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Cap-Binding Protein (Eukaryotic Initiation Factor 4E) and 4E-Inactivating Protein BP-1 Independently Regulate Cap-Dependent Translation

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Cap-dependent protein synthesis in animal cells is inhibited by heat shock, serum deprivation, metaphase arrest, and infection with certain viruses such as adenovirus (Ad). At a mechanistic level, translation of capped mRNAs is inhibited by dephosphorylation of eukaryotic initiation factor 4E (eIF-4E) (cap-binding protein) and its physical sequestration with the translation repressor protein BP-1 (PHAS-I). Dephosphorylation of BP-1 blocks cap-dependent translation by promoting sequestration of eIF-4E. Here we show that heat shock inhibits translation of capped mRNAs by simultaneously inducing dephosphorylation of eIF-4E and BP-1, suggesting that cells might coordinately regulate translation of capped mRNAs by impairing both the activity and the availability of eIF-4E. Like heat shock, late Ad infection is shown to induce dephosphorylation of eIF-4E. However, in contrast to heat shock, Ad also induces phosphorylation of BP-1 and release of eIF-4E. BP-1 and eIF-4E can therefore act on cap-dependent translation in either a mutually antagonistic or cooperative manner. Three sets of experiments further underscore this point: (i) rapamycin is shown to block phosphorylation of BP-1 without inhibiting dephosphorylation of eIF-4E induced by heat shock or Ad infection, (ii) eIF-4E is efficiently dephosphorylated during heat shock or Ad infection regardless of whether it is in a complex with BP-1, and (iii) BP-1 is associated with eIF-4E in vivo regardless of the state of eIF-4E phosphorylation. These and other studies establish that inhibition of cap-dependent translation does not obligatorily involve sequestration of eIF-4E by BP-1. Rather, translation is independently regulated by the phosphorylation states of eIF-4E and the 4E-binding protein, BP-1. In addition, these results demonstrate that BP-1 and eIF-4E can act either in concert or in opposition to independently regulate cap-dependent translation. We suggest that independent regulation of eIF-4E and BP-1 might finely regulate the efficiency of translation initiation or possibly control cap-dependent translation for fundamentally different purposes.

In this study, we used heat shock and adenovirus (Ad) infection to independently block eukaryotic initiation factor 4E (eIF-4E) phosphorylation and cell protein synthesis. We determined that translation regulated by the phosphorylation state of eIF-4E is not controlled coordinately with that of the 4E-inactivating protein, known as PHAS-I or BP-1, which sequesters eIF-4E. eIF-4E (cap-binding protein) is one of three mammalian protein factors that comprise initiation factor eIF-4F: eIF-4E, a 28-kDa m^7GTP (cap)-binding protein; eIF-4A, a 46-kDa ATP-dependent RNA helicase; and p220 (eIF-4G), a 220-kDa protein of unknown function (36). Collectively, eIF-4E, eIF-4A, and eIF-4G form the cap-dependent RNA helicase complex known as initiation factor eIF-4F. eIF-4F mediates the unwinding of RNA structure near the 5′ cap, which in turn facilitates binding by the 43S ribosomal subunit, thereby promoting translation initiation (reviewed in reference 44). The activity of eIF-4F is thought to confer discriminatory and regulatory activities to the process of translation initiation (40, 41). These results are supported by studies which indicate that minimal secondary structure in the 5′ end of an mRNA correlates with an enhanced ability to initiate translation, particularly when the amount of active eIF-4F is low (14, 30).

eIF-4F is a major target for the regulation of protein synthesis in the mammalian cell. The activity of eIF-4F is generally regulated by altering the phosphorylation state of the eIF-4E component. Increased phosphorylation of eIF-4E correlates with enhanced translation in cells stimulated with mitogens, growth factors, or serum (28, 35, 38) and in cells transformed with the *ras* or *src* oncogene (17). Reduced phosphorylation of eIF-4E correlates with inhibition of protein synthesis during heat shock (10, 11, 47), mitosis (5, 25), and serum depletion (9) and in Ad- and influenza virus-infected cells (12, 25).

