The Association of Initiation Factor 4F with Poly(A)-binding Protein Is Enhanced in Serum-stimulated Xenopus Kidney Cells*

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Serum stimulation of cultured Xenopus kidney cells results in enhanced phosphorylation of the translational initiation factor (eIF) 4E and promotes a 2.8-fold increase in the binding of the adapter protein eIF4G to eIF4E, to form the functional initiation factor complex eIF4F. Here we demonstrate the serum-stimulated co-isolation of the poly(A)-binding protein (PABP) with the eIF4F complex. This apparent interaction of PABP with eIF4F suggests that a mechanism should be shown to be important in the control of translation in the yeast Saccharomyces cerevisiae also operates in vertebrate cells. We also present evidence that the signaling pathways modulating eIF4E phosphorylation and function in Xenopus kidney cells differ from those in several mammalian cell types studied previously. Experiments with the immunosuppressant rapamycin suggest that the mTOR signaling pathway is involved in serum-promoted eIF4E phosphorylation and association with eIF4G. Moreover, we could find little evidence for regulation of eIF4E function via interaction with the specific binding proteins 4E-BP1 or 4E-BP2 in these cells. Although rapamycin abrogated serum-enhanced rates of protein synthesis and the interaction of eIF4G with eIF4E, it did not prevent the increase in association of eIF4G with PABP. This suggests that serum stimulates the interaction between eIF4G and PABP by a distinct mechanism that is independent of both the mTOR pathway and the enhanced association of eIF4G with eIF4E.

Control of polypeptide synthesis plays an important role in cell proliferation. The physiological regulation of protein synthesis is almost always exerted at the level of polypeptide chain initiation (reviewed in Refs. 1 and 2), influenced by elements in the 5'- and 3'-untranslated regions of the mRNA (3). The initiation phase is regulated, in part, by the phosphorylation and association of initiation factors involved in binding mRNA to the 40S ribosomal subunit (reviewed in Refs. 1 and 4–8). The cap structure present at the 5'-end of mRNA facilitates its binding to the ribosome, a process mediated by at least three initiation factors (eIF4A, eIF4B, and eIF4F) and ATP hydrolysis. eIF4F is a cap-binding protein complex composed of three polypeptides; eIF4E, which specifically recognizes the cap structure, eIF4A, a single strand RNA-binding protein with helicase activity (9), and eIF4G, which acts as a bridging molecule between eIF4E and the 40S ribosome via eIF3 (8, 10–14). It is believed that eIF4F functions to unwind secondary structure in the mRNA 5'-untranslated region to facilitate binding to the 40S ribosomal subunit (4). Recent studies in the yeast Saccharomyces cerevisiae have indicated that a further association occurs between eIF4G and poly(A)-binding protein (Pab1p), which binds to the 3' poly(A) tail of mRNA. This interaction allows functional interaction of the 5'- and 3'-ends of the mRNAs that is essential for transmitting the stimulatory signal of the poly(A) tail on translation to the cap structure (8, 15–21).

eIF4E activity can be regulated by both its phosphorylation and by its availability to participate in the initiation process. Increased levels of eIF4E phosphorylation have been directly correlated with the enhancement of translation that follows mitogenic stimulation of mammalian cells (1, 7, 22). Parallel increases in eIF4E phosphorylation and interaction of the factor with eIF4G have been observed in a number of cellular systems (23–27), and the phosphorylated form of eIF4E is reported to exhibit increased affinity for the cap structure in vitro (28). Another important mechanism regulating the interaction between eIF4E and eIF4G is exerted by the eIF4E-binding proteins 4E-BP1 and 4E-BP2 (PHAS-I and PHAS-II). In resting cells, 4E-BP1 and 4E-BP2 are hypophosphorylated and bound to eIF4E (7, 29–32). Stimulation of cells with growth factors or hormones increases the phosphorylation of these eIF4E-binding proteins to disrupt their association with eIF4E, liberating eIF4E to interact with a conserved hydrophobic region of eIF4G. A similar sequence found in 4E-BP1 is involved in binding to eIF4E and competes with eIF4G for eIF4E binding (33). Insight into the signaling pathways regulating the phosphorylation of 4E-BP1/BP2 has been provided by the use of the immunosuppressant, rapamycin (34, 35). This drug binds to its cytosolic receptor (FK-506-binding protein), which then interacts with and inhibits the activity of a protein known variously as RAFT-1/FKAP/RAFT-1 or mTOR (36). As a consequence of this, rapamycin causes the selective inhibition of two parallel signaling pathways downstream of mTOR; the p70 S6 kinase (p70S6K) signaling pathway and the phosphorylation of 4E-BP1 (36–38). Thus in mammalian cells rapamycin is thought to impair the enhancement of protein synthesis initiation in response to serum stimulation by stabilizing the interaction between eIF4E and 4E-BP1 (1, 2, 7, 32). In contrast, rapamycin does not prevent the phosphorylation of eIF4E during the early phases of T cell activation (26), hormone-induced maturation of Xenopus oocytes (25), or the responses of Chinese pancreas sulfonic acid; PAGEL, polyacrylamide gel electrophoresis; VSIEF, vertical slab isoelectric focusing; PABP, poly(A)-binding protein.
hamster ovary cells to insulin (39) or NIH 3T3 cells to serum (27, 40).

