Molecular Cross-talk between MEK1/2 and mTOR Signaling during Recovery of 293 Cells from Hypertonic Stress*

Received for publication, May 4, 2004, and in revised form, August 3, 2004 Published, JBC Papers in Press, August 3, 2004, DOI 10.1074/jbc.M404945200

Susanne Naegle and Simon J. Morley‡

From the Biochemistry Laboratory, School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, United Kingdom

To investigate the role for initiation factor phosphorylation in de novo translation, we have studied the recovery of human kidney cells from hypertonic stress. Previously, we have demonstrated that hypertonic shock causes a rapid inhibition of protein synthesis, the disaggregation of polysomes, the dephosphorylation of eukaryotic translation initiation factor (eIF)-4E, 4E-BP1, and ribosomal protein S6, and increased association of 4E-BP1 with eIF4E. The return of cells to isotonic medium promotes a transient activation of Erk1/2 and the phosphorylation of initiation factors, promoting an increase in protein synthesis that is independent of a requirement for eIF4E phosphorylation. As de novo translation is associated with the phosphorylation of 4E-BP1, we have investigated the role of the signaling pathways required for this event by the use of cell-permeable inhibitors. Surprisingly, although rapamycin, RAD001, wortmannin, and LY294002 inhibited the phosphorylation of 4E-BP1 and its release from eIF4E, they did not prevent the recovery of translation rates. These data suggest that only a small proportion of the available eIF4F complex is required for maximal translation rates under these conditions. Similarly, prevention of Erk1/2 activity alone with low concentrations of PD184352 did not impinge upon de novo translation until later times of recovery from salt shock. However, U0126, which prevented the phosphorylation of Erk1/2, ribosomal protein S6, TSC2, and 4E-BP1, attenuated de novo protein synthesis in recovering cells. These results indicate that the phosphorylation of 4E-BP1 is mediated by both phosphatidylinositol 3-kinase-dependent rapamycin-sensitive and Erk1/2-dependent signaling pathways and that activation of either pathway in isolation is sufficient to promote de novo translation.

Mammalian mRNAs have a unique cap structure at their 5′ terminus, which has a strong stimulatory effect on the translation of mRNA. The cap structure facilitates the recruitment of eukaryotic translation initiation factors (eIFs) to the mRNA to allow ribosome binding and initiation at the correct start site (reviewed in Refs. 1–3). eIF4E, a protein whose three-dimensional structure resembles a cupped hand, specifically interacts directly with the cap via its concave surface (4, 5). It also forms a complex on its convex surface with the scaffold protein, eIF4G (6), which recruits other initiation factors, such as eIF3, eIF4A, and poly(A)-binding protein (PABP) to the 5′ end of the mRNA, allowing for efficient unwinding of secondary structure in the 5′-untranslated region and the functional circularization of mRNA believed to promote efficient translation (reviewed in Refs. 1–3 and 7–9).

Human eIF4E undergoes regulated phosphorylation on Ser-209 (10, 11–13) mediated via multiple signaling pathways (1–3, 14, 15). Two protein kinases, Mnk1 and Mnk2, which act at the convergence point of extracellular-signal regulated kinase and stress-activated p38 mitogen-activated protein kinase (p38MAPK)-phosphorylate eIF4E at the physiological site in vitro and in vivo (reviewed in Refs. 1–3 and 7–9). Mnk1/Mnk2 interact directly with eIF4G (13, 16) bringing them in close proximity to eIF4E within the eIF4F complex. In addition, eIF4E binds a family of regulatory proteins, 4E-binding proteins (4E-BPs), which compete with eIF4G for sites on the convex surface of eIF4E (5, 17) and inhibit formation of initiation complexes. Association of 4E-BPs with eIF4E is modulated by phosphorylation events controlled via the target of rapamycin (mTOR) signaling pathway, integrating signals from mitogens, nutrients, and energy availability with the translational apparatus (1–3, 7–9, 18, 19). Current models suggest that hypophosphorylated 4E-BP1 binds to eIF4E to inhibit cap-dependent translation, a process readily reversed following 4E-BP1 phosphorylation. The dissociation of hyperphosphorylated 4E-BP1 from eIF4E permits the binding of eIF4G to eIF4E and the initiation of protein synthesis.

Recent work has suggested a role for the hamartin-tuberin complex (TSC1-TSC2) in the regulation of mTOR activity (19–22). TSC2, a GTPase-activating protein, has activity toward the Ras family small GTPase, Rheb (Ras homolog enriched in brain), which promotes mTOR signaling by an ill-defined mechanism. TSC1/2 antagonizes the mTOR signaling pathway by stimulating the hydrolysis of GTP associated with Rheb (23) thereby reducing its activity. Thus, TSC1/2 and Rheb have pivotal roles in mediating growth factors, nutrient, and energy sensing signals to mTOR-dependent targets (19, 21, 23–25). PI3-kinase-dependent signaling serves to inhibit the TSC1-TSC2 complex through direct Akt-mediated phosphorylation of tuberin at Ser-939 and Thr-1462, relieving the inhibitory activity of the complex on Rheb. In addition, it has been demonstrated recently that the protein kinase C-induced extracellular-signal regulated kinase pathway can also modulate the phosphorylation of tuberin at Thr-1462 (19, 23, 26). This signaling to TSC1/TSC2 is sensitive to both protein kinase C inhibitors and the MEK1/2 inhibitor, U0126, with both the PI3-K-dependent and protein kinase C-dependent pathways sensitive to rapamycin (18, 22), which causes a rapid dephos-
Translation during Recovery from Hypertonic Shock

Exposure of mammalian cells to hypertonic stress is a very effective means of inducing a transient fall in protein synthesis, which is rapidly reversible upon restoration of isotonic medium (27, 28). Because polysomes become completely disaggregated during hypertonic stress, the resumption of translation during recovery represents de novo recruitment of mRNA. We have used this system as a tool to study the function of the eIF4F complex during de novo recruitment of mRNA in intact cells and to demonstrate that eIF4E phosphorylation is not required for de novo protein synthesis (28). Here we have employed hypertonic stress and cell-permeable inhibitors to investigate the signaling pathways involved in de novo translation. Our findings indicate that although inhibition of mTOR activity during recovery prevented the hyperphosphorylation of 4E-BP1 and its release from eIF4E, it did not prevent the restoration of translation rates. Furthermore, prevention of the transient activation of Erk1/2 with PD184352 did not impinge upon translation rates during the recovery phase. However, U0126 (which prevented Erk1/2 activity, S6 phosphorylation, TSC2 phosphorylation, and the phosphorylation of 4E-BP1) attenuated the restoration of translation rates in recovering cells. Our results, therefore, provide evidence that the phosphorylation of 4E-BP1 following hypertonic shock is mediated by both mTOR-dependent, and Erk1/2-dependent signaling pathways and that inhibition of both pathways is required to prevent de novo translation.

EXPERIMENTAL PROCEDURES

Chemicals and Biochemicals—Materials for tissue culture were from Invitrogen and fetal calf serum was from Labtech International. [3H]methionine was from ICN, and antisera for total ERK1/2 and 4E-BP1 were from Cell Signaling Technology, as were the following phospho-specific antisera: eIF4E (Ser-209), eIF4G (Ser-1108), p38 MAP kinase (Thr-180/Tyr182), 4E-BP1 (Ser-65), 4E-BP1 (Thr-37/Thr-46), Akt (Ser-473), TSC2 (Thr-1462), Erk1/2 (Thr-202/Tyr-204), MEK1/2 (Ser-176/Ser-211) and ribosomal protein S6 (Ser-240/Ser-244). Antiserum to p70S6K (C-18) was from Santa Cruz Biotechnology and polyvinylidine difluoride and mGTP-Sepharose were from Amersham Biosciences. LY294002 and U0126 were from Alexis Corp., rapamycin was gift from Dr. J. Kay (Sussex, UK), wortmannin was from Sigma, PD184352 was from Pfizer, Inc., and CGP57380 and RAD001 were from Novartis Pharma AG. Unless otherwise stated, all other chemicals were from Sigma (UK).