A cellular protein with the ability to regulate the activity of eIF-4F by physically sequestering the eIF-4E component into an inactive complex has been recently identified and characterized. This protein, termed PHAS-I or BP-1, was originally identified in rat adipocytes as a prominent insulin-stimulated phosphoprotein (23, 31, 32) and was found through interaction cloning techniques to bind to eIF-4E (39). BP-1 binding to eIF-4E inhibits cap-dependent translation of mRNA in vitro and in vivo by preventing the formation of eIF-4F (3, 32, 34, 39). Studies indicate that when eIF-4E is bound to BP-1, it no longer forms an eIF-4F complex (34). However, in response to insulin or growth factors, BP-1 becomes heavily phosphorylated and releases eIF-4E (31, 39), presumably allowing it to form a complex with eIF-4A and eIF-4G, leading to assembly of eIF-4F and the stimulation of translation. A critical question which we sought to address in this study concerns whether cap-dependent translation is coordinately controlled by eIF-4E phosphorylation and its sequestration with BP-1, or whether they constitute independent mechanisms for regulating protein synthetic rates. This question is important because studies conducted to date have found a consistent role for BP-1 sequestration of eIF-4E in inhibiting cap-dependent translation, including translation inhibition during quiescence (serum

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starvation) and infection by various picornaviruses (3, 18, 32, 34, 39). It was therefore possible that inhibition of cap-dependent translation might always involve sequestration of eIF-4E by BP-1 but only under certain conditions would involve dephosphorylation of eIF-4E.

The shutoff of cellular protein synthesis in heat-shocked (stressed) cells, and during the late phase of Ad infection, provided a useful approach for investigating the respective roles of BP-1 and eIF-4E in controlling cap-dependent translation. Ad is temporally organized into early and late phases (reviewed in references 19, 42, and 43). During the early phase of Ad infection, only small amounts of virus-specific mRNA and protein are synthesized, and no dramatic changes occur in cellular metabolism. The late phase of Ad infection is marked by suppression of host cell protein synthesis and preferential translation of late viral mRNAs (reviewed in references 42 and 43). Most late Ad mRNAs are transcribed from the viral major late promoter and share a common 5′ noncoding region of 200 nucleotides, known as the tripartite leader. The tripartite leader is required for translation of mRNAs at late but not early times after Ad infection (4, 33). The suppression of host cell translation by Ad, and selective translation of late tripartite leader mRNAs, involves a virus-induced block to eIF-4E phosphorylation, which in turn impairs or alters eIF-4F (25). The tripartite leader provides late Ad mRNAs with the ability to translate with little requirement for eIF-4F (7, 8), by promoting an unusual form of initiation known as ribosome jumping or shunting (46), thereby permitting exclusive translation of late Ad mRNAs after virus-mediated dephosphorylation of eIF-4E (24, 25, 48). In summary, Ad blocks the phosphorylation of eIF-4E and the activity of eIF-4F during the late phase of infection, which in turn prevents translation of cellular but not late viral mRNAs. Heat shock treatment of cells also blocks eIF-4E phosphorylation and cellular protein synthesis (10, 47) in a manner that may be similar to that of Ad infection (13).