Using Xenopus kidney cells in culture, we have utilized rapa-
mycin to examine the signal transduction pathways involved
in the enhanced phosphorylation of eIF4E, its recruitment to
eIF4F complexes, and its association with other initiation fac-
tors. In contrast to the situation with the mammalian cells
described above, rapamycin prevented the stimulus-stimulated
increase in eIF4E phosphorylation in these cells. Although
rapamycin inhibited eIF4F complex formation, this did not
appear to be due to stabilization of the interaction of eIF4E
with 4E-BP1 or 4E-BP2. In addition, we show that serum
stimulated the association between eIF4F and PABP via a
rapamycin-insensitive pathway. These data indicate that mul-
tiple signaling pathways converge at the level of eIF4F complex
formation to influence the interactions between eIF4E, eIF4G,
and PABP during the stimulation of cell growth.

EXPERIMENTAL PROCEDURES

Chemicals and Biochemicals—Materials for tissue culture were from
Life Technologies; [32P]orthophosphate and [35S]methionine were from
ICN; Immobilon polyvinylidene difluoride was from Millipore; m7GTP
Sepharose was from Amersham Pharmacia Biotech; Microcystin was
from Calbiochem; and rapamycin was a kind gift from Dr. J. Kay
(University of Sussex, United Kingdom). Unless otherwise stated, all
other chemicals were from Sigma.

Cell Culture—Xenopus laevis kidney B 3.2 cells (41) were grown in
10-cm dishes at room temperature (20–24 °C), containing 60% Leibo-
vitz L-15 medium, 30% H2O, 10% fetal calf serum supplemented with 2
mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. For
serum starvation, cultures were incubated with 0.5% fetal calf serum
for 24 h prior to treatment with serum (10%), as described in individual
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In Vivo [32P]Orthophosphate Labeling—Cells were serum-starved for
24 h in phosphate-free Leibovitz L-15 medium and incubated with
[32P]orthophosphate (200 μCi/ml) for 2 h prior to treatment with serum
(10%). Extracts were prepared as described below.

Preparation of Cell Extracts—Cultures were placed on ice, and cells
were scraped into 1 ml of Buffer A (80 mM β-glycerophosphate, pH 7.2,
2 mM benzamidine), isolated by centrifugation, the resin was washed three
times with Buffer C (50 mM Mops/KOH, pH 7.4, 100 mM NaCl, 0.5 mM EDTA,
0.5 mM EGTA, 1 mM microcystin, 50 mM β-glycerophosphate, 50 mM
NaF, 2 mM benzamidine, 7 mM 2-mercaptoethanol, 0.1 mM GTP), and
recovered protein was eluted directly into sample buffer for either
SDS-PAGE or VSIEF, prior to analysis (25, 27). In a similar manner, to
directly isolate PABP and associated proteins, cell extracts were sub-
exposed to immunoaffinity chromatography using poly(A)-Sepharose, and the
resin washed in Buffer C prior to elution with SDS-PAGE sample
buffer. Immunoprecipitation of eIF4F and associated proteins from cell
extracts was as described previously, except the resin was washed five
times (with 1 ml of buffer each time) (27, 43).

Antiserum to eIF4F Protein—Rabbit antisera specific to eIF4E (peptide
sequence, TATKSGSTSSTKNRFV) and eIF4A (peptide sequence, DLPTANREnymhirgrgrfgrk) were prepared as described previously (25, 27, 43); rabbit antisera to the C terminus of eIF4G was generated
following expression of eIF4G920–1396 in bacteria, as described (44);
anisutrin specific to the phosphorylated form of eIF4E was generated
in rabbits using the peptide TATKSggs/gptsttknrFV (prepared by Dr.
D. G. Bloomberg, Department of Biochemistry, University of Bristol,
United Kingdom) and affinity-purified on the same peptide. In all cases,
detection was within the linear response of the antisera to the
protein.