Cell Culture—Human 293 kidney cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Actively growing cells were incubated in complete growth medium for 60 min in the absence or presence of 150 mM additional NaCl, and recovery was in complete medium as described (28). When used, drugs or vehicle alone were included for the final 20 min of the salt shock treatment and were present in all subsequent incubations.

Protein Synthesis Measurements—Cells were incubated with [3H]methionine in complete growth medium at the specific activity and for the times indicated in the individual figure legends. Cells were harvested and the incorporation of methionine into protein determined by trichloroacetic acid (TCA) precipitation using aliquots of extract containing equal amounts of protein as described previously (28–32).

Preparation of Cell Extracts—Following treatment, cells were scraped into phosphate-buffered saline containing 40 mM β-glycerophosphate and 2 mM benzamidine and isolated in a cooled microcentrifuge. Cell pellets were resuspended in 200 µl of ice-cold Buffer A (20 mM MOPS-KOH, pH 7.2, 10% (v/v) glycerol, 20 mM sodium fluoride, 1 µM microcystin, 75 mM KCl, 2 mM MgCl2, 2 mM benzamidine, 0.1 mM GTP, 2 mM Na2VO4, complete protease inhibitor mix (- EDTA (Roche Applied Science)) and lysed by the addition of 0.5% (by volume) each of IgGel and deoxycholate. Cell debris and nuclei were removed by centrifugation in a microcentrifuge for 5 min at 4 °C, and the resultant supernatants were frozen in liquid N2.

SDS-PAGE, Iso-electric Focusing, and Immunoblotting—Samples containing equal amounts of protein were resolved by SDS-PAGE and processed as described previously (28–32). Antiserum specific for the C-terminal domain of eIF4G1 and those specific to eIF4E, PABP, and eIF4A were visualized with alkaline phosphatase-conjugated secondary antibodies, whereas the determinations using commercial phosphospecific antisera employed ECL for detection. In all cases, care was taken to ensure that detection was within the linear range of the response.

RESULTS

Recruitment of Protein Synthesis Rates following Salt Stress Is Associated with the Activation of Multiple Signaling Pathways—Exposure of mammalian cells to hypertonic stress induces a transient fall in protein synthesis that is rapidly reversible upon restoration of isotonic medium (Fig. 1A). Whereas the integrity of eIF4F is critical for the re-programming of translation during recovery from hypertonic stress (27), our previous data have shown that increased phosphorylation of eIF4E at Ser-209 is not obligatory (28). In this work, we have employed hypertonic stress and cell-permeable inhibitors to investigate the role of individual signaling pathways for de novo translation in 293 cells. Fig. 2B (lane 2 versus lane 1) shows that salt shock induces a dephosphorylation and the inactivation of a number of signaling intermediates that impinge upon the translational machinery, including components of the mTOR (Akt, TSC2, ribosomal protein S6, and 4E-BP1) and the Erk pathways (MEK1/2 and Erk1/2). Under these conditions, dephosphorylation of a key phosphorylation site in 4E-BP1 (Ser-65) was complete within 15 min, whereas that of eIF4F (Ser-1108), Akt (Ser-473), S6 (Thr-421/Ser-424), and eIF4E (Ser-209, (28)) occurred at a slower rate and were maximal at 60 min (data not shown and lane 2 versus lane 1). There was no change in the overall levels of initiation factors recovered in the extracts after this time and (in agreement with published data) little change in the phosphorylation status of eIF2α ((28) and data not shown). In contrast, salt shock promotes an increase in the phosphorylation (and activation) of p38 MAP kinase (Fig. 1B, lane 2 versus lane 1). Concomitant with a recovery of translation rates (Fig. 1A), returning cells to isotonic medium promotes a transient phosphorylation (and activation) of MEK1/2, with Erk1/2 phosphorylation being more persistent (Fig. 1B, quantified in the lower panel). MEK1/2 phosphorylation was evident within 10 min and decreased to basal levels after 20 min of recovery (lanes 2–5). In contrast, release from salt stress promotes a more sustained phosphorylation of Akt (Fig. 1B, lower panel), S6, and 4E-BP1 (upper panel, lanes 3–8), along with a gradual inactivation of
Translation during Recovery from Hypertonic Shock

4E-BP1 Phosphorylation Is Not Required for the Recovery of Translation Rates following Hypertonic Shock—Association of 4E-BPs with eIF4E is modulated by phosphorylation events controlled via the mTOR signaling pathway (1–3, 19–22), integrating signals from mitogens, nutrients, and energy availability with the translational apparatus. Current models suggest that hypophosphorylated 4E-BP1 binds to eIF4E to inhibit cap-dependent translation, a process readily reversed following 4E-BP1 phosphorylation (1, 2). To investigate the role of the PI3-kinase and mTOR pathways in recovery from salt shock, cells were incubated with wortmannin or rapamycin, respectively, during the last 20 min of salt treatment and throughout the recovery period and extracts prepared. SDS-PAGE analysis (Fig. 2A) shows that as expected wortmannin (lanes 6–8 versus lanes 3–5) but not rapamycin (lanes 9–11 versus lanes 3–5) prevents the phosphorylation of Akt on recovery in isotonic medium. However, whereas both inhibitors abrogate the phosphorylation of ribosomal protein S6 and 4E-BP1 (lanes 6–11 versus lanes 3–5), consistent with a role for PI3-kinase and mTOR in signaling to these target proteins (18, 23, 35), neither impinge upon the phosphorylation of Erk1/2 (data not shown).

In agreement with our previous findings (28), isolation of eIF4E and associated factors with m7GTP-Sepharose (lane 2 versus lane 1) shows that hypertonic shock causes increased binding of 4E-BP1 to eIF4E. In the presence of wortmannin (lanes 6–8 versus lanes 3–5) or rapamycin (lanes 9–11), 4E-BP1 remains associated with eIF4E during recovery. In addition, Fig. 2B (lane 3, quantified in the right panel) shows that (relative to the salt-shocked cells (lane 2)) the co-isolation of eIF4G with eIF4E is increased at the 30 min recovery point, co-incident with the loss of 4E-BP1 binding (Fig. 2A, lane 4).

Using a more detailed time course (Fig. 1C, quantified in the lower panel), we have determined that the phosphorylation of eIF4G and 4E-BP1 is detectable within 5–10 min of returning cells to isotonic medium. The phosphorylation of eIF4E is evident at 10 min of recovery at a time when translation rates are increased (Fig. 1A).

p38MAPK (lanes 2–8). Using a more detailed time course (Fig. 1C, quantified in the lower panel), we have determined that the phosphorylation of eIF4G and 4E-BP1 is detectable within 5–10 min of returning cells to isotonic medium. The phosphorylation of eIF4E is evident at 10 min of recovery at a time when translation rates are increased (Fig. 1A).

4E-BP1 Phosphorylation Is Not Required for the Recovery of Translation Rates following Hypertonic Shock—Association of 4E-BPs with eIF4E is modulated by phosphorylation events controlled via the mTOR signaling pathway (1–3, 19–22), integrating signals from mitogens, nutrients, and energy availability with the translational apparatus. Current models suggest that hypophosphorylated 4E-BP1 binds to eIF4E to inhibit cap-dependent translation, a process readily reversed following 4E-BP1 phosphorylation (1, 2). To investigate the role of the PI3-kinase and mTOR pathways in recovery from salt shock, cells were incubated with wortmannin or rapamycin, respectively, during the last 20 min of salt treatment and throughout the recovery period and extracts prepared. SDS-PAGE analysis (Fig. 2A) shows that as expected wortmannin (lanes 6–8 versus lanes 3–5) but not rapamycin (lanes 9–11 versus lanes 3–5) prevents the phosphorylation of Akt on recovery in isotonic medium. However, whereas both inhibitors abrogate the phosphorylation of ribosomal protein S6 and 4E-BP1 (lanes 6–11 versus lanes 3–5), consistent with a role for PI3-kinase and mTOR in signaling to these target proteins (18, 23, 35), neither impinge upon the phosphorylation of Erk1/2 (data not shown).

