-regulated sites in vivo (Figures 1B, 1C, and 1D), we next examined whether the TOS motif is required for mTOR phosphorylation of 4E-BP1 in vitro. We compared the phosphorylation of recombinant wild-type GST-4E-BP1and GST-4E-BP1-F114A by AU-tagged mTOR immunoprecipitated from cells transfected with AU-mTOR in the absence or presence of myc-raptor. Importantly, we prepared cell extracts under conditions that retain the mTOR/raptor complex, and, indeed, myc-raptor coimmunprecipitatetd with AU-mTOR (Figure 3A; [25]) (We have noted that the myc-antibody cross-reacts with a weak nonspecific band that runs at about the size of raptor). AU-mTOR phosphorylated wild-type GST-4E-BP1 to a much greater extent than GST-4E-BP1-F114A (Figure 3A); this finding indicates that direct phosphorylation of 4E-BP1 by mTOR requires an intact TOS motif. As previously reported, AU-mTOR/myc-raptor immunocomplexes significantly enhance in vitro phosphorylation of wild-type GST-4E-BP1 (Figure 3A; [26]). Interestingly, AU-mTOR/myc-raptor immunocomplexes did not enhance the phosphorylation of GST-4E-BP1-F114A as potently as wild-type GST-4E-BP1 under the same conditions in vitro (approximately 8 times lower). Importantly, the kinase-inactive mutant of mTOR (AU-mTOR-KD) did not phosphorylate GST-4E-BP1 or GST-4E-BP1-F114A (Figure 3A), indicating that mTOR, rather than an associated kinase, directly phosphorylates GST-4E-BP1 in this assay. In contrast, activated recombinant ERK2 phosphorylated both wild-type 4E-BP1 [17] and the TOS motif mutant F114A to a similar extent (Figure 3B), indicating that the proline-directed sites in both recombinant proteins are accessible for phosphorylation. As expected, we did not detect phosphorylation of GST alone by mTOR or ERK2 (Figures 3A and 3B). Immunoprecipitated endogenous mTOR from HEK293E cells also phosphorylated wild-type GST-4E-BP1 much more efficiently than the F114A mutant (data not shown).

Mutation of the TOS Motif Strengthens the Binding of 4E-BP1

Next, we investigated whether the 4E-BP1-F114A mutant could inhibit eIF4E function under conditions in which wild-type 4E-BP1 does not, as during insulin stimulation. We compared the association of wild-type 4E-BP1 and 4E-BP1-F114A to eIF4E by a m^7GTP-Sepharose