RESULTS

Rapamycin Prevents the Serum-induced Phosphorylation of eIF4E in Xenopus Kidney Cells—In several mammalian cell
systems, it has been possible to show that physiological stim-
ulation of protein synthesis and cell growth results in parallel
enhancement of the phosphorylation of eIF4E and eIF4G (23–
27) and the association of these polypeptides to form the eIF4F
complex (45, 46). To examine this relationship further, we have
analyzed the activation of protein synthesis in Xenopus kidney
cells in culture, using a cell line well established for studies of
serum stimulation of both general translation and of specific
translation of mRNAs encoding ribosomal proteins (41). Rela-
tive to control cells, addition of serum resulted in a 20% in-
crease in the rate of total protein synthesis within 30 min,
reaching a 40% increase by 3 h (Fig. 1A). Consistent with
previous studies with this cell line (41), the early activation of
protein synthesis was accompanied by a significant increase in
the proportion of ribosomes in polysomes (data not shown).
This modest effect on the translation rate at early times after
stimulation is similar in extent to that reported for insulin-
stimulated (39, 46–48) and serum-stimulated mammalian
cells in culture (49). To determine the effect of serum on the
phosphorylation of eIF4E, extracts were prepared from control
or stimulated cells, and the level of phosphorylation was di-
rectly monitored by immunoblotting using a phosphospecific
antisera; the recovery of eIF4E was monitored using anti-
serum specific to eIF4E. The effect of serum on the
phosphorylation of eIF4E and eIF4G (23–
27) was measured by immunoblotting using antibodies that
recognize eIF4E irrespective of its phosphoryla-
tion status (24, 25, 27, 42). As shown in Fig. 1B, serum stimulation
resulted in a 40% increase in the phosphorylation of total
eIF4E, which was maximal within 30 min. Because Xenopus
kidney cells contain two distinct forms of eIF4E (25, 50), we have
analyzed the phosphorylation of these independently following
m7GTP-Sepharose affinity purification, VSIEF, and immuno-
 blotting (Fig. 1C). With this system, for each form of the pro-
tein, the more phosphorylated eIF4E variant is the upper im-
munoreactive band (25, 27, 42, 43). These data show that the
addition of serum resulted in a 50% increase in the total phos-
phorylation of eIF4E within 30 min; the proportion of eIF4E in
the phosphorylated form did not increase any further upon
prolonged incubation of the cells (data not shown). In most
experiments, the enhancement of phosphorylation was similar
for both forms of eIF4E, but in some cases the effect on phos-
phorylation of the upper form (resolved by VSIEF) was greater
(see Fig. 2A). The data on overall eIF4E phosphorylation were
confirmed by prelabeling of cells with [32P]orthophosphate:
extracts were prepared from serum-starved control and treated
cells, and eIF4E and associated proteins were isolated by af-
finity chromatography. Serum induced greater than a 50%
crease in the phosphate labeling of total eIF4E, without
affecting the amount of eIF4E protein present in the cell
(Fig. 1D). By virtue of its association with eIF4E, eIF4G was also
recovered following m7GTP-Sepharose chromatography. Fol-

m7GTP-Sepharose, Poly(A)-Sepharose Chromatography, and Immu-
precipitation of eIF4G—For the isolation of eIF4E and associated
proteins, cell extracts of equal protein concentration were subjected
to m7GTP-Sepharose chromatography, the resin was washed three
times with Buffer C (50 mM Mops/KOH, pH 7.4, 100 mM NaCl, 0.5 mM EDTA,
0.5 mM EGTA, 1 mM microcystin, 50 mM β-glycerophosphate, 50 mM
NaF, 2 mM benzamidine, 7 mM 2-mercaptoethanol, 0.1 mM GTP), and
recovered protein was eluted directly into sample buffer for either
SDS-PAGE or VSIEF, prior to analysis (25, 27). In a similar manner, to
directly isolate PABP and associated proteins, cell extracts were sub-
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Fig. 1. Serum stimulation of Xenopus kidney cells enhances the phosphorylation of eIF4E and eIF4F complex formation. A, Xenopus kidney B3.2 cells were serum-starved for 24 h prior to the addition of serum (10%) and 10 μCi/ml [35S]methionine. At the times indicated, cells were harvested, and the labeling of total protein was determined as described under “Experimental Procedures.” The effect of serum on translation is expressed as percentage of change relative to the serum-starved cells. The experiment was carried out three times, each in triplicate, and the error bars indicate the S.E. B, cells were incubated in the absence (lane 1) or presence (lane 2) of 10% (v/v) serum for 30 min prior to the preparation of extracts, as described. Aliquots of extracts containing equal amounts of protein were resolved directly by SDS-PAGE, followed by immunoblotting with antisera specific to total eIF4E (27) (top panel) or with antisera that specifically recognizes the phosphorylated form of eIF4E (bottom panel; see under “Experimental Procedures”). C, aliquots of extracts containing equal amounts of protein were subjected to m’GTP-Sepharose affinity chromatography to isolate total eIF4E. The two forms of eIF4E present in Xenopus cells were resolved by VSIEF and visualized by immunoblotting with antisera specific to total eIF4E. The migration of the more phosphorylated variant of each form of eIF4E resolved by VSIEF is indicated (eIF4E (P), D, serum-starved cells were incubated with 200 μCi/ml [32P]orthophosphate in phosphate-free medium for 2 h prior to further incubation in the absence (lane 1) or presence (lane 2) of serum (10%) for 30 min. Extracts were prepared, and eIF4E and associated proteins were isolated by m’GTP-Sepharose chromatography and resolved by SDS-PAGE. The resulting autoradiograph is presented, with the bottom panel showing the phosphorylation of eIF4F and the top panel showing the labeling of associated eIF4G. E, in parallel cultures to those described in D, extracts were prepared, but without [32P]orthophosphate labeling. Unfractionated cell extracts containing equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antisera to either total eIF2α or with antisera specific to the phosphorylated form of eIF2α (bottom panel). In all cases, these data are representative of those obtained in at least three separate experiments.