In this report, we first demonstrate that heat shock simultaneously induces the dephosphorylation of both BP-1 and eIF-4E, which in turn causes BP-1 to sequester the pool of eIF-4E. Cap-dependent translation is therefore inhibited during heat shock through the dephosphorylation of eIF-4E, which inhibits or alters its activity, as well as the physical sequestration of eIF-4E by BP-1. We then show that Ad infection of cells induces phosphorylation (inactivation) of BP-1 and release of sequestered eIF-4E during early and late times of infection, despite the fact that Ad also mediates dephosphorylation of eIF-4E. The Ad-induced phosphorylation of BP-1 is shown to induce the continuous turnover of phosphate on BP-1 protein, to release eIF-4E from the BP-1–eIF-4E complex, and to stimulate cap-dependent translation of both cellular and viral early mRNAs during the early phase of infection. Ad-induced phosphorylation (inactivation) of BP-1 therefore provided an experimental system in which it was demonstrated that physical inactivation of eIF-4E by BP-1 sequestration is not coupled to functional inactivation of eIF-4E by dephosphorylation. Moreover, both eIF-4E sequestered by BP-1 and free eIF-4E were equally dephosphorylated during late Ad infection and during heat shock treatment of uninfected cells. Thus, the mechanisms of regulation of protein synthesis controlled by the phosphorylation state of eIF-4E and BP-1 are not always linked, and BP-1 sequestration of eIF-4E is not an obligatory event in inhibition of cap-dependent translation. Instead, these two systems represent independent mechanisms for controlling cap-dependent mRNA translation which can act either in concert or in opposition to one another. Additionally, studies indicate that BP-1 and eIF-4E phosphorylation is ultimately regulated by nonoverlapping protein kinase signalling pathways. Ad-induced phosphorylation of BP-1 is shown to be mediated by a rapamycin-sensitive pathway that involves the p70^{s6k} Rsk kinase signalling cascade, in common with activation of BP-1 phosphorylation by hormonal signals (3, 21, 32), but stimulation of eIF-4E phosphorylation is not.

MATERIALS AND METHODS

Viruses and cells. Adenovirus type 5 *dl309* (Ad5*dl*309) is a phenotypically wild-type virus that contains a series of altered restriction enzyme cleavage sites (26). 293 cells are a human embryonic kidney cell line transformed with the E1 region of Ad5 (20). Chinese hamster ovary (CHO) and 293 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Titers of virus stocks were determined on 293 cells. Heat shock of cells was carried out at 44°C for 3 to 4 h as described previously (46).

Characterization of eIF-4E. For in vivo labeling of eIF-4E with $^{32}PO_4$, cells were washed in phosphate-free DMEM and incubated for 2 h at 37°C in 1 ml of phosphate-free DMEM containing 100 μCi of carrier free $^{32}PO_4$ (New England Nuclear) per ml. For heat-shocked cells, $^{32}PO_4$ was added 1 h after heat shock for 1 h. Preparation of ^{32}P-labeled extracts for affinity chromatography on m^{7}GTP-Sepharose was carried out as described previously (25). Briefly, equal numbers of cells were resuspended in lysis buffer (100 mM KCl, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 0.2 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.5% Triton X-100) and centrifuged at 10,000 × *g* at 4°C, and the S10 supernatant was collected. Equal amounts of the supernatant protein were then incubated with a slurry of m^{7}GTP-Sepharose at 4°C for 1 h. The Sepharose was washed with lysis buffer followed by 0.6 ml of 1 mM GTP in lysis buffer, and the eIF-4E was eluted with 100 μM m^{7}GTP. Eluted protein was recovered by precipitation with 6 volumes of acetone at −20°C and prepared for sodium dodecyl sulfate (SDS)–15% polyacrylamide gel electrophoresis (15% PAGE) and autoradiography.

Characterization of BP-1. Samples of whole cell extract were dissolved in SDS sample buffer and subjected to SDS–15% PAGE (29). Proteins were electrophoretically transferred to nitrocellulose and immunoblotted with an antibody to BP-1 (gift of J. Lawrence, Washington University, St. Louis, Mo.) at 3 μg/ml. For in vivo labeling of BP-1 with $^{32}PO_4$, cells were labeled as described above, and BP-1 was immunoprecipitated as described previously (32) and then subjected to SDS-PAGE followed by autoradiography. Rapamycin was used at 20 ng/ml starting 1 h after Ad infection unless otherwise noted.

eIF-4E–BP-1 complexes. The association of BP-1 and eIF-4E was analyzed by quantitating and characterizing the forms of BP-1 recovered with eIF-4E that were purified by m^{7}GTP-Sepharose chromatography and by measuring the amount of eIF-4E that coimmunoprecipitated with BP-1, using a specific antibody (provided by J. Lawrence). eIF-4E and BP-1 were resolved by SDS–15% PAGE and subjected to specific immunoblot analysis.