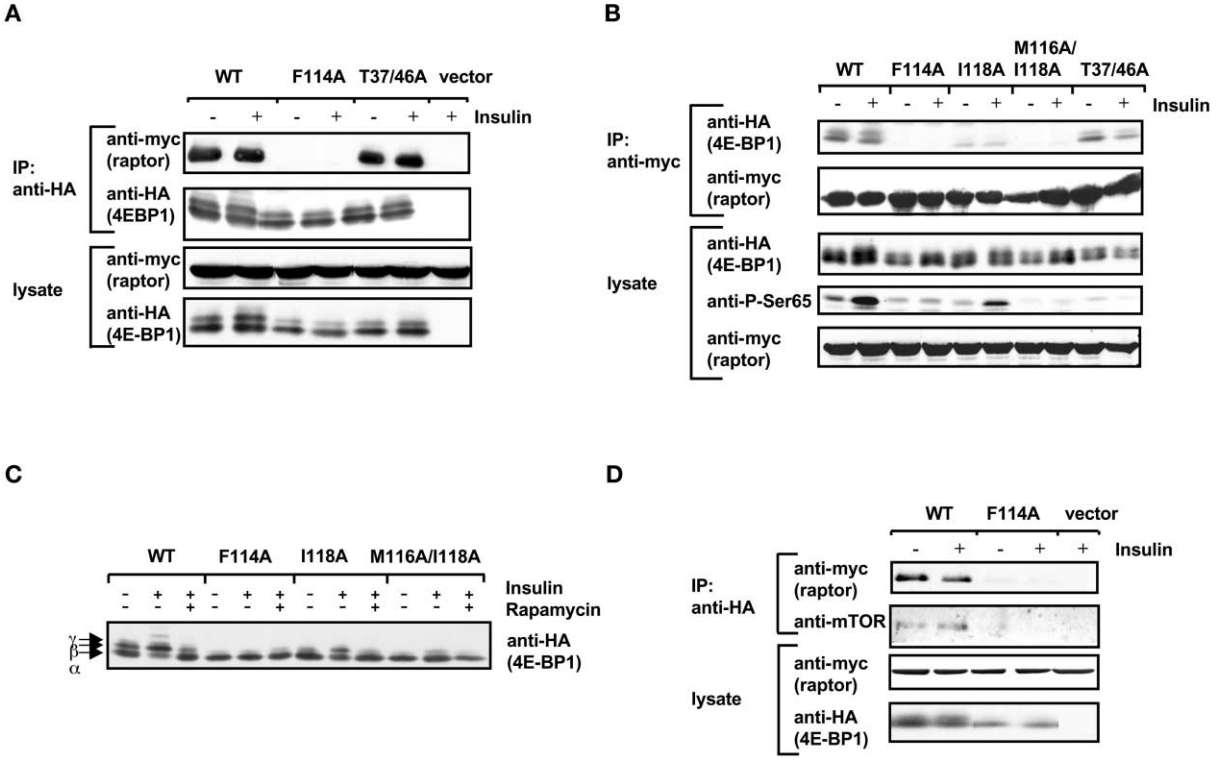

Figure 2. A Functional TOS Motif Is Required for 4E-BP1 to Bind to Raptor

(A) HEK293E cells were cotransfected with myc-raptor and either wild-type HA-4E-BP1 (WT), HA-4E-BP1-F114A (F114A), HA-4E-BP1-T37/46A (T37/46A), or the empty pACTAG-2 vector (vector). Transfected cells were starved and stimulated as described in the Experimental Procedures and lysed in buffer A as described in the Supplemental Data. Anti-HA immunoprecipitations were analyzed by anti-HA (4EBP1) and anti-myc (myc-raptor) immunoblotting. Myc-raptor and HA-4E-BP1 expression levels was assayed by immunoblotting of cell lysate with anti-myc antibody and anti-HA antibody, respectively.

(B) HEK293E cells were cotransfected with myc-raptor and either wild-type HA-4E-BP1 (WT), HA-4E-BP1-F114A (F114A), HA-4E-BP1-I118A (I118A), or HA-4E-BP1-M116A/I118A (M116A/I118A). Transfected cells were starved and stimulated as described in the Experimental Procedures and lysed in buffer A as described in the Supplemental Data. α-myc immunoprecipitations were analyzed by anti-HA (4E-BP1) and anti-myc (myc-raptor) immunoblotting. Protein expression levels were assayed by immunoblotting cell lysate with anti-myc antibody. Ser65 phosphorylation was evaluated by immunoblotting with anti-4E-BP1-P-Ser65 phosphopeptide-specific antibody.

(C) HEK293E cells were transfected with wild-type HA-4E-BP1 (WT), HA-4E-BP1-F114A (F114A), HA-4E-BP1-I118A (I118A), and HA-4E-BP1-M116A/I118A (M116A/I118A). Transfected cells were starved, rapamycin treated, stimulated as described in the Experimental Procedures and lysed in buffer A as described in the Supplemental Data. 4E-BP1 was detected by immunoblotting with anti-HA antibody.

(D) HEK293E cells were cotransfected with myc-raptor and either wild-type HA-4E-BP1 (WT), HA-4E-BP1-F114A (F114A), or the empty pACTAG-2 vector (vector). Transfected cells were starved and stimulated as described in the Experimental Procedures and lysed in buffer B containing 0.2% CHAPS as detergent and the chemical crosslinker DSP as described in the Supplemental Data. Anti-HA immunoprecipitations were analyzed by anti-myc (myc-raptor) and anti-mTOR immunoblotting. Myc-raptor expression levels were assayed by immunoblotting of cell lysate with anti-myc antibody.

pull-down assay. m^7GTP mimics the cap structure of mRNA and can be used to affinity purify eIF4E and its associated proteins. In serum-starved HEK293E cells, wild-type 4E-BP1 associated with eIF4E, which was abrogated upon insulin stimulation but was recovered when cells were pretreated with rapamycin, as expected (Figure 4). The 4E-BP1-F114A mutant displayed stronger association to eIF4E than wild-type 4E-BP1 in serum-starved or rapamycin-treated HEK293E cells; this finding is consistent with decreased phosphorylation on the 4E-BP1-F114A mutant (Figures 1B, 1C, and 4). The amount of 4E-BP1-F114A bound to eIF4E decreased upon insulin stimulation of serum-starved HEK293E cells, but significant binding was still detectable. Indeed, the association of 4E-BP1-F114A to eIF4E in insulin-stimulated cells was significantly higher than association of wild-type 4E-BP1 to eIF4E in serum-starved cells (Figure 4). As a negative control, we mutated the eIF4E binding site (4E-BP1-Y54A/L59A) on 4E-BP1 [31]. Binding of both wild-type and 4E-BP1-F114A to eIF4E was abrogated by Y54A/L59A mutations (Figure 4). These data suggest that 4E-BP1-F114A binds to eIF4E specifically via the defined eIF4E binding site and therefore functions as an inhibitor of eIF4E. Similar data were observed by using U2OS cells (data not shown).