Below this step, an 80% increase in the phosphate labeling of eIF4E recovered in association with eIF4E was observed (Fig. 1D). Although we have observed modest increases in the phosphorylation of total cellular eIF4G following serum stimulation (data not shown), the results presented here mainly reflect the enhanced recovery of eIF4E protein associated with eIF4E following serum activation of cells (see Fig. 3A). Conversely, by comparing the signals on Western immunoblots probed with antisera recognizing either total or phosphorylated eIF2α, we have been able to demonstrate that serum stimulation caused a reduction in the phosphorylation of this factor (Fig. 1E).

By using the immunosuppressant rapamycin in conjunction with VSIEF and immunoblotting, we have also begun to examine the intracellular signaling pathways modulating the enhanced phosphorylation of eIF4E and the activation of protein synthesis in response to serum. It is now well established that in a number of mammalian cell types, rapamycin prevents the activation by serum or insulin of the p70s6k signaling pathway (36) and the phosphorylation of eIF4E-binding proteins (4E-BP1 and 4E-BP2) (7, 27, 29–32, 51, 52). These effects are thought to explain the partial abrogation by this agent of the stimulation of translation (27, 40, 49, 53, 54). As described above, serum stimulation resulted in an increase in total eIF4E phosphorylation from 16 to 42% within 30 min of addition (Fig. 2A, top panel, compare lanes 2 and 1; quantified in the bottom panel), a finding confirmed by in vivo labeling of eIF4E with [32P]orthophosphate prior to isolation (middle panel). Preincubation of Xenopus kidney cells with rapamycin strongly inhibited the activation of p70s6k (data not shown), and surprisingly, the serum-stimulated phosphorylation of eIF4E monitored by immunoblotting or phosphate labeling in vivo (Fig. 2A, lane 1 versus lane 2). Under these conditions, rapamycin had no obvious effect on the phosphate labeling of total eIF4G (data not shown). The effect of rapamycin on the serum-induced activation of protein synthesis was monitored by pulse labeling of cells with [35S]methionine prior to harvest. Fig. 2B shows that rapamycin abrogated the early serum-induced increase in the rate of protein synthesis. Similar effects on translation rates and eIF4E phosphorylation were observed with wortmannin and LY294002 (data not shown). These data are in
incubated in the absence or presence of rapamycin (50 nM) for 1 h, prior to the addition of serum (10%). Following a 30-min incubation, extracts were prepared and adjusted to equal protein concentration, and total eIF4E was isolated by m7GTP-Sepharose chromatography prior to analysis by VSIEF and immunoblotting with anti-eIF4E antiserum. In a parallel culture, cells were prelabeled with [32P]orthophosphate as described in Fig. 1.

Serum Stimulation Promotes the Co-isolation of Poly(A)-binding Protein with the eIF4F Complex—mRNA is posttranslationally modified with a cap structure at the 5'-end and a poly(A) tail at the 3'-end, both of which, individually and in concert, play essential roles in the regulation of translation (reviewed in Refs. 8, 12, 21, 55, and 56). Cap structure-dependent initiation of translation involves the assembly of the eIF4F complex (eIF4E/eIF4A/eIF4G) at the 5'-end of mRNA. Recent studies in the yeast S. cerevisiae have indicated a further interaction between eIF4G and poly(A)-binding protein (Pab1p), which results in circularization of the mRNA (17–20). Evidence has also been presented for interactions between poly(A) or poly(A)-binding protein and polypeptides of the eIF4F and eIF (iso)4F complexes in the wheat germ system (57, 58). Furthermore, synergistic effects of the cap and poly(A) tail on translational efficiency have been demonstrated in mammalian cells as well as in yeast and plant systems (3, 21, 55).