In agreement with our previous findings (28), isolation of eIF4E and associated factors with m7GTP-Sepharose (lane 2 versus lane 1) shows that hypertonic shock causes increased binding of 4E-BP1 to eIF4E. In the presence of wortmannin (lanes 6–8 versus lanes 3–5) or rapamycin (lanes 9–11), 4E-BP1 remains associated with eIF4E during recovery. In addition, Fig. 2B (lane 3, quantified in the right panel) shows that (relative to the salt-shocked cells (lane 2)) the co-isolation of eIF4G with eIF4E is increased at the 30 min recovery point, co-incident with the loss of 4E-BP1 binding (Fig. 2A, lane 4).

Using a more detailed time course (Fig. 1C, quantified in the lower panel), we have determined that the phosphorylation of eIF4G and 4E-BP1 is detectable within 5–10 min of returning cells to isotonic medium. The phosphorylation of eIF4E is evident at 10 min of recovery at a time when translation rates are increased (Fig. 1A).

p38MAPK (lanes 2–8). Using a more detailed time course (Fig. 1C, quantified in the lower panel), we have determined that the phosphorylation of eIF4G and 4E-BP1 is detectable within 5–10 min of returning cells to isotonic medium. The phosphorylation of eIF4E is evident at 10 min of recovery at a time when translation rates are increased (Fig. 1A).
versus lane 2). However, in the presence of rapamycin (Fig. 2B, lane 4) or wortmannin (data not shown), the binding of eIF4G to eIF4E was quantified in the right panel is strongly reduced, indicative of a decrease in eIF4F complex levels. Rapamycin treatment also reduces the association of PABP (lanes 1–4) and eIF4A (data not shown) with the eIF4F complex, albeit to a lesser extent than observed with eIF4G. In all experiments, rapamycin and RAD001 (36, 37) were more effective than wortmannin at preventing eIF4F complex assembly (data not shown). However, despite increased binding of 4E-BP1 and decreased binding of eIF4G to eIF4E, pulse-labeling with [35S]methionine shows that general translation rates during recovery are not significantly different to those observed in control cells (Fig. 2C, lane 10 and 11 versus lanes 4 and 5). Furthermore, neither inhibitor affected the rate of recruitment of ribosomes into polysomes at early times of recovery (data not shown). Therefore, these data suggest that the activity of the mTOR pathway is not absolutely required for de novo translation or that other, rapamycin-insensitive pathways can influence translation rates under these conditions.

Recovery of Translation Rates Occurs in the Absence of Both eIF4E and 4E-BP1 Phosphorylation—Previously we have shown that inhibition of eIF4E phosphorylation with the Mnk1 inhibitor, CGP57380, did not prevent the recovery of translation rates following salt shock (28). To address whether the simultaneous inhibition of 4E-BP1 and eIF4E phosphorylation influences translation rates upon recovery from salt stress, cells were incubated with CGP57380 alone or in conjunction...
Translation during Recovery from Hypertonic Shock

FIG. 3. Recovery of translation rates in the absence of both eIF4E and 4E-BP1 phosphorylation. 293 cells were incubated in complete growth medium for 60 min without (lane 1) or with addition of 150 mM NaCl (lanes 2–7). During the last 20 min of the salt shock, cells were supplemented with vehicle alone (lane 3), 20 μM CGP57380 alone (lane 4), or 20 μM CGP57380 in conjunction with 10 μM LY294002 (lane 5), 50 nM rapamycin (lane 6), or 100 nM wortmannin (lane 7). Cultures (lanes 3–7) were rinsed in fresh, complete medium and incubated in the absence of added salt but in the presence of vehicle or inhibitors (as indicated) for 30 min. For the last 5 min of this period, [35S]methionine (5 μCi/ml) was added and cell extracts were prepared as described above. Aliquots of extract containing equal amounts of protein were resolved by SDS-PAGE with 4E-BP1 (upper panel) and eIF4E visualized by immunoblotting (middle panel). Signals obtained for total eIF4E serves as a loading control. Results are representative of those obtained in three separate experiments. The incorporation of methionine into protein was determined by TCA precipitation in aliquots of extracts containing equal amounts of protein (lower panel). The presented data are the means and range of values (bars) from three separate experiments, each performed in triplicate. Statistical analysis (data not shown) indicates no significant differences in translation rates at any times analyzed.

with LY294002, wortmannin, or rapamycin for 30 min during the recovery from salt shock (Fig. 3). SDS-PAGE analysis of initiation factors shows that CGP57380 alone abrogates eIF4E phosphorylation during recovery from salt stress without affecting 4E-BP1 phosphorylation (lane 4 versus lane 3). However, as predicted, the additional presence of LY294002 (lane 5), rapamycin (lane 6), or wortmannin (lane 7) prevents both 4E-BP1 and eIF4E phosphorylation. Despite these events, translation rates during the recovery period are unaffected (lanes 4–7 versus lane 3), showing that neither 4E-BP1 nor eIF4E phosphorylation is required for protein synthesis under these conditions.

Inhibition of MEK1/2 Prevents 4E-BP1 and S6 Phosphorylation, Delays Polysome Formation, and Inhibits Translation Rates during Recovery from Salt Shock—As the activity of neither the mTOR nor the Mnk1 pathway is apparently required for recovery of translation rates during recovery from salt stress, we have investigated the role of the Erk1/2 pathway in this response. To this end, we have used U0126 and PD184352, recognized inhibitors of this signaling pathway (38–42). To determine the correct conditions for this investigation, cells were incubated in the presence of 150 mM NaCl for 60 min and allowed to recover in the presence of increasing concentrations of U0126. Fig. 4A shows that, relative to cells exposed to Me2SO alone (lane 1), the presence of 10–50 μM U0126 (lanes 4–6) prevents the phosphorylation of ribosomal protein S6 without preventing the phosphorylation of Akt. Furthermore, immunocomplex kinase assays (Fig. 4B) demonstrate that this results from an inhibition of p70S6K activity, albeit to a lesser extent than that observed with rapamycin. In addition, inhibition of MEK1/2 activity with U0126 strongly reduces the phosphorylation of 4E-BP1 at Ser-65 (Fig. 4A, lanes 4–6), suggesting a molecular cross-talk between the Erk1/2 and mTOR pathways. Isolation of eIF4E and associated factors with m7GTP-Sepharose (Fig. 4A, lower panel) shows that, at concentrations greater than 10 μM, U0126 also promotes increased binding of 4E-BP1 to eIF4E, conditions in which 4E-BP1 phosphorylation is reduced.

Previously, we have shown that salt shock results in the disaggregation of polysomes that rapidly reform upon the return of cells to isotonic medium (28). To determine the effect of U0126 on ribosome recruitment into polysomes, cells were salt-shocked and allowed to recover for 30 min in the absence or presence of 25 μM U0126. Fig. 4C shows that, relative to control cells (middle panel), inhibition of the Erk1/2 signaling pathway delays the recruitment of ribosomes into polysomes (right panel), an effect characteristic of a defect in translation initiation.