Overexpression of 4E-BP1-F114A Reduces Cell Size

Reduction of raptor expression by siRNA [25] or inhibition of mTOR-dependent signaling with rapamycin [2] results in reduced cell size, suggesting that raptor mediates mTOR-dependent regulation of cell growth. To de-

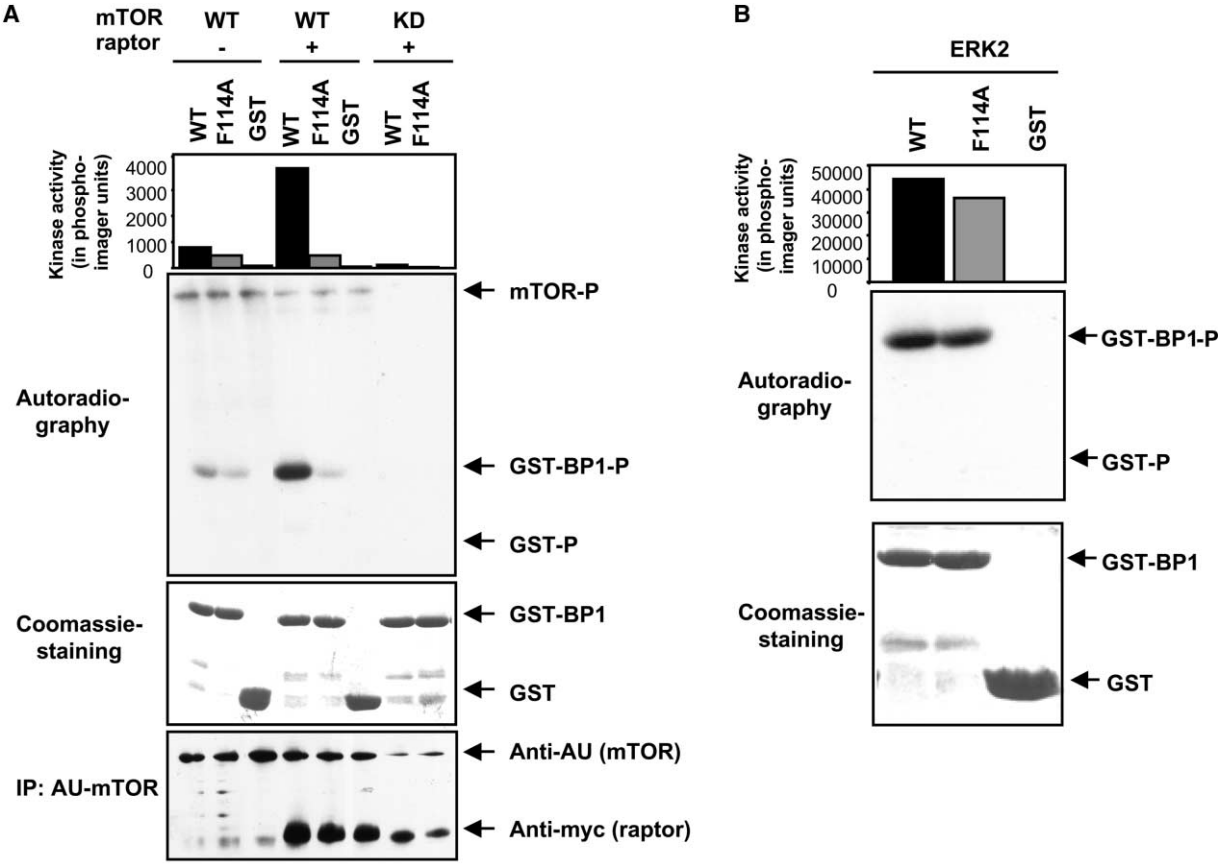

Figure 3. TOS Motif Is Required for 4E-BP1 In Vitro Phosphorylation by mTOR, but not ERK2

(A) AU-mTOR wild-type (WT) or kinase-dead (KD) coexpressed in HEK293E cells with myc-raptor or vector control were purified by anti-AU immunoprecipitation and were subjected to an in vitro mTOR kinase assay with recombinant wild-type GST-4E-BP1, GST-4E-BP1-F114A, or GST as substrates as described in the Supplemental Data. Quantification of the kinase assay (first panel) and autoradiogram of phosphorylated proteins (second panel) is shown. Coomassie staining of the SDS-PAGE gel reveals the relative amounts of each fusion protein in the assay (third panel). An anti-AU immunoblot of the kinase assay (fourth panel) is shown.