Contrary to investigations with mammalian cells, in which the enhancement of phosphorylation of eIF4E has been found to be resistant to attenuation by rapamycin (25–27, 39, 40, 45, 49).

FIG. 2. Rapamycin abrogates the serum-induced phosphorylation of eIF4E in Xenopus kidney cells. A, serum-starved B3.2 cells were incubated in the absence or presence of rapamycin (50 nM) for 1 h, prior to the addition of serum (10%). Following a 30-min incubation, extracts were prepared and adjusted to equal protein concentration, and total eIF4E was isolated by m7GTP-Sepharose chromatography prior to analysis by VSIEF and immunoblotting with anti-eIF4E antiserum. In a parallel culture, cells were prelabeled with [32P]orthophosphate as described in Fig. 1D prior to incubation in the absence or presence of serum (10%) and rapamycin (50 nM). Total eIF4E was isolated as described and resolved by SDS-PAGE. The resulting autoradiograph is presented (middle panel). Using densitometric scanning, the proportion of total eIF4E in the phosphorylated form was quantified from analysis of cell extracts by VSIEF and immunoblotting (bottom panel). Lane 1, control cells; lane 2, serum-stimulated cells; lane 3, serum-stimulated in the presence of rapamycin. The experiment was carried out four times, and the error bars indicate S.E. B, cells were serum-starved for 24 h prior to the addition of serum (10%) and 10 μCi/ml [35S]methionine for 30 min. The level of total protein synthesis was determined as described under “Experimental Procedures,” and the effect of serum and rapamycin on early translation rates is expressed as percentage of change relative to the serum-starved cells. These data are representative of those obtained in at least three separate experiments.

To address this, we have analyzed eIF4E/eIF4G complex formation and the association of PABP with eIF4G in serum-stimulated Xenopus kidney cells. Extracts were prepared from cells, and eIF4E and associated proteins were isolated by m7GTP-Sepharose chromatography (in the presence of GTP to reduce nonspecific interactions between proteins and resin) and visualized by immunoblotting. Fig. 3A, lane 1, shows that even in the starved state, there is some association of eIF4E with eIF4A, eIF4G, and PABP. The specificity of this isolation procedure for eIF4E and associated proteins was demonstrated by the finding that under these assay conditions, none of these proteins were retained by Sepharose lacking the m7GTP moiety (Fig. 3A, lane 4) and inclusion of m7GTP in the extraction buffer prevents recovery of eIF4E and associated proteins (data not shown). This association of eIF4G, eIF4A, and PABP with eIF4E was enhanced by at least 2-fold in extracts derived from cells treated with serum for 30 min (Fig. 3A, lane 2; quantified in Fig. 3B), and was unaffected by ribonuclease treatment of the extract prior to affinity chromatography (data not shown). Preincubation of the cells with rapamycin (Fig. 3A, lane 3) prevented the serum-induced enhancement of association between eIF4E and eIF4G, indicating a role for the p70sk signaling pathway in modulating eIF4F complex formation. The recovery of eIF4A and PABP on the m7GTP-Sepharose affinity matrix essentially paralleled that of eIF4G. To examine more closely the association between eIF4G and PABP, eIF4G was immunoprecipitated from extracts, and the level of associated eIF4E and PABP was visualized by immunoblotting (Fig. 3C). Extracts from serum-stimulated cells showed approximately a
Fig. 3. Serum promotes the co-isolation of poly(A)-binding protein with eIF4F. A, cell extracts were prepared as in Fig. 2A and adjusted to equal protein concentration, and eIF4E and associated proteins were isolated by m’GTP-Sepharose chromatography (lanes 1–3) or Sepharose alone (lane 4). Recovered proteins were resolved by SDS-PAGE and visualized by immunoblotting using the specific antiserum indicated. The data
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2-fold increase in recovery of both eIF4E and PABP associated with eIF4G (Fig. 3C, compare lanes 1 and 2); in the absence of antiserum, little eIF4G, PABP, and eIF4E was recovered with the protein A-Sepharose (lane 4). As the antiserum to eIF4G did not react with purified eIF4E or PABP (data not shown), these data suggest that serum treatment of cells promoted the interaction of eIF4G with eIF4E and PABP. In agreement with the data where eIF4G was recovered via eIF4E (Fig. 3D), pre-treatment of cells with rapamycin (lane 3) andwortmannin or LY294002 (data not shown) abrogated the serum-stimulated interaction of eIF4E with eIF4G. However, in contrast, the enhanced co-isolation of PABP in eIF4G immunoprecipitates was still apparent in extracts from cells stimulated in the presence of either agent (Fig. 3C; quantified in bottom panel), indicating that serum stimulates the interaction between eIF4G and PABP by a distinct mechanism that is independent of both the mTOR signaling pathway and the association of eIF4G with eIF4E. To confirm these results, we also examined the recovery of associated proteins with PABP selected from extracts using the poly(A)-Sepharose affinity matrix (Fig. 3D). Again, these data indicated that serum stimulated the co-isolation of eIF4G (and associated eIF4E) with PABP by approximately 2-fold (Fig. 3D, lane 1 versus lane 2), whereas eIF4G, PABP, and eIF4E do not associate with the Sepharose resin lacking the selection moiety (Fig. 3D, lane 4). The serum-stimulated increase in the association between eIF4G and PABP was largely unaffected by rapamycin (Fig. 3D, lane 3), whereas the association between eIF4G and eIF4E was reduced by inhibition of mTOR signaling (quantified in Fig. 3D, bottom panel). The quantitative differences in recovery of PABP associated with eIF4G using the different resins may reflect, in part, the enhanced stability of the eIF4F complex when recovered via eIF4E (28, 59, 60) or that the association of PABP with eIF4G is not as stable as that for eIF4E with eIF4G.