Analysis of translation. Cells were labeled with 50 μCi of [^{35}S]methionine for 1 h (Express Label; New England Nuclear) per ml in DMEM without methionine. Cells were lysed in lysis buffer at 4°C, and specific activity was determined by trichloroacetic acid precipitation of equal amounts of protein. Equal counts per minute of protein were subjected to SDS–15% PAGE analysis and fluorography. eIF-4E-dependent (pCR3/βgal) and -independent (pCMV-Ad/βgal) β-galactosidase reporter mRNA constructs were developed by linking the β-galactosidase coding region to the pSK+ Bluescript polylinker (Stratagene) at the *Sal*I site and to the late Ad tripartite leader 5′ noncoding region, respectively.

RESULTS

BP-1 phosphorylation and sequestration of eIF-4E during heat shock. Previous studies demonstrated a role for BP-1 in translational regulation in specialized cells by its ability to sequester eIF-4E into inactive complexes in the absence of mitogen (insulin) stimulation (31, 39). We examined whether inactivation of eIF-4E by sequestration with BP-1 is linked to functional inactivation of eIF-4E by dephosphorylation. Coordinate physical and functional inactivation of eIF-4E might then constitute a unified cellular mechanism for the inhibition of cap-dependent mRNA translation. Both heat shock and late Ad infection inhibit eIF-4E phosphorylation and cap-dependent protein synthesis. We therefore probed the phosphorylation state and potential role of BP-1 in shutoff of protein synthesis during heat shock. Phosphorylation of BP-1 can be detected electrophoretically because phosphorylated BP-1 displays slower migration by SDS-PAGE (31, 39). Most cells contain multiple electrophoretic forms of BP-1 corresponding to different phosphorylation states, ranging from a fast-migrat-

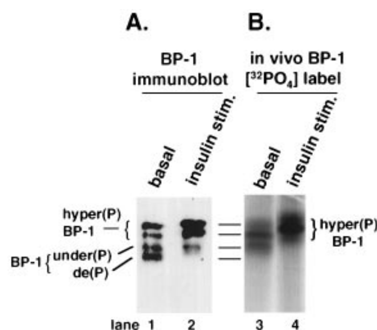

FIG. 1. Characterization of BP-1 hyperphosphorylated and underphosphorylated forms by immunoblotting and in vivo $^{32}PO_4$ labeling. Confluent 293 cells were untreated (basal) or stimulated with 20 nM insulin for 60 min. (A) Equal whole cell protein amounts were resolved by SDS–15% PAGE, transferred to nitrocellulose, and immunoblotted with antisera specific for BP-1. (B) Cells were labeled in vivo with 100 μCi of $^{32}PO_4$ for 1 h during confluence or insulin treatment; equal protein amounts were immunoprecipitated with BP-1-specific antisera and coelectrophoresed with unlabeled cell extracts in panel A. BP-1 protein was visualized by autoradiography. (P), phosphorylated.

ing band (dephosphorylated) to a slowly migrating band (hyperphosphorylated). The dephosphorylated and underphosphorylated forms of BP-1 can be observed in resting or serum-starved cells in a complex with eIF-4E (31, 39).