(B) In vitro phosphorylation of recombinant wild-type GST-4E-BP1, GST-4E-BP1-F114A, and GST by activated $(His)_6$-ERK2 was performed as described in the Supplemental Data. Quantification of the kinase assay (first panel) and an autoradiogram of phosphorylated proteins (second panel) are shown. Coomassie staining of the SDS-PAGE gel reveals the relative amounts of each fusion protein in the assay (third panel).

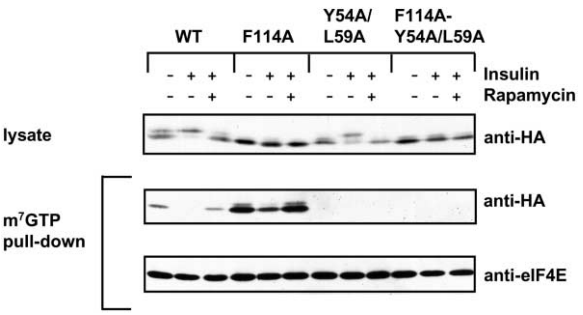

Figure 4. Mutation of the TOS Motif Induces Stronger Binding to eIF4E

HEK293E cells overexpressing wild-type HA-tagged 4E-BP1 (WT) and HA-tagged 4E-BP1 mutants (F114A, Y54A/L59A, or F114A-Y54A/L59A) were starved, rapamycin treated, stimulated, and lysed as described in the Experimental Procedures. Protein expression levels were assayed by immunoblotting with anti-HA antibody (top panel). m^7GTP-Sepharose pull down was analyzed by immunoblotting for anti-HA (HA-4E-BP1) (middle panel) and anti-eIF4E (bottom panel).

termine whether TOS-mediated 4E-BP1 phosphorylation by the mTOR/raptor complex is important for the control of cell growth, we tested whether overexpression of 4E-BP1-F114A would reduce the size of proliferating U2OS cells. We used U2OS cells for this study as their cell size is fairly homogenous and they have been well characterized for cell size regulation by mTOR signaling [2]. Furthermore, we have shown that the regulation of 4E-BP1 phosphorylation is similar in U2OS and HEK293 cells (Figures 1B and 1C). The relative size of G_1 phase cells was measured by flow cytometry with the parameter forward scatter-height. We first compared the FSC-H histogram of cells transfected with pACTAG-2 vector control to those treated with rapamycin or transfected with 4E-BP1-T37/46, -WT, or -F114A (Figure 5A). Overexpression of 4E-BP1-F114A induced a shift to reduced cell size compared to vector control (Figure 5A), and this is similar to the previously reported effect that rapamycin or the dominant 4E-BP1-T37/46A mutant has on reducing cell size [2]. 4E-BP1-WT had a minimal effect, which was also previously reported. Graphical representation of the same experiment whereby