Increased eIF4F Complex Formation Is Not Mediated by Dissociation of 4E-BP1 from eIF4E—In addition to phosphorylation, the ability of eIF4E to participate in the initiation process can also be modulated by its availability, mediated by its interaction with specific binding proteins, 4E-BP1 and 4E-BP2, identified as downstream signaling targets of rapamycin-sensitive pathways (7, 37, 61). Following our finding that serum enhanced the association of eIF4E with eIF4G in Xenopus kidney cells (Fig. 3), we examined the effects of serum on the phosphorylation of 4E-BP1 and 2E and their association with eIF4E, using separate independent sources of anti-4E-BP antisera. In several cell types, the phosphorylation of either 4E-binding protein has been shown to result in a characteristic mobility shift on SDS-PAGE analysis and their total release from eIF4E (27, 29, 30, 40, 51, 61–63). We were therefore surprised to find that addition of serum to starved Xenopus kidney cells did not appear to influence the distribution of either total 4E-BP1 (Fig. 4A, top panel) or total 4E-BP2 (bottom panel) between phosphorylated and non-phosphorylated forms. This was further illustrated by the lack of effect of rapamycin on the mobility of these proteins on SDS-PAGE (Fig. 4A, lane 3). To further confirm this finding, the migration on SDS-PAGE of 4E-BP1 isolated from starved (Fig. 4B, lane 1) or serum-stimulated (lane 2) cells was compared with that of 4E-BP1 isolated from NIH 3T3 cells following incubation in the absence (lane 3) or presence (lane 4) of serum, as described previously (27). These data show that although serum induced a characteristic mobility shift of 4E-BP1 to the more phosphorylated form (γ) in the NIH3T3 cell system (27) (Fig. 4B, lane 4 versus lane 3), there was no such mobility shift in the Xenopus cell system (lane 2 versus lane 1). Similar results were found when antiserum specific to 4E-BP2 was used (data not shown). In addition, although [32P]phosphate labeling of 4E-BP1 and 4E-BP2 was increased by serum in vivo (Fig. 4B, bottom panel, lane 2 versus lane 1), again there was no evidence for a phosphorylation-induced mobility shift of these proteins, and the increased phosphorylation of 4E-BP1 and BP2 was unaffected by rapamycin, suggesting phosphorylation on rapamycin-in-sensitive sites (Ref. 61 and data not shown). Fig. 4C shows that the addition of serum had little or no influence on the association of 4E-BP1 with eIF4E, although the amount of associated 4E-BP-2 did decrease by up to 30% in some experiments (compare lanes 2 and 1; quantified in panel D). In general, the latter effect was more variable between experiments and was often accompanied by decreased recovery of 4E-BP-2 in the cell extracts following serum stimulation (data not shown). The reasons for this are unclear. Direct isolation of 4E-BP1 by immunoprecipitation confirmed that serum had little or no effect on the association of this protein with eIF4E (data not shown). Preincubation of cells with rapamycin (Fig. 4C, lane 3), which is known to increase the level of the eIF4E/BP complex above basal levels in mammalian cells (7, 29–32), did not significantly influence the recovery of 4E-BP1 associated with total eIF4E (quantified in Fig. 4D). However, although rapamycin promoted the interaction between eIF4E and 4E-BP-2, inhibition of mTOR signaling did not increase the level of this complex above basal levels.