Results presented in Fig. 1 first confirm the identification of fast-migrating and slowly migrating forms of BP-1 with under- and hyperphosphorylated forms of the protein, respectively. In vivo $^{32}PO_4$-labeled BP-1 and unlabeled BP-1 were coelectrophoresed and detected by autoradiography or immunoblot analysis (Fig. 1). The fast-migrating species, detected by immunoblotting (Fig. 1A) but not by in vivo $^{32}PO_4$ labeling (Fig. 1B), were present only in resting cells in the absence of insulin stimulation. Accumulation of the slowly migrating forms of BP-1 with insulin treatment corresponded to an increase in the phosphorylated forms of the protein. The effects of heat shock on BP-1 phosphorylation and eIF-4E sequestering activities were next investigated. Within 2 to 3 h of treatment, heat shock induced a strong dephosphorylation and underphosphorylation of BP-1, indicated by the appearance of one or two new fast-migrating forms of the protein on immunoblots compared with control (growing) non-heat-shocked cells (Fig. 2A; compare lanes 1 and 3). Phosphorylation of BP-1 can be inhibited by rapamycin, which blocks signal transduction pathways that involve the p70^{s6k} Rsk group of protein kinases (21, 31, 32). Rapamycin treatment of control cells produced one or two fast-migrating protein bands identical to those induced by heat shock (Fig. 2A, lanes 2 and 3). These results confirm that heat shock mediated the appearance of a dephosphorylated form of BP-1. The newly induced fast-migrating forms of BP-1 correspond to fully dephosphorylated and largely underphosphorylated proteins shown in Fig. 1 that cannot be labeled in vivo with $^{32}PO_4$. As expected, heat shock induced strong dephosphorylation of eIF-4E and inhibition of cell protein synthesis (Fig. 2B and Table 1). This was determined by analyzing eIF-4E phosphorylation in cells labeled in vivo with $^{32}PO_4$; then eIF-4E was purified by m^{7}GTP-Sepharose affinity chromatography and examined by SDS-PAGE. The effect of heat shock on cellular protein synthesis was examined by labeling control and heat-shocked cells with [^{35}S]methionine, and protein synthetic rates were determined (Table 1). The strong dephosphorylation of BP-1 induced by heat shock was also found to be associated with sequestration of eIF-4E (Fig. 2C). This was examined by immunoprecipitation of BP-1 from equal amounts of protein extracts; precipitates were resolved by SDS-PAGE, and both BP-1 and associated eIF-4E protein were immunoblotted with antisera specific for each. BP-1 purified from heat-shocked cells (Fig. 2A) was complexed with ~15-fold-greater levels of eIF-4E than in control (non-heat-shocked) cells. Importantly, treatment of heat-shocked cells with rapamycin blocked BP-1 phosphorylation even more strongly than either treatment alone (Fig. 2A, lane 4) and increased the amount of eIF-4E associated with BP-1 by about twofold (Fig. 2C; compare lanes 3 and 4). However, rapamycin treatment of heat-shocked cells did not significantly influence

TABLE 1. Protein synthesis during heat shock

Treatment	Protein synthetic activity[a]	
	Sp act (10^2 cpm/μg of protein/h, mean ± SE)	% Change[b] from untreated
None (untreated)	1.00 ± 0.10	
Heat shock	0.05 ± 0.02	−95
None + rapamycin (4 h)	0.92 ± 0.20	−8
Heat shock + rapamycin (4 h)	0.35 ± 0.05	−65

[a] Calculated from three independent studies.
[b] Ratio of treated sample to untreated (1 − treated/untreated × 100).