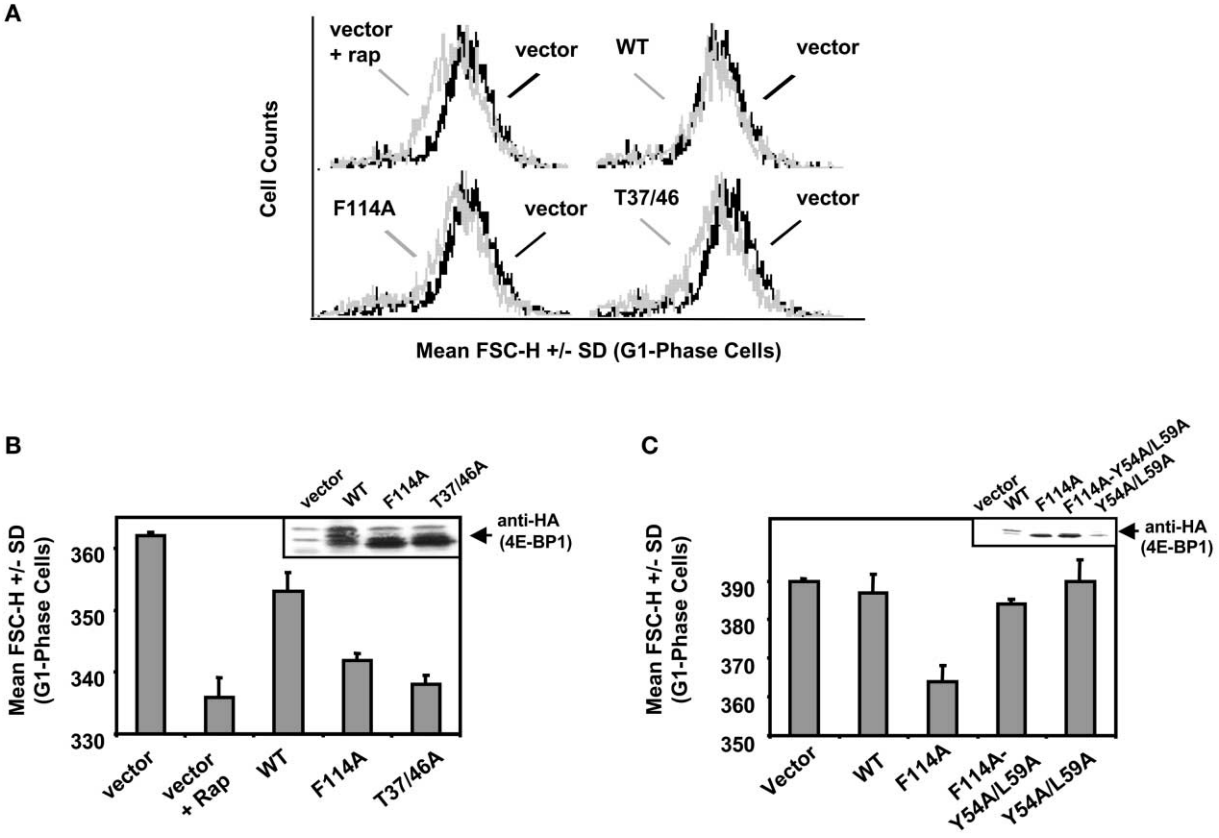

Figure 5. Overexpression of 4E-BP1-F114A Causes Reduction of Cell Size

(A–C) U2OS cells were cotransfected with 1 μg CD20 and 10 μg of either the empty pACTAG-2 vector (vector), wild-type HA-4E-BP1 (WT), HA-4E-BP1-F114A (F114A), or HA-4E-BP1-T37/46A (T37/46A) (A and B) or with 1 μg CD20 and either 10 μg of the empty pACATG-2 vector (vector), wild-type HA-4E-BP1 (WT), or the following HA-4E-BP1 mutants: F114A, F114A-Y54A/L59A, or Y54A/L59A (C). After overnight transfection, cells were split to new plates containing DMEM/FBS to allow subconfluent proliferation for 72 hr and were assayed by flow cytometry to determine cell size. In (A) and (B), vector-transfected cells were cultured in the absence or presence of rapamycin (rap); in (B) and (C), the mean FSC-H ± SD of triplicate transections is shown. Panel (A) depicts FSC-H histograms of representative samples shown in (B).

each construct was transfected in triplicate shows that the reduction in cell size induced by 4E-BP1-F114A expression is significant (Figure 5B). Importantly, mutations of the eIF4E binding domain (Y54A/L59A) on 4E-BP1 abrogated the ability of the TOS motif mutant 4E-BP1-F114A to reduce cell size (Figure 5C), demonstrating that the growth inhibitory effect caused by 4E-BP1-F114A occurs specifically through eIF4E binding and thus inhibition of eIF4E function. These data indicate that the TOS motif is important for regulation of 4E-BP1's biological function, to control cell growth.

Discussion

The role of the recently identified TOS motif within 4E-BP1 to regulate its phosphorylation and function has not been defined. We find that the TOS motif is absolutely required for efficient phosphorylation of 4E-BP1 at all the identified mTOR-regulated sites, namely, Thr37/46, Ser65, and Thr70 in vivo (Figures 1B, 1C, and 1D). Only 4E-BP1 with an intact TOS motif coimmunoprecipitates with raptor (Figures 2A, 2B, and 2D) and is efficiently phosphorylated by mTOR in vitro (Figure 3A), indicating that the TOS motif is required for 4E-BP1 phosphorylation by the mTOR/raptor complex. Additionally, we demonstrate that mTOR signaling via the TOS motif is important for the complete insulin-stimulated release of 4E-BP1 from eIF4E, which is a prerequisite for proper cell growth control (Figures 4 and 5A–5C). Consistently, expression of 4E-BP1 containing a mutated TOS motif reduces cell size (Figures 5A–5C).