These data suggest that in serum-stimulated Xenopus kidney cells, the increase in association of eIF4G and PABP with eIF4E does not correlate well with control at the level of dissociation of 4E-binding proteins from eIF4E. These data are distinct from those obtained with mammalian cells, but the reasons for such a difference are unclear. At this time, we cannot discount some regulation at the level of 4E-BP-2, but this does not appear to involve its phosphorylation. Also, there is the possibility that as yet unknown eIF4E interacting proteins may play a role in the effects we observe. Another binding protein, 4E-BP3, has been identified (75), but our attempts to analyze the interaction between eIF4E and this protein have been inconclusive as the rabbit antiserum reacted poorly with the Xenopus protein (data not shown). One possibility is that the availability of eIF4E may not be limiting in these cells; attempts to quantify the relative levels of eIF4E and 4E-BPs have proven difficult due to the existence of multiple forms of these proteins in Xenopus cells. On the other hand, studies with recombinant proteins have indicated that both forms of
FIG. 4. Serum-stimulated eIF4F complex formation occurs without concomitant dissociation of 4E-BP1 from eIF4E. A, serum-starved B3.2 cells were incubated in the absence (lanes 1 and 2) or presence (lane 3) of rapamycin (50 nM) for 1 h prior to the addition of serum (10%) for 30 min (lanes 2 and 3). Extracts were prepared and adjusted to equal protein concentration, and 4E-BPs were isolated as described under “Experimental Procedures.” Total 4E-BP1 (top panel) and 4E-BP2 (bottom panel) was visualized by SDS-PAGE and immunoblotting; for 4E-BP1, the antisera (from A. A. Thomas, Utrecht, The Netherlands) was specific to this protein, whereas the antisera to 4E-BP-2 (from N. Sonenberg, Montreal, Canada), also cross-reacted with 4E-BP-1. B, top panel, extracts prepared from Xenopus kidney cells as above (lanes 1 and 2) or from serum-starved (lane 3) or stimulated (lane 4) NIH 3T3 cells, as described (27), were resolved by SDS-PAGE, and 4E-BP1 was visualized by immunoblotting as in A. In addition, cells were incubated with [32P]orthophosphate as described in Fig. 1, prior to incubation in the absence (lane 1) or presence (lane 2) of serum. Extracts were prepared and adjusted to equal protein concentration, 4E-BPs were isolated as described under “Experimental Procedures,” and the resulting autoradiograph is shown. The identity of 4E-BP1 and 4E-BP2 was confirmed by immunoblotting (data not shown). C, extracts were prepared as in A, but eIF4E and associated proteins were isolated by mGTP-Sepharose chromatography prior to resolution by SDS-PAGE. Recovered proteins were visualized by immunoblotting using the specific antisera described in A. D, the amount of 4E-BP recovered in association with eIF4E (C) was quantified by scanning densitometry and normalized for recovery of eIF4E, and it is expressed as a percentage of control. The experiment was carried out six times, and the error bars indicate the S.E. eIF4E found in Xenopus cells interact to the same extent with 4E-BP1 or 4E-BP2, suggesting that inhibition of phosphorylation of eIF4E does not involve the selective interaction of one variant of eIF4E with 4E-BPs (data not shown). As a consequence, the mechanism by which rapamycin inhibits eIF4F complex formation in the Xenopus kidney cells remains unclear. It is clear, however, from work using mammalian cells that the phosphorylation of eIF4E and 4E-BPs can be regulated independently and that each may play a distinct role in translation initiation in vivo (27, 64).

DISCUSSION

The stimulation of protein synthesis plays a central role in the activation of cell growth (1, 2, 7). Studies using rapamycin as a pharmacological probe have implicated the signaling pathway governed by mTOR in the parallel activation of p70S6K and the increase in cap structure-dependent translation in mitogen-activated cells (7, 31, 37, 38, 40, 51, 52, 62, 65). Rapamycin attenuates the stimulation of cap structure-dependent translation in response to serum re-feeding, such that the overall protein synthesis rate is decreased by 15–40% (27, 40, 49, 53, 54, 66, 67). Current models suggest that rapamycin stabilizes the eIF4F/4E-BP interaction and thus prevents the mitogen-induced liberation of eIF4E and consequent increase in the availability of eIF4F complexes (1, 27, 32). However, even in mammalian systems, this may be a simplistic view, as studies have shown a poor temporal correlation between the inhibition of phosphorylation of 4E-BP1, the inhibition of translation, and eIF4F complex formation (27, 40, 68). We now show that Xenopus kidney cells in culture do not seem to exhibit regulation of eIF4E by 4E-BP-1.