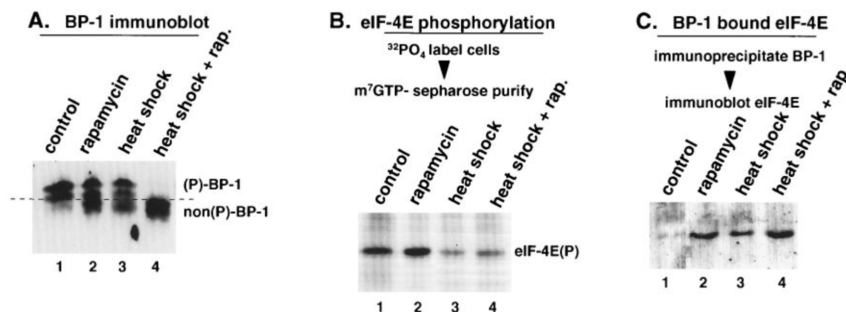

FIG. 2. Effect of heat shock on BP-1 and eIF-4E phosphorylation. 293 cells were heat shocked for 3 to 4 h at 44°C, which was found to be optimal for induction in these cells (46). Control cells were treated with rapamycin (rap.; 20 ng/ml) for 4 h. Rapamycin was added to heat-shocked cells for 4 h during a 4-h heat shock. (A) Phosphorylation state of BP-1 during heat shock. Equal amounts of protein extracts were resolved by SDS–15% PAGE and transferred to nitrocellulose, and BP-1 was detected by immunoblotting with BP-1-specific antisera. (B) Phosphorylation of eIF-4E during heat shock. Control, rapamycin-treated, and heat-shocked 293 cells were labeled with $^{32}PO_4$ for 2 h starting 2 h after heat shock, equal protein amounts were subjected to m^{7}GTP-Sepharose chromatography, and eIF-4E was eluted and resolved by SDS–15% PAGE. (C) Sequestration of eIF-4E with BP-1 during heat shock. Equal amounts of protein extract were immunoprecipitated with antisera specific for BP-1, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antisera specific for eIF-4E. Autoradiograms were quantitated by phosphor image analysis or densitometry. (P), phosphorylated.

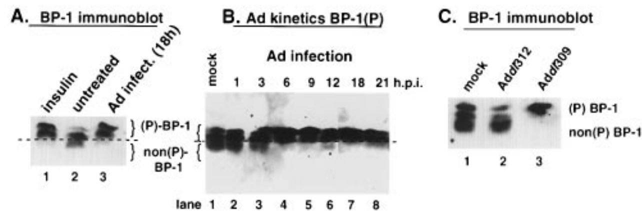

FIG. 3. Effect of Ad infection on BP-1 phosphorylation. 293 cells were infected with phenotypically wild-type Ad5*dl*309 at 25 PFU per cell. (A) Equal protein amounts of whole cell lysates were prepared from 18-h Ad-infected cells and compared with those from confluent control cells and cells treated with insulin. (B) Equal amounts of protein extracts were prepared from uninfected and Ad-infected cells at the indicated times. Extracts were resolved by SDS–15% PAGE and transferred to nitrocellulose, and BP-1 was detected by immunoblotting with BP-1-specific antisera. Hyperphosphorylated BP-1 was detected by a slower electrophoretic mobility; underphosphorylated BP-1 was detected by faster mobility. (C) Hyperphosphorylation of BP-1 by E1A protein. CHO cells were infected with 25 PFU of phenotypically wild-type Ad5*dl*309 or mutant Ad*dl*312, which does not synthesize E1A proteins, per cell. At 6 h after infection, equal amounts of cell lysates were resolved by SDS-PAGE, and BP-1 was immunoblotted with specific antisera. h.p.i., hours postinfection; (P), phosphorylated.

the ~7-fold reduction in eIF-4E phosphorylation induced by heat shock (Fig. 2B; compare lanes 3 and 4). Heat shock therefore induced both dephosphorylation of eIF-4E and its sequestration by BP-1. Identical results were also obtained for CHO cells (data not shown).

Rapamycin treatment of cells for 4 h during the course of heat shock only partially prevented the shutoff of protein synthesis (Table 1; ~65% reduction) and did not prevent heat-shock induced dephosphorylation of eIF-4E. These results therefore indicate that although eIF-4E is sequestered by BP-1 during heat shock, eIF-4E must also be dephosphorylated to fully block protein synthesis in the cells studied. It is not known why heat shock or rapamycin treatment alone did not induce complete dephosphorylation of BP-1 and complete sequestration of eIF-4E. These results suggest that there may be redundant phosphorylation sites in BP-1 that have similar abilities to interfere with eIF-4E binding and are responsive to different signalling cascades. For example, the mitogen-activated protein (MAP) kinase and $p70^{s6k}$ Rsk pathways might be differentially blocked by heat shock or rapamycin, and inhibition of only one pathway might not be sufficient to induce full sequestration of eIF-4E by BP-1. Regardless, since both eIF-4E phosphorylation and BP-1 phosphorylation were largely blocked by heat shock, studies were carried out to determine whether the control of cap-dependent protein synthesis always involves the coordinated phosphorylation and dephosphorylation of eIF-4E and BP-1.