Which phosphorylation sites on 4E-BP1 are regulated by mTOR signaling remains controversial. Recent data demonstrate that raptor is important for the regulation of mTOR's function [25, 32]. Hara et al. [26] suggest that raptor functions as a scaffolding protein, recruiting mTOR to its substrates S6K1 and 4E-BP1. Kim et al. [25] suggest an additional function for raptor as a bidirectional modulator of mTOR: raptor association is required for mTOR function under nutrient-rich conditions but represses mTOR kinase activity through a second high-affinity interaction under nutrient-poor conditions. Based on the data presented here and on previous observations [25, 26, 33], we propose the following model (Figure 6): the TOS motif mediates 4E-BP1 binding to raptor, which in turn recruits mTOR to the 4E-BP1/raptor complex. At this time, we do not know whether 4E-BP1 directly binds to raptor or whether the binding is indirect

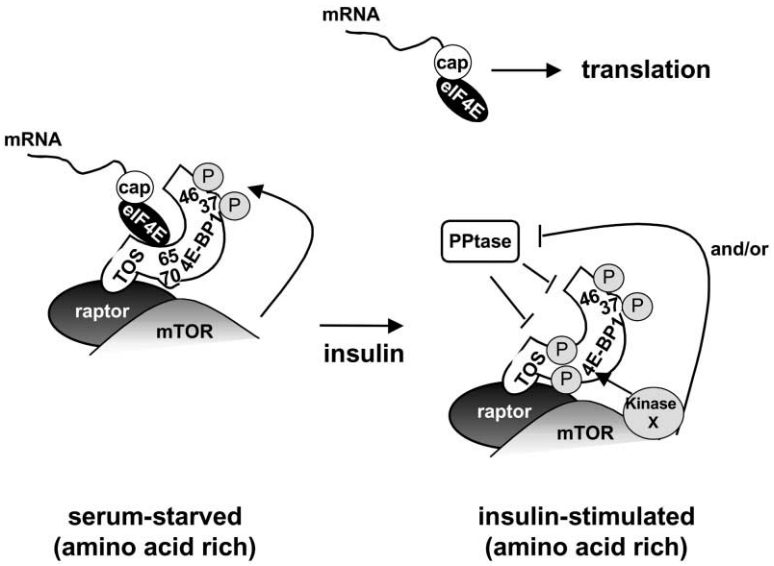

Figure 6. Model of 4E-BP1 Regulation by mTOR Signaling

The mTOR/4E-BP1/raptor complex exists in two forms under amino acid-rich conditions. In serum-starved cells, mTOR directly phosphorylates the 4E-BP1 priming sites Thr37/46. Insulin/serum stimulation leads to phosphorylation of Ser65 and Thr70 by an mTOR-regulated kinase (kinase X) and/or by inhibition of an mTOR-regulated phosphatase (PPtase). Phosphorylation of Thr37/46, Ser65, and Thr70 induces dissociation of eIF4E from 4E-BP1. At this point, it is not known whether the association of 4E-BP1 with raptor is direct or indirect.

and thus mediated by another molecule or molecules in the complex. This recruitment of mTOR enables direct and/or indirect phosphorylation of 4E-BP1 at Thr37/46, Ser65, and Thr70. In support of this model, the TOS motif 4E-BP1 mutant (4E-BP1-F114A) fails to coimmunoprecipitate with raptor, resulting in reduced in vitro phosphorylation of 4E-BP1 by mTOR (Figures 2A, 2B, 2D, and 3A) and reduced phosphorylation of 4E-BP1 at Thr37/46, Ser65, and Thr70 in vivo (Figures 1B and 1C).

The high basal phosphorylation of Thr37/46 in serum-starved cells is significantly reduced in the 4E-BP1-F114A mutant (Figure 1B); this finding suggests that the TOS motif is absolutely required for Thr37/46 phosphorylation under serum-starved conditions. Under amino acid-rich but serum-starved conditions, mTOR likely forms a constitutively active complex with raptor that directly phosphorylates 4E-BP1 at the Thr37/46 priming sites to allow for additional mitogen-stimulated phosphorylation (see model: Figure 6; [26]). Consistently, mTOR immunoprecipitates have been shown to phosphorylate 4E-BP1 at Thr37/46 in vitro [10, 34, 35]. The residual insulin-stimulated phosphorylation on 4E-BP1-F114A could be mediated by an insulin/mitogen-stimulated kinase that phosphorylates Thr37/46 independently of the TOS motif (Figures 1B and 1C). The existence of a serum-stimulated, mTOR-independent kinase for Thr37/46 has also been previously suggested [19, 36].