To address the effect of inhibition of signaling through Erk1/2 on translation rates more directly, a more detailed time course was carried out, allowing cells to recover for various times in the absence or presence of 25 μM U0126. As shown in Fig. 4D, U0126 prevents the transient activation of Erk1/2 observed at 15–30 min of recovery and results in a severe inhibition of ribosomal protein S6 phosphorylation (lanes 6 and 7 versus lanes 3 and 4). Inhibition of Erk1/2 signaling with U0126 also delays (lane 6 versus lane 3) but does not prevent Thr37/Thr46 phosphorylation of 4E-BP1 (lane 8 versus lane 5) and reduces levels of 4E-BP1 Ser-65 phosphorylation at later times (lanes 7 and 8 versus lanes 3 and 4). These data are consistent with a role for Erk1/2 signaling in modulating the activity of mTOR (1–3). As predicted, decreased levels of 4E-BP1 phosphorylation under these conditions result in a stabilization of the 4E-BP1-eIF4E complex, which readily dissociates in the absence of inhibitor (Fig. 4E, lanes 6–8 and lanes 3–5 versus lane 2). However, to our surprise, abrogation of Erk1/2 signaling with U0126 also results in a significant inhibition of translation rates during recovery (Fig. 4F, lane 7 versus lane 4, **, p = 0.0011; lane 8 versus lane 5, ***, p = 0.0015). This inhibition was always evident at later times of incubation with rates of translation during the initial 15 min not significantly different (Fig. 4F and data not shown).

PD184352 Inhibits Erk1/2 Signaling without Affecting Translation Rates during Recovery from Salt Shock—To confirm these data, we have used another, unrelated inhibitor of the Erk1/2 pathway, PD184352 (40, 41). At low micromolar concentrations PD184352 is specific for Erk1/2, whereas at higher concentrations it will also inhibit Erk5, a known additional target for U0126 (40, 42). Cells were incubated with 150 mM NaCl for 60 min and allowed to recover in isotonic medium in the absence or presence of U0126 or PD184352 for various
FIG. 4. Inhibition of MEK1/2 prevents 4E-BP1 and S6 phosphorylation, delays polysome formation, and inhibits translation rates during recovery from salt shock. 293 cells were incubated in complete growth medium for 60 min in the presence of 150 mM NaCl, washed, and allowed to recover for 30 min in the absence (lane 1) or presence of 0.1 μM U0126 (lane 2), 1 μM U0126 (lane 3), 10 μM U0126 (lane 4), 25 μM U0126.
times. Extracts were prepared and the phosphorylation of target proteins was visualized by SDS-PAGE and immunoblotting. Fig. 5A shows that relative to Me2SO alone (lanes 3–5), both U0126 (lanes 6–8) and PD184352 at either concentration (lanes 9–14) prevent the transient phosphorylation of Erk1/2. However, differences were observed between these inhibitors in their ability to impinge upon mTOR signaling. Although U0126 did not affect the phosphorylation of Akt in our cells under these conditions (Fig. 4A), it proved effective at reducing the phosphorylation of ribosomal protein S6 (lanes 6–8 versus lanes 3–5) with 2 μM PD184352 having little effect (lanes 9–11 versus lanes 3–5) and 20 μM PD184352 causing a partial reduction (lanes 12–14 versus lanes 3–5). These results were confirmed with immunocomplex kinase assays (Fig. 5C), which demonstrate the ability of U0126 (lane 3 versus lane 2) and 20 μM PD184352 (lane 5 versus lane 2) to inhibit p70S6k activity, albeit to a lesser extent than observed with rapamycin (lane 6 versus lane 2, quantified in the lower panel). In a similar manner, whereas U0126 is effective at reducing the phosphorylation of 4E-BP1 on Ser-65 (lanes 6–8 versus lanes 3–5), 2 μM PD184352 (lanes 9–11 versus lanes 3–5) and 20 μM PD184352 are less effective (lanes 12–14 versus lanes 3–5) and did not delay or prevent the phosphorylation of 4E-BP1 on Thr-37/Thr-46 (data not shown). Significant differences are also observed in the effects of these inhibitors on the rateemof de novo protein synthesis. Fig. 5D shows that whereas U0126 is effective at delaying protein synthesis rates at 15 min (lane 6 versus lane 3, **, \(p = 0.002\)), 30 min (lane 7 versus lane 4, ***, \(p = 0.0001\)), and 60 min of recovery (lane 8 versus lane 5, ***, \(p = 0.0005\)), 2 μM PD184352 has a lesser but significant impact on translation rates only at 60 min (lane 11 versus lane 5, * \(p = 0.0189\)). However, in contrast to U0126 (Fig. 4C), the recruitment of ribosomes into polyosomes is unaffected by PD184352 (data not shown). Similarly, at 20 μM PD184352, there is a small but significant inhibition of translation rates evident only at later times of recovery (lane 14 versus lane 5, **, \(p = 0.0043\)). A weak effect on translation rates at later times of recovery is also seen with a number of inhibitors that target protein kinase C and p90rsk (BIM1, Go6796, and Ro31–8220, data not shown), kinases also functioning on the Erk1/2 pathway (3, 7, 22).

To determine whether these differences can be ascribed to differential effects on Erk5 signaling (42), we have also monitored Erk5 phosphorylation during the recovery from hypertonic shock. As shown in Fig. 5D, although Erk5 phosphorylation (visualized as a mobility shift on SDS-PAGE) is clearly seen in HeLa cells stimulated with EGF, Erk5 is not phosphorylated in 293 cells under these conditions. These data were confirmed in 293 cells by the lack of a signal with antisemur specific to phospho-Erk5 (data not shown). Therefore, these data suggest that by virtue of its ability to inhibit both Erk1/2 and mTOR signaling, U0126 can prevent the recovery of translation rates following salt shock. In contrast, PD184352, which inhibits Erk1/2 but is less efficient at inhibiting mTOR signaling, does not influence translation rates in the same manner.

Incubation of Cells with Rapamycin and PD184352 Reduces Translation Rates to a Similar Level as Observed with U0126 Alone—In agreement with studies using growth factors and phorbol ester in transfected cells (22), our data indicate that during recovery from cell stress both the PI3-kinase-dependent pathway and the Erk1/2 pathway impinge on 4E-BP1 and S6 phosphorylation. This could result from a convergence of signaling pathways at the level of mTOR activity or reflect an input from parallel pathways. In an attempt to address this, we have investigated the phosphorylation (on Thr-1462) of endogenous TSC2 in our system, a component of the TSC1-TSC2 (hamartin-tuberin) complex that functions upstream of and indirectly regulates mTOR activity (19, 23, 43). Fig. 6A shows that during salt shock, TSC2 becomes less phosphorylated on Thr-1462 (lane 2 versus lane 1, quantified in the lower panel) with phosphorylation recovering within 15–30 min on returning cells to isotonic medium (lanes 3–5, quantified in the lower panel). As predicted, when adjusted for protein loading, the phosphorylation of TSC2 during recovery from salt shock is significantly inhibited by the PI3-kinase inhibitor, LY294002 at 15 min (lane 6 versus lane 3, ***, \(p = 0.0054\)), 30 min (lane 7 versus lane 4, **, \(p = 0.0042\)), and 60 min of recovery (lane 8 versus lane 5, **, \(p = 0.0027\)). Similar results were obtained with another PI3-kinase inhibitor, wortmannin (data not shown). Fig. 6B shows that whereas U0126 has no significant effect on TSC-2 phosphorylation at 15 min of recovery (lane 6 versus lane 3), it prevents the increase in phosphorylation of TSC2 at both 30 min (lane 7 versus lane 4, **, \(p = 0.0095\)) and 60 min of recovery (lane 8 versus lane 5, ***, \(p = 0.007\)). Therefore these data suggest that under these assay conditions Erk1/2 signaling can modulate TSC2 phosphorylation in 293 cells. As there appears to be no role for Erk5 activation in this response (Fig. 5D), we have asked whether the lack of effect of PD184352 on translation rates can be ascribed to its inability to prevent TSC2 phosphorylation during recovery from salt shock. Fig. 6C shows that this was indeed the case; when analyzed relative to the Erk1/2 loading control, U0126 caused a 76% (\(\pm 8\%\), \(n = 2\)) reduction in TSC2 phosphorylation (lane 4), whereas rapamycin (lane 5) or PD184352 at either concentration (lanes 6 and 7) were without significant effect.