BP-1 phosphorylation and sequestration of eIF-4E during Ad infection. Since both heat shock and late Ad infection induce the dephosphorylation of eIF-4E, the influence of Ad infection on BP-1 phosphorylation and activity was also examined. Moreover, infection of cells with picornaviruses, which like Ad block cap-dependent protein synthesis, involves the sequestration of eIF-4E by dephosphorylated BP-1 (18). The effect of heat shock and picornavirus infection on BP-1 could seemingly suggest that inhibition of cap-dependent translation generally involves dephosphorylation (activation) of BP-1. As a control for these studies, uninfected quiescent cells were treated with insulin, which induced conversion of the underphosphorylated electrophoretically fast form of BP-1 to one or two electrophoretically slower (hyperphosphorylated) forms (Fig. 3A), as shown previously (31, 39). Late Ad infection (18 h postinfection) unexpectedly induced the hyperphosphorylation of BP-1, marked by a slower electrophoretic mobility. The kinetics for phosphorylation of BP-1 protein during the course of Ad infection was examined by Western blot (immunoblot) analysis (Fig. 3B). Phosphorylation of BP-1 was found to be strongly induced early during Ad infection, initiating within 3 h and completed by 6 h in this experiment, and to be maintained throughout the course of the viral life cycle, including late phase. Identical results were obtained when Ad infection was examined in Chang and CHO cells, which are unrelated to 293 cells (data not shown).

The Ad early gene product that induces phosphorylation of BP-1 during infection was found to be the E1A protein, shown previously to induce phosphorylation of the ribosomal protein S6 in transfected cells (6). CHO cells were infected with wild-type Ad5*dl*309 or the E1A deletion mutant Ad*dl*312, and the effect on BP-1 phosphorylation was determined 6 h after infection (Fig. 3C). Whereas wild-type Ad5*dl*309 induced phosphorylation of BP-1, the E1A mutant Ad*dl*312 did not. Transfection of CHO cells with E1A expression plasmids also induced BP-1 phosphorylation (data not shown). The E1A gene expresses two related major forms of the E1A protein early during infection, a 289-amino-acid form and a truncated 249-amino-acid form, which share conserved regions 1 and 2 (CR1 and CR2) (15). Activation of the $p70^{s6k}$ pathway and phosphorylation of BP-1 during Ad infection are consistent with the results from E1A transfection, which demonstrated that the CR1 transforming region of E1A stimulates S6 protein phosphorylation (6). Identical results with wild-type and E1A mutant Ad were obtained in 293 cells (data not shown). Although 293 cells constitutively express the E1A proteins, they do not display the entire repertoire of E1A protein activities (15). Thus, it is not unusual to detect additional E1A activities during infection that are not expressed in 293 cells.

Studies next determined whether the phosphorylation of BP-1 protein during early Ad infection correlates with release of sequestered eIF-4E. The eIF-4E protein was purified from equal amounts of extracts after Ad infection by m^7GTP-Sepharose resin affinity chromatography, and the phosphorylation state of associated BP-1 was examined (Fig. 4). Only the electrophoretically fast-migrating (under- and dephosphorylated) forms of BP-1, present prior to infection, copurified with eIF-4E (lane 1). A small amount of BP-1 was not bound to eIF-4E at the zero time point, suggesting but not proving that BP-1 maybe present in excess of eIF-4E (lane 7). Phosphorylated BP-1 (slow and intermediate electrophoretic forms [lanes 8 to 12]) were not bound to eIF-4E. These results confirm that