In contrast, phosphorylation of Ser65 and Thr70 appears to be more dependent on mTOR signaling, since phosphorylation of these sites in the TOS motif mutant (4E-BP1-F114A) is more strongly reduced under serum-starved and insulin-stimulated conditions (Figures 1B and 1C). Mimicking Thr37/46 phosphorylation with acidic amino acid substitutions does not rescue Ser65 and Thr70 phosphorylation in the 4E-BP1-F114A mutant (Figure 1D); this finding demonstrates that the TOS motif's effect on Ser65 and Thr70 is not merely a secondary consequence of its effect on Thr37/46 phosphorylation. Ser65 and Thr70 are not likely directly phosphorylated by mTOR, given that mTOR is not active on these sites in vitro (Figure 6; [34]). Phosphorylation of Ser65 and Thr70 has been suggested to be regulated by an mTOR-regulated kinase [35] (kinase X in model in Figure 6) and/or by a mTOR-regulated phosphatase [29] (PPtase in model in Figure 6).

The RAIP motif in the N terminus of 4E-BP1 and 4E-BP2 has been found to be critical for 4E-BP1 phosphorylation on mTOR-regulated sites [37]. In contrast to the TOS motif mutant, which has a more pronounced inhibitory effect on the phosphorylation of Ser65 and Thr70, deletion or mutation of the RAIP motif significantly inhibits mitogen-stimulated phosphorylation of the priming sites Thr37/46. Therefore, it is likely that the RAIP motif mediates phosphorylation of Thr37/46 by a mitogen-stimulated kinase.

The TOS motif on S6K1 was also found to be important for phosphorylation of several structurally diverse sites, and this importance is consistent with the possibility that mTOR also dynamically regulates S6K1 via activation of a kinase and via inhibition of a phosphatase [28]. It has been reported that S6K1 activation requires raptor association [25, 26], making it likely that the TOS motif within S6K1 is required for S6K1/raptor complex formation. Consistent with the results reported by Hara et al. [26], we also detect weak binding of wild-type S6K1 to raptor (data not shown). Unfortunately, this weak interaction makes it experimentally difficult for us to characterize the contribution of the S6K1 TOS motif to raptor binding. This difference in raptor binding affinity to S6K1 and 4E-BP1 could be caused by the different compositions of mTOR/S6K1/raptor or mTOR/4E-BP1/raptor complexes, with additional molecules found specifically in one or the other complex, or as a result of the existence of additional binding determinants in 4E-BP1.

4E-BP1 functions to regulate cell growth/cell size and cellular proliferation [2, 38]. Overexpression of 4E-BP1 antagonizes transformation induced by eIF4E overexpression [24], and overexpression of dominant-negative 4E-BP1 mutants in cycling mammalian U2OS cells [2] or *Drosophila* [38] reduces cell size. Here, we demonstrate that 4E-BP1 regulation by mTOR via the TOS motif is required for 4E-BP1-mediated control of cell size. Over-

expression of 4E-BP1-F114A reduced the size of G_1 phase-gated U2OS cells in a manner similar to the dominant-negative 4E-BP1 mutant 4E-BP1-T37/46A (Figures 5A and 5B). The effect of 4E-BP1-F114A overexpression on cell growth is consistent with the lack of 4E-BP1 phosphorylation by mTOR via the TOS motif, as several of these phosphorylation events are required to release eIF4E and promote cap-dependent translation [16, 39]. Mutation in the eIF4E binding site on 4E-BP1-F114A (4E-BP1-F114A-Y54A/L59A), although still hypophosphorylated, abrogated its ability to bind to eIF4E and to reduce cell size (Figure 5C). Thus, the growth inhibitory effect of 4E-BP1-F114A is mediated via its constitutive binding to and inhibition of eIF4E.

Conclusions

In this study, we identify a docking site on 4E-BP1 that is required for raptor association and thus clarify the mechanism by which the mTOR substrate 4E-BP1 is regulated by the mTOR/raptor complex. TOS motif-mediated 4E-BP1 complex formation with raptor recruits 4E-BP1 to the mTOR kinase. The association of 4E-BP1 with the mTOR/raptor complex is required for efficient phosphorylation of 4E-BP1 at several diverse sites, probably via different mTOR-dependent mechanisms. Importantly, we demonstrate that regulation of 4E-BP1 phosphorylation by the TOS motif is biologically significant: expression of 4E-BP1-F114A reduces cell size, indicating that the TOS motif is crucial for regulation of cell growth by mTOR/raptor signaling. The TOS motif appears to function as a general motif that allows raptor to bring downstream substrates to mTOR. In the future, we hope to identify novel mTOR substrates by using our mechanistic knowledge of the TOS motif.