Based on these findings, it would be predicted that the si-

(lane 1), or 50 μM U0126 (lane 6). For the last 5 min of this period, [\(^{35}\)S]methionine (5 μCi/ml) was added and cell extracts were prepared as described above. A (upper), aliquots of extracts containing equal amounts of protein were resolved by SDS-PAGE and the phosphorylation status of Akt, ribosomal protein S6, and 4E-BP1 (Ser-65) was visualized by immunoblotting. Signals obtained for PABP serve as a loading control. (lower), aliquots of extract containing equal amounts of protein were also subjected to mGTP-Sepharose chromatography and eIF4E, and associated proteins were resolved by SDS-PAGE. The recovery of 4E-BP1 and eIF4E was visualized by immunoblotting. Results are representative of those obtained in three separate experiments. B, 293 cells were incubated as in A and allowed to recover for 30 min in the presence of 50 mM rapamycin or 25 μM U0126. Aliquots of extracts containing equal amounts of protein were used for immunocomplex kinase assays for p70S6k as described. The top shows the results from a single experiment, whereas the presented data in the lower panel are the means and S.D. (bars) of three separate experiments, each performed in duplicate. C, ongoing cultures (left panel) or those incubated with 150 mM NaCl were allowed to recover for 30 min in the absence (middle panel) or presence of 25 μM U0126 (right panel). Aliquots of extract containing equal amounts of protein were resolved by sucrose gradient centrifugation as described. Sedimentation is from left to right, and the migration of the 80 S ribosome is indicated by an arrow. D, 293 cells were incubated in complete growth medium for 60 min in the absence (lane 1) or presence of 150 mM NaCl (lanes 2–8), washed, and allowed to recover for the times indicated in the absence (lanes 3–5) or presence of 25 μM U0126 (lanes 6–8). For the last 5 min of this period, [\(^{35}\)S]methionine (5 μCi/ml) was added and cell extracts were prepared as described above. Aliquots of extract containing equal amounts of protein were resolved by SDS-PAGE, and the phosphorylation of Erk1/2, S6, and 4E-BP1 (Ser-65 and Thr-37/Thr-46) was visualized by immunoblotting. Signals obtained for total Erk1/2 serve as a loading control, and the migration of Erk1/2-P is indicated. Results are representative of those obtained in five separate experiments. E, aliquots of extracts containing equal amounts of protein were also subjected to mGTP-Sepharose chromatography, and eIF4E and associated proteins were resolved by SDS-PAGE. The recovery of 4E-BP1 and eIF4E was visualized by immunoblotting. Results are representative of those obtained in three separate experiments. F, the incorporation of [\(^{35}\)S]methionine into protein was determined by TCA precipitation in aliquots of extracts containing equal amounts of protein. The presented data are the means and S.D. (bars) of three separate experiments, each performed in triplicate (*, \(p = 0.011\), **, \(p = 0.0015\)).
FIG. 5. PD184352 inhibits Erk1/2 signaling without affecting translation rates during recovery from salt shock. 

**A**, 293 cells were incubated in complete growth medium for 60 min without (lane 1) or with the addition of 150 mM NaCl (lanes 2–14). During the last 20 min of the salt shock, cells were supplemented with vehicle (lanes 3–5), 25 μM U0126 (lanes 6–8), 2 μM PD184352 (lanes 9–11), or 20 μM PD184352 (lanes 12–14). Cultures (lanes 3–14) were then rinsed in fresh, complete medium and incubated in the absence of added salt but in the presence of vehicle or inhibitors for the times indicated. For the last 5 min of this period, [35S]methionine (5 μCi/ml) was added and cell extracts were prepared as described above. Aliquots of extract containing equal amounts of protein were resolved by SDS-PAGE, and the phosphorylation of Erk1/2, S6, and 4E-BP1 was visualized by immunoblotting. Signals obtained for total Erk1/2 and eIF4E serve as loading controls, and arrows indicate the migration of Erk1/2-P. Results are representative of those obtained in three separate experiments.

**B**, the incorporation of [35S]methionine into protein was determined by TCA precipitation in aliquots of extracts containing equal amounts of protein. The presented data are the means and S.D. (bars) of three separate experiments, each performed in triplicate. Lane 6 versus lane 3, ***, p = 0.0002; lane 7 versus lane 4, ***, p = 0.0001; lane 8 versus lane 5, ***, p = 0.0005; lane 11 versus lane 5, *, p = 0.0189; lane 14 versus lane 5, ***, p = 0.0043. Statistical analysis (data not shown) indicates no significant differences in translation rates between lanes 9 and 12 and lane 3 or between lanes 10 and 13 and lane 4. C, 293 cells were incubated for 60 min with 150 mM NaCl and harvested immediately (lane 1), or allowed to recover for 30 min in the absence (lane 2) or presence of 25 μM U0126 (lane 3), 2 μM PD184352 (lane 4), 20 μM PD184352 (lane 5) or 50 nM rapamycin (lane 6). Aliquots of extracts containing equal amounts of protein were used for immunocomplex kinase assays for p70S6K as described. The top shows the phosphorylation of S6 from a single experiment, whereas the presented data in the lower panel are expressed as the percentage of phosphorylation obtained in cells recovered in the absence of inhibitors (set at 100%). The presented data are the means and S.D. (bars) of three separate experiments, each performed in duplicate.

**D**, serum-starved HeLa cells (lanes 1 and 2) were incubated in the absence (lane 3) or presence (lanes 4–8) of 10 ng/ml EGF for 10 min and then harvested, and the extracts were prepared. In addition, 293 cells were incubated in the absence (lane 3) or presence (lanes 4–8) of 150 mM NaCl for 60 min. Cultures (lanes 3–8) were rinsed in fresh, complete medium and allowed to recover for 5 min (lane 5), 15 min (lane 6), 30 min (lane 7) or 60 min (lane 8), and cell extracts were prepared. Aliquots of extracts containing equal amounts of protein were resolved by SDS-PAGE, and Erk5 was visualized by immunoblotting. Results are representative of those obtained in four separate experiments.
Translation during Recovery from Hypertonic Shock

A. 293 cells were incubated in complete growth medium for 60 min without (lane 1) or with the addition of 150 mM NaCl (lanes 2–8). During the last 20 min of the salt shock, cells were supplemented with vehicle (lanes 3–5) or 10 μM LY294002 (lanes 6–8). Cultures (lanes 2–8) were rinsed in fresh, complete medium and incubated in the absence of added salt but in the presence of vehicle or inhibitor for the times indicated, and the cell extracts were prepared. Aliquots of extracts containing equal amounts of protein were resolved by SDS-PAGE, and phospho-TSC2 was visualized by immunoblotting. Signals obtained for eIF4G serve as a loading control with results representative of those obtained in three separate experiments. The lower panel shows quantification of the phosphorylation of TSC2 (adjusted for protein levels using eIF4G) as determined by densitometric scanning. These data are expressed as the percentage of phosphorylation obtained in untreated cells (set at 100%). The presented data are the means and S.D. (bars) of three separate experiments. Lane 6 versus lane 3, **, p = 0.0054; lane 7 versus lane 4, **, p = 0.0042; lane 8 versus lane 5, **, p = 0.0027. B, cells were incubated as in A except that recovery was in the absence (lanes 3–5) or presence of 25 μM U0126 (lanes 6–8). Signals obtained for total eIF4E serve as a loading control. These data are from a single experiment and the phosphorylation of TSC2 relative to untreated cells (set at 100%) is indicated on the figure. Results were representative of those obtained in two separate experiments. C, cultures (lanes 3–7) were rinsed in fresh, complete medium and incubated in the absence of added salt but in the presence of vehicle or inhibitor for 30 min. Aliquots of extracts containing equal amounts of protein were resolved by SDS-PAGE, and phospho-TSC2 was visualized by immunoblotting. Signals obtained for total Erk1/2 serve as a loading control. These data are from a single experiment and the phosphorylation of TSC2 relative to untreated cells (set at 100%) is indicated on the figure. Results were representative of those obtained in two separate experiments. D, cultures (lanes 3–9) were rinsed in fresh, complete medium and incubated in the absence of added salt but in the presence of vehicle or inhibitor for 40 min. For the last 5 min of this period, [35S]methionine (2.5 μCi/ml) was added and cell extracts were prepared as described above. The incorporation of methionine into protein was determined by TCA precipitation in aliquots of extracts containing equal amounts of protein. The presented data are the means and S.D. (bars) of three separate experiments, each performed in duplicate. Lane 6 versus lane 3, **, p = 0.0001; lane 7 versus lane 3, **, p = 0.0001; lane 8 versus lane 3, *, p = 0.03; lane 9 versus lane 3, ***, p = 0.0001. Statistical analysis (data not shown) indicates no significant differences in TSC2 phosphorylation between lanes 3, 4 and 5 (lower). Aliquots of extract containing equal amounts of protein were resolved by SDS-PAGE, and the phosphorylation of 4E-BP1 (Ser-65) was visualized by immunoblotting. Results are representative of those obtained in three separate experiments. E, aliquots of extract containing equal amounts of protein were also subjected to m7GTP-Sepharose chromatography and eIF4E, and the recovery of 4E-BP1 and eIF4E was visualized by SDS-PAGE and immunoblotting. Results are representative of those obtained in three separate experiments.