Here we have characterized the effect of rapamycin on the phosphorylation of eIF4E and its interaction with eIF4G following serum stimulation in the Xenopus kidney cell line B3.2. In these cells, the activation of protein synthesis is associated with enhanced phosphorylation of eIF4E and eIF4G and decreased phosphorylation of eIF2a (Fig. 1). In mammalian tumor cells, decreased phosphorylation of eIF2a, with concomitant enhancement of eIF2B function, has been reported to play a role in serum stimulation of translation (69). However, to date, our attempts to investigate changes in the activity of eIF2B in extracts from Xenopus kidney cells have been unsuccessful. Our data on the phosphorylation of eIF4E and eIF4G are consistent with earlier studies using Xenopus oocytes and primary T cells (25, 26), in which the recovery of phosphorylated eIF4G associated with eIF4E was increased in response to hormones or the activation of cell growth. As phosphorylation of eIF4E (28) and eIF4G (59) has been shown to increase the ability of these proteins to interact with the cap structure, and
the association of eIF4E with eIF4G enhances the binding of eIF4E to the cap structure (60), these data indicate that eIF4F phosphorylation and complex formation are important in the regulation of translation in these cells.

Unlike several other systems examined to date (25–27, 40), inhibition of mTOR or PI3-kinase signaling blocked the enhancement of protein synthesis and the phosphorylation of both forms of eIF4E in serum-stimulated Xenopus kidney cells (Fig. 2). A similar observation has been reported for IL-3-starved, insulin receptor substrate-1-transfected cells treated with insulin (67). Together, these data indicate that eIF4F phosphorylation can occur downstream of mTOR in some cell types and may stabilize the eIF4F complex (23–28, 59). One explanation for this is that activation of mTOR causes the dissociation of 4E-BPs from eIF4E, making the latter available as a kinase substrate. Indeed, the association of eIF4E with 4E-BP1 abrogates its phosphorylation by protein kinase C in vitro (70). However, this is an unlikely explanation for the data presented here, because we find no evidence for the enhanced phosphorylation of 4E-BP1 or BP2 and only small effects on the dissociation of the latter from eIF4E following serum addition to Xenopus kidney cells (Fig. 4). These data are in agreement with studies in Chinese hamster ovary cells, in which it was found that eIF4E associated with 4E-BP1 was phosphorylated to the same extent as total eIF4E (39).

During these studies, we have demonstrated an increase in the co-isolation of eIF4F with PABP following the activation of protein synthesis (Fig. 3). PABP is found in all eukaryotes and mediates the stimulatory effects of the poly(A) tail on translation (reviewed in Refs. 12, 21, 55, and 56). In S. cerevisiae, Pab1p has been shown to interact directly (via its RNA recognition motif 2) (71) with eIF4F through a specific sequence N-terminal to the eIF4E binding site (17, 20). This association is believed to mediate the circularization of mRNA and promote the poly(A) and PABP-dependent stimulation of mRNA translation (12, 21, 55, 56, 72). The synergistic effects of the poly(A) tail and the mRNA cap structure on translational efficiency and its hormonal stimulation have been demonstrated in mammalian cells (3). Our data show that in conjunction with the phosphorylation of eIF4E and eIF4G, there is a reproducible increase in the recovery of PABP and eIF4F associated with eIF4F following serum stimulation of Xenopus kidney cells. The enhanced association of PABP with eIF4F was confirmed by co-precipitation with antisera specific to eIF4F and following isolation of PABP with poly(A)-Sepharose (Fig. 3C). Whereas inhibition of mTOR signaling prevented the activation of p70^S6K, the phosphorylation of eIF4E, the association between eIF4E and eIF4G, and the activation of protein synthesis, the binding of PABP to eIF4E was less sensitive to this inhibition. To our knowledge, this represents the first demonstration of physiological regulation of the interaction between PABP and proteins of the eIF4F complex. At this time, we do not know whether the interaction between eIF4F and PABP is direct or indirect. However, work completed after our study has now demonstrated that PABP can interact directly with eIF4F via a newly discovered N-terminal motif in mammalian systems, although the strength of this interaction remains to be determined.2 3 Our previous attempts to delineate the site of interaction between eIF4F and PABP with bacterially expressed proteins were unsuccessful, as the widely used eIF4F clone (10, 11) lacks the N-terminal motif required for this interaction (73).

The data presented here indicate that more than one signal-

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