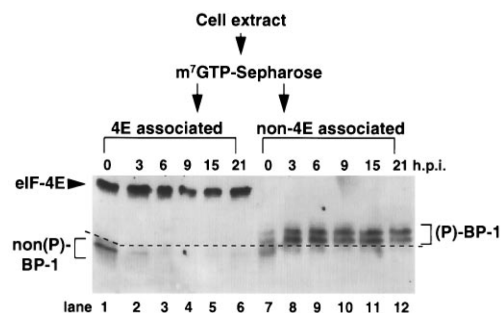

FIG. 4. Association of eIF-4E and BP-1 during Ad infection. Equal protein amounts of 293 cell extracts from Ad-infected cells were prepared at various times and resolved by m^7GTP-Sepharose chromatography, and the retained (4E-associated) and flowthrough (non-4E-associated) fractions were resolved by SDS–15% PAGE. eIF-4E and BP-1 were identified by coimmunoblotting with specific antisera for each. h.p.i., hours postinfection; (P), phosphorylated.

TABLE 2. Protein synthesis during Ad infection

Treatment	Protein synthetic activity[a]	
	Sp act (10^2 cpm/μg of protein/h, mean ± SE)	% Change[b] from untreated
None (uninfected)	1.00 ± 0.10	
Ad infected (6 h)	1.70 ± 0.20	+70
None + rapamycin (5 h)	0.95 ± 0.15	−5
Ad (6 h) + rapamycin (5 h)	0.90 ± 0.12	−10
Insulin (30 min)	1.30 ± 0.23	+30
Insulin + rapamycin (30 min)	0.92 ± 0.15	−10

[a] Calculated from three independent studies.
[b] Ratio of treated sample to untreated (1 − treated/untreated × 100).

the quantitative phosphorylation and inactivation of BP-1 occurred during the first 6 h after Ad infection, because the fast-migrating (under- and dephosphorylated) forms of BP-1 protein were no longer bound to eIF-4E. These data also confirm that BP-1 remains phosphorylated during the late phase of the viral life cycle (after 15 h). The Ad-induced phosphorylation of BP-1 therefore released eIF-4E that was sequestered prior to infection, which continued during the late phase. Inhibition (or alteration) of eIF-4F activity mediated by Ad therefore involves only dephosphorylation of eIF-4E and not its inactivation by sequestration with BP-1. Most importantly, these results show that although heat shock and late Ad infection both inactivate eIF-4E by dephosphorylation, this process is not always linked to the dephosphorylation of BP-1 and the sequestration of eIF-4E. The phosphorylation of BP-1 and its binding to eIF-4E in these two systems were oppositely regulated despite the fact that both induce dephosphorylation of eIF-4E. The results for Ad-infected and heat-shocked cells therefore indicate that the phosphorylation and dephosphorylation of eIF-4E and BP-1 are not controlled coordinately. Cap-dependent translation is therefore independently regulated by the phosphorylation state of eIF-4E and BP-1.

The phosphorylation of BP-1 during early Ad infection and the release of sequestered eIF-4E were next shown to increase eIF-4E-dependent but not eIF-4E-independent protein synthesis. Exponentially growing 293 cells were either mock or Ad infected for 6 h and then labeled for 30 min with [^{35}S]methionine in the presence or absence of rapamycin. The specific activities (protein synthetic rates) were measured and averaged for three independent experiments (Table 2). Early Ad infection induced a 70% (±10%) increase in total protein synthesis, which was blocked by treatment with rapamycin. Ad therefore induced a significant increase in total cellular protein synthesis by inactivating (phosphorylating) BP-1 and releasing eIF-4E. Although one recent study found that a 20-h treatment with rapamycin induced a 25 to 50% reduction in total protein synthesis (3), only a slight reduction was found here during a 6-h incubation of 293 cells or CHO cells (not shown), at a time when BP-1 was largely dephosphorylated. The dephosphorylation