FIG. 6. Inhibition of both mTOR and Erk1/2 signaling is required to prevent recovery of translation following salt shock. A, 293 cells were incubated in complete growth medium for 60 min without (lane 1) or with the addition of 150 mM NaCl (lanes 2–8). During the last 20 min of the salt shock, cells were supplemented with vehicle (lanes 3–5) or 10 μM LY294002 (lanes 6–8). Cultures (lanes 2–8) were rinsed in fresh, complete medium and incubated in the absence of added salt but in the presence of vehicle or inhibitor for the times indicated, and the cell extracts were prepared. Aliquots of extracts containing equal amounts of protein were resolved by SDS-PAGE, and phospho-TSC2 was visualized by immunoblotting. Signals obtained for eIF4G serve as a loading control. These data are expressed as the percentage of phosphorylation obtained in untreated cells (set at 100%). The presented data are the means and S.D. (bars) of three separate experiments. Lane 6 versus lane 3, **, p = 0.0054; lane 7 versus lane 4, **, p = 0.0042; lane 8 versus lane 5, **, p = 0.0027. B, cells were incubated as in A except that recovery was in the absence (lanes 3–5) or presence of 25 μM U0126 (lanes 6–8). Signals obtained for total eIF4E serve as a loading control. These data are from a single experiment and the phosphorylation of TSC2 relative to untreated cells (set at 100%) is indicated on the figure. Results were representative of those obtained in two separate experiments. C, cultures (lanes 3–7) were rinsed in fresh, complete medium and incubated in the absence of added salt but in the presence of vehicle or inhibitor for 30 min. Aliquots of extracts containing equal amounts of protein were resolved by SDS-PAGE, and phospho-TSC2 was visualized by immunoblotting. Signals obtained for total Erk1/2 serve as a loading control. These data are from a single experiment and the phosphorylation of TSC2 relative to untreated cells (set at 100%) is indicated on the figure. Results were representative of those obtained in two separate experiments. D, cultures (lanes 3–9) were rinsed in fresh, complete medium and incubated in the absence of added salt but in the presence of vehicle or inhibitor for 40 min. For the last 5 min of this period, [35S]methionine (2.5 μCi/ml) was added and cell extracts were prepared as described above. The incorporation of methionine into protein was determined by TCA precipitation in aliquots of extracts containing equal amounts of protein. The presented data are the means and S.D. (bars) of three separate experiments, each performed in duplicate. Lane 6 versus lane 3, **, p = 0.0001; lane 7 versus lane 3, **, p = 0.0001; lane 8 versus lane 3, *, p = 0.03; lane 9 versus lane 3, ***, p = 0.0001. Statistical analysis (data not shown) indicates no significant differences in TSC2 phosphorylation between lanes 3, 4 and 5 (lower). Aliquots of extract containing equal amounts of protein were resolved by SDS-PAGE, and the phosphorylation of 4E-BP1 (Ser-65) was visualized by immunoblotting. Results are representative of those obtained in three separate experiments. E, aliquots of extract containing equal amounts of protein were also subjected to m7GTP-Sepharose chromatography and eIF4E, and the recovery of 4E-BP1 and eIF4E was visualized by SDS-PAGE and immunoblotting. Results are representative of those obtained in three separate experiments.
multaneous inhibition of Erk1/2 and mTOR signaling by PD184352 and rapamycin should reduce translation rates during recovery from salt shock in a manner similar to that described for U0126. Fig. 6D shows that rapamycin alone (lane 4 versus lane 3) or 2 µM PD184352 alone (lane 5 versus lane 3) has little effect on translation rates. In agreement with the data presented in Fig. 5, 20 µM PD184352 causes a small inhibition of translation rates at later times of recovery (lane 8 versus lane 3, * p = 0.03). However, when rapamycin and PD184352 are present together (lane 6 versus lane 3, ** p = 0.0002; lane 9 versus lane 3, *** p = 0.0001), a significant and robust inhibition of translation is observed similar to that obtained with U0126 alone (lane 7 versus lane 3, ** p = 0.0001). Results obtained using combinations of RAD001 or LY294002 and PD184352 (data not shown) confirmed this finding. To determine that these inhibitors were functioning as predicted, the phosphorylation status of 4E-BP1 on Ser-65 (Fig. 6D) was monitored. These data show that rapamycin (lane 4) and U0126 (lane 7) but not PD184352 (lanes 4 and 8) reduce the phosphorylation of 4E-BP1 (Fig. 6D) and increase its binding to eIF4E (Fig. 6E). Additional studies (Fig. 5A and data not shown) also indicate that PD184352 but not rapamycin prevents the transient phosphorylation of Erk1/2 under these conditions.

**DISCUSSION**

Exposure of mammalian cells to hypertonic stress induces a drop in protein synthesis rates (Fig. 1A) with concomitant disaggregation of polysomes (28) and induces a dephosphorylation of translation initiation factors (Fig. 1, B and C). This treatment is also associated with the reversible inactivation of a number of signaling intermediates that impinge upon the translational machinery. Previously, we have shown that the rapid recovery in translation rates seen upon returning cells to isotonic medium does not require phosphorylation of eIF4E (28). In the present study, we have extended our work to show that components of the mTOR (Akt, TSC2, ribosomal protein S6, 4E-BP1) and the Erk pathways (MEK1/2, Erk1/2) are inactivated by salt stress. However, the role for these signaling pathways in de novo translation during recovery from salt stress is unresolved. To redress this, we have used a variety of cell-permeable inhibitors to investigate these individual signaling pathways in further detail.

Phosphorylation events controlled via the mTOR signaling pathway clearly play a central role in regulating 4E-BP1 phosphorylation and translation rates in a number of cell systems (1–3, 19–22, 44). To investigate the requirement for 4E-BP1 phosphorylation in the recovery of translation rates following hypertonic stress, we have used the PI3-kinase inhibitor, wortmannin, and the mTOR inhibitor, rapamycin. As expected, both inhibitors prevented the phosphorylation of ribosomal protein S6 and 4E-BP1 during recovery (Fig. 2), consistent with a role for PI3-kinase and mTOR in signaling to these target proteins (18, 23, 35). However, whereas rapamycin, LY294002, RAD001 (data not shown) and wortmannin stabilized the interaction between 4E-BP1 and eIF4E and reduced eIF4F complex assembly, we were surprised to find that inhibition of mTOR signaling did not influence translation rates over this recovery period (Fig. 2 and data not shown). Similar results were obtained when both eIF4E phosphorylation and mTOR signaling were simultaneously inhibited (Fig. 3), suggesting that only a small proportion of the available eIF4F complex is required for maximal translation rates under these conditions. However, we cannot discount the possibility that there is a localized pool of eIF4E that functions during recovery from salt shock which is not subject to regulation by 4E-BP1. An alternative explanation is that other parallel or rapamycin-insensitive pathways may be influencing translation rates under these conditions.