Experimental Procedures

Plasmids and Fusion Proteins

pcDNA3/AU-mTOR encoding wild-type (WT) and kinase-dead (KD, D2338A) mTOR were kindly provided by Robert Abraham (Burnham Institute, San Diego, CA) and have been described [10]. pACTAG-2/3HA-4E-BP1-WT (4E-BP1), pACTAG-2/3HA-4E-BP1-T37/46E (4E-BP1-EE), and pACTAG-2/3HA-4E-BP1-T37/46A (4E-BP1-T37/46A) were generously provided by Nahum Sonenberg (McGill University, Montreal, Quebec, Canada) and are described in [15]. Andy R. Tee (Harvard Medical School, Boston) generated GST-4E-BP1 by subcloning human 4E-BP1 into pGEX-2T/GST. Point mutations of 4E-BP1 were generated by site-directed mutagenesis as described in the manufacture's protocol (Stratagene). pRK5/myc-raptor (human) has been described [25]. Recombinant wild-type GST-4E-BP1 and GST-4E-BP1-F114A were purified from *E. coli*. pET$(His)_6$/ERK2 was provided by M.H. Cobb [40] and was purified as described in [41].

Antibodies

Anti-myc monoclonal antibodies were purchased from Charles River Laboratories. Anti-HA monoclonal antibodies were kindly provided by Margaret Chou (University of Pennsylvania, Philadelphia). The anti-4E-BP1-phospho-Thr37/46, -phospho-Ser65, -phospho-Thr70, and anti-eIF4E antibodies were obtained from Cell Signaling Technology. The anti-AU1 antibody was purchased from Covance, and the anti-MAPK antibodies have been described [42]. Anti-mTOR antibodies were purchased from Santa Cruz Biotechnology. For immunoblotting, anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Amersham and Chemicon, respectively.

Cell Culture and Transfection

HEK293E were cultured, transfected, and lysed for immunoblotting and coimmunoprecipitation as described previously [43], and human U2OS osteosarcoma cells were cultured, transfected, and lysed as described in [2]. Human U2OS osteosarcoma cells were seeded on 60-mm dishes 1 day prior to transfection with Fugene (Roche) overnight according to the manufacturer's directions by using 3–10 μg total DNA, depending on the experiment. After 20 hr of starvation in serum-free DMEM, cells were pretreated for 30 min with rapamycin (provided by S.N. Seghal, Wyeth-Ayerst) (20 ng/ml) or ethanol vehicle, then stimulated with 100 nM insulin (HEK293E) or 10% FBS (U2OS) for 30 min.

Immunoblots

Proteins were resolved by SDS-12% polyacrylamide gel electrophoresis (PAGE), transferred to nitro-cellulose (Schleicher and Schuell), probed with the indicated antibodies, and then detected with HRP-conjugated secondary antibody.

Coimmunoprecipitations

The coimmunoprecipitation assays were carried out as described in the Supplemental Data available with this article online.

Immune Complex Kinase Assay

The immune complex kinase assay was carried out as described in the Supplemental Data available with this article online.

m^7GTP Cap Binding Assays

Cell extracts were incubated with 30 μl m^7GTP-Sepharose CL4B beads (Amersham Pharmacia Biotech) at 4°C for 1 hr, then washed twice in 1 ml buffer E (10 mM Tris, 100 mM NaCl, 1% NP-40, 1 mM EDTA, 0.5% sodium deoxycholate, 2 mM DTT, 1 mM sodium orthovanadate, 40 mg/ml PMSF, 10 μg/ml leupeptin, 5 μg/ml pepstatin [pH 7.2]) and once in 1 ml ST. Sepharose beads were resuspended in Laemmli sample buffer with 2% β-ME and were resolved on SDS-PAGE.

Flow Cytometry

Relative cell size was determined by using the parameter mean forward scatter-height (FSC-H) using a Becton Dickinson FACS Calibur flow cytometer with Cell Quest software, as previously described [2]. Briefly, 4×10^5 U2OS cells were plated to 60-mm plates 1 day prior to transfection with Fugene reagent (Roche) by using 10 μg pACTAG2 plasmids and 1 μg CD20. After overnight transfection, 60-mm plates were split 1:4 to 10-cm plates to allow subconfluent proliferation for 72 hr. After 72 hr, cells were harvested for FACS analysis by staining with anti-CD20-FITC antibodies, followed by fixation in ethanol. Immediately prior to FACS analysis, samples were treated with propidium iodide and RNaseA.

Supplemental Data

Supplemental Data including a detailed description of the coimmunoprecipitation procedure and the immune complex kinase assay are available at http://images.cellpress.com/supmat/supmatin.htm.

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