To address the latter, we have also investigated the effect of inhibiting signaling through the Erk1/2 pathway on translation rates during the recovery phase. As expected, U0126, which acts as non-competitive inhibitor of MEK1/2 (39), prevented the transient increase in Erk1/2 (Fig. 4D) and MEK1/2 phosphorylation (data not shown). As Erk5 was not apparently activated in 293 cells under these conditions (Fig. 5D), additional effects of this inhibitor on Erk5 can be discounted (39–42). However, inhibition of Erk1/2 signaling with U0126 also resulted in a decrease in 4E-BP1 phosphorylation of Ser-65 with a concomitant increase in its binding to eIF4E (Fig. 4A) without affecting the phosphorylation state of Akt. This was associated with decreased levels of p70S6K activity and decreased S6 phosphorylation, albeit to a lesser extent than that observed with rapamycin (Fig. 4B). In contrast, whereas rapamycin did not influence translation rates (Fig. 2C) or polysome formation during recovery (data not shown), inhibition of Erk1/2 signaling with U0126 caused a decrease in protein synthesis (Fig. 4F) and in the recruitment of ribosomes into polysomes (Fig. 4C). Although we cannot discount any effect of this compound on polypeptide chain elongation (45, 46), the accumulation of 80 S ribosomes in the presence of U0126 is suggestive of a dominant effect on the initiation phase of translation. Although the p38MAPK inhibitor, SB203580, was without effect, results obtained with another MEK1/2 inhibitor, PD98059, and those obtained with U0126 in NIH3T3 cells following hypertonic shock were similar to those described above (data not shown). Therefore, in agreement with published findings (22, 47–50), the delayed phosphorylation of 4E-BP1 on Thr-37/Thr-46 (Fig. 4A, (51)) and the partial inactivation of p70S6K in the presence of U0126 are consistent with molecular cross-talk between the Erk1/2 and mTOR signaling pathways during recovery from salt shock. Indeed, over-expression of a constitutively active form of MEK stimulated the rate of phosphorylation of 4E-BP1 during recovery but was unable to further influence rates of translation (data not shown).

To confirm these data, we have also used another, unrelated Erk1/2 pathway inhibitor, PD184352 (38–42). In contrast to U0126, low concentrations of PD184352, which specifically inhibit Erk1/2 signaling, did not delay or prevent the phosphorylation of 4E-BP1 on Ser-65 (Fig. 5A) or Thr-37/Thr-46 (data not shown). Higher concentrations of inhibitor, which can also inhibit Erk5 signaling (38–42), did partially prevent 4E-BP1 phosphorylation and S6 phosphorylation and did inhibit p70S6K activity (Fig. 5, A and C). Surprisingly, although U0126 was effective at delaying protein synthesis, PD184352 at either concentration had no significant impact on translation rates (Fig. 5B) or the recruitment of ribosomes into polysomes (data not shown). Addition of PD184352 and rapamycin together, however, was as effective as U0126 at reducing translation rates (Fig. 6D), suggesting that inhibition of both Erk1/2 and mTOR signaling is required to prevent the increase in de novo translation during recovery from salt shock.

To understand how the Erk1/2 pathway can influence signaling through mTOR in our cells, we investigated the phosphorylation of endogenous TSC2 on Thr-1462 in the RXXR(S/T) consensus site, an event shown to inactivate the hamartin/tuberin heterodimer (22, 23). Fig. 6 shows that Thr-1462 was phosphorylated during recovery from salt shock.

Phosphorylation of endogenous TSC2 on Thr-1462 correlated with a reduction in 4E-BP1 phosphorylation (Fig. 6D) and a decrease in eIF4E binding (Fig. 6E) and S6 phosphorylation (Fig. 6F). The regulation of this phosphorylation event by inhibition of both Erk1/2 and mTOR signaling suggests that this is an important mechanism for the inhibition of translation during recovery from salt shock.

**CONCLUSION**

The data presented here show that the Erk1/2 and mTOR signaling pathways play a dominant role in the regulation of translation during recovery from salt shock. Inhibition of these pathways by rapamycin or MEK1/2 inhibitors is associated with a decrease in p70S6K activity and S6 phosphorylation, indicating that these inhibitors reduce initiation of translation. However, inhibition of Erk1/2 signaling alone is insufficient to reduce translation rates, suggesting that other parallel or rapamycin-insensitive pathways may be influencing translation rates under these conditions.
FIG. 7. Schematic model depicting the PI3-K/Akt-dependent and U0126-sensitive phosphorylation of TSC2 in the control of protein synthesis during recovery from hypertonic shock. This figure shows the activation of the mTOR and Erk1/2 signaling pathways following release of 293 cells from salt shock. Increased rates of de novo translation are associated with the activation of the Erk1/2 pathway, and the phosphorylation of TSC2 at a consensus (RXRX/X/S/T) phosphorylation site (Thr-1462), leading to inactivation of the hamartin-tuberin (TSC1-TSC2) complex (18, 19, 22, 23, 26, 52, 53). Inactivation of TSC2 phosphorylation provides a plausible explanation as to why U0126 can prevent both Erk1/2 signaling and inhibit the de novo protein synthesis during recovery from hypertonic shock. This inactivation of TSC2, whereas PD184352 only prevents Erk1/2 signaling during the initial 15 min unaffected (Fig. 4 C). These data suggest an uncharacterized, early Erk1/2 and mTOR-independent phase of translation sufficient to recruit a population of ribosomes onto small polysomes (Fig. 4C), but insufficient to attain maximal translation rates. The identity of the critical downstream target(s) of Erk1/2 signaling that influence translation rates during this period is unknown. One possible target is the guanine nucleotide exchange factor, eIF2B (46). However, although the phosphorylation of eIF2B did not appear to be regulated under conditions of hypertonic shock, we have been unsuccessful in determining whether there is any change in eIF2B activity during recovery (data not shown). Further work is required to investigate the nature of such downstream targets and to clarify whether the U0126-sensitive input into TSC2 is caused by modulation of a novel Thr-1462 kinase and/or phosphatase activity directed to this site.

does not target Thr-1462 (22). However, although both rapamycin (data not shown) and PD184352 were without effect, inhibition of Erk1/2 phosphorylation with U0126 did prevent Thr-1462 phosphorylation (Fig. 6). Therefore, an effect at the level of TSC2 phosphorylation provides a plausible explanation as to why U0126 can prevent both Erk1/2 signaling and inhibit the phosphorylation of targets downstream of mTOR (Fig. 7). However, without the use of overexpression studies, we cannot discount the possibility that U0126 indirectly interferes with the TOS-mediated mTOR/raptor binding of 4E-BP1 and p70S6K to prevent their phosphorylation (54).

It appears that signaling through either pathway is sufficient to allow de novo translation with efficient inhibition of both Erk1/2 and mTOR signaling required to inhibit translation rates during recovery from salt shock (Fig. 6). This inhibition was always evident at later times with rates of translation during the initial 15 min unaffected (Fig. 4F and data not shown). These data suggest an uncharacterized, early Erk1/2 and mTOR-independent phase of translation sufficient to re-

REFERENCES

1. Gingras, A. C., Raught, B., and Sonenberg, N. (1999) Ann. Rev. Biochem. 68, 913–963
2. Gingras, A. C., Raught, B., and Sonenberg, N. (2001) Prog. Mol. Subcell. Biol. 27, 143–174
3. Morley, S. J. (2001) in Signaling Pathways for Translation (Rhoads, R. E. ed) pp. 1–37, Springer-Verlag, Berlin
4. Marcottigiano, J., Gingras, A. C., Sonenberg, N., and Burley, S. K. (1997) Cell 89, 951–961
5. Marcottigiano, J., Gingras, A. C., Sonenberg, N., and Burley, S. K. (1999) Mol. Cell 3, 707–716
6. Mader, S., Lee, H., Pause, A., and Sonenberg, N. (1995) Mol. Cell. Biol. 15, 4990–4997
7. Proug, C. G. (1999) Eur. J. Biochem. 269, 5327–5349
8. Sonenberg, N., and Dever, T. E. (2003) Curr. Opin. Struct. Biol. 13, 56–63
9. Preiss, T., and Hentze, M. W. (2003) BioEssays 25, 1201–1211
10. Waskiewicz, A. J., Flynn, A., Proud, C. G., and Cooper, J. A. (1997) EMBO J. 16, 1909–1920
11. Waskiewicz, A. J., Johnson, J. C., Penn, B., Mahalingam, M., Kimball, S. R., and Cooper, J. A. (1999) Mol. Cell. Biol. 19, 1871–1880
12. Schepfer, G. C., van Kollenburg, B., Hu, J. Z., Loo, Y. J., Goss, D. J., and Proud, C. G. (2002) J. Biol. Chem. 277, 33035–33049
13. Parra-Palau, J. L., Scheper, G. C., Wilson, M. L., and Proud, C. G. (2003) J. Biol. Chem. 278, 44197–44204
14. Ronen, S. (2000) Biochem. Pharmacol. 60, 1237–1243
15. Raught, B., Gingras, A. C., and Sonenberg, N. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 245–294, Cold Spring Harbor Laboratory Press, New York
16. Pyronnet, S., Imataka, H., Gingras, A.-C., Fukunaga, R., Hunter, T., and Sonenberg, N. (1999) EMBO J. 18, 270–279
17. Hagihata, A., Mader, S., Pause, A., and Sonenberg, N. (1995) EMBO J. 14, 701–709
18. Blenis, J., Fingar, D., Richardson, C., Schalm, S., and Teo, A. (2003) FASEB J. 17, A149–A159
19. Tee, A. R., Manning, B. D., Roux, P. P., Cantley, L. C., and Blenis, J. (2003) Curr. Biol. 13, 1259–1268
20. Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J., and Cantley, L. C. (2002) Mol. Cell 10, 151–162
21. Krymskaya, V. P. (2003) Cell Signal. 15, 729–739
22. Tee, A. R., Anjum, R., and Blenis, J. (2003) J. Biol. Chem. 278, 37288–37296
23. Li, Y., Corradetti, M. N., Inoki, K., and Guan, K. L. (2004) Trends Biochem. Sci. 29, 32–38
24. Inoki, K., Li, Y., Xu, T., and Guan, K. L. (2003) Genes Dev. 17, 1829–1834
25. Kwiatkowski, D. J. (2003) Cancer Biol. Ther. 2, 471–476
26. Dan, H. C., Sun, M., Yang, L., Feldman, R. I., Sui, X. M., Ou, C. C., Nellist, M., Yeong, R. S., Hailey, D. J. J., Nissim, S. Y., Pledger, W. J., and Cheng, J. Q. (2002) J. Biol. Chem. 277, 35364–35370
27. Nova, I., and Carrascos, I. (1999) Mol. Cell. Biol. 19, 2445–2454
28. Morley, S. J., and Naegele, S. (2002) J. Biol. Chem. 277, 32855–32859
29. Morley, S. J., and McKendrick, L. (1997) J. Biol. Chem. 272, 17887–17893
30. Fraser, C. S., Pain, V. M., and Morley, S. J. (1999) Biochem. J. 342, 519–526
31. Morley, S. J., and Naegele, S. (2003) Cell. Signal. 15, 741–749
32. Bushell, M., Wood, W., Clemens, M. J., and Morley, S. J. (2000) Eur. J. Biochem. 267, 1083–1091
33. Lane, H. A., Morley, S. J., Dorée, M., Kozma, S. C., and Thomas, G. (1992) EMBO J. 11, 1743–1749
34. Morley, S. J., and Pain, V. M. (1995) J. Cell Sci. 108, 1751–1760
35. Garani, A., Zwarzhkuis, F. J. T., Nohukuni, T., Joaquin, M., Roccio, M., Stocker, H., Kozma, S. C., Hafen, E., Jos, L. J., and Thomas, G. (2003) Mol. Cell 11, 1457–1468
36. Brown, V. I., Barr, R., Akorn, K., and Grupp, S. A. (2003) Pediatr. Res. 53, 1637
37. Huang, S., and Houghton, P. J. (2003) Curr. Opin. Pharmacol. 3, 371–377
38. Cohn, P. (1999) Philos. Trans. R. Soc. Lond. B Biol. Sci. 354, 485–495
39. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) Biochem. J. 351, 95–105
40. Mody, N., Leitch, J., Armstrong, C., Dixon, J., and Cohen, P. (2001) FEBS Lett. 502, 21–24
41. Allen, L. F., Sebolt-Leopold, J., and Meyer, M. B. (2003) Semin. Oncol. 30, 105–116
42. Squires, M. S., Nixon, P. M., and Cook, S. J. (2002) Biochem. J. 366, 673–680
43. Fingar, D. C., Richardson, C. J., Tee, A. R., Cheatham, L., Tsou, C., and Blenis, J. (2004) Mol. Cell. Biol. 24, 200–216
44. Gingras, A. C., Raught, B., Gygi, S. P., Niedzwiecka, A., Miron, M., Burley, S. K., Polakiewicz, R. D., Wyslouch-Cieszyńska, A., Aebersold, R., and Sonenberg, N. (2001) Genes Dev. 15, 2852–2864
45. Wang, L. J., and Proud, C. G. (2002) FEBS Lett. 531, 285–289
46. Proud, C. G. (2002) Eur. J. Biochem. 269, 5338–5349
47. Wang, L. J., Gout, I., and Proud, C. G. (2001) J. Biol. Chem. 276, 32670–32677
48. Iijima, Y., Laser, M., Shiraishi, H., Willey, C. D., Sundaravadivel, B., Xu, L., McDermott, P. J., and Kuppuswamy, D. (2002) J. Biol. Chem. 277, 23065–23075
49. Lehman, J. A., and Gomez-Cambronero, J. (2002) Biochem. Biophys. Res. Comm. 293, 463–469
50. Shi, Y. Y., Hsu, J. H., Ha, L. P., Gera, J., and Lichtenstein, A. (2002) J. Biol. Chem. 277, 15712–15720
51. Gingras, A. C., Gygi, S. P., Raught, B., Polakiewicz, R. D., Abraham, R. T., Hoekstra, M. F., Aebersold, R., and Sonenberg, N. (1999) Genes Dev. 13, 1422–1437
52. Li, Y., Inoki, K., Vacratsis, P., and Guan, K. L. (2003) J. Biol. Chem. 278, 13663–13671
53. Li, Y., Inoki, K., Yeung, R., and Guan, K. L. (2002) J. Biol. Chem. 277, 44593–44596
54. Schalm, S. S., Fingar, D. C., Sabatini, D. M., and Blenis, J. (2003) Curr. Biol. 13, 797–806
Molecular Cross-talk between MEK1/2 and mTOR Signaling during Recovery of 293 Cells from Hypertonic Stress
Susanne Naegele and Simon J. Morley

J. Biol. Chem. 2004, 279:46023-46034. doi: 10.1074/jbc.M404945200 originally published online August 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404945200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 23 of which can be accessed free at http://www.jbc.org/content/279/44/46023.full.html#ref-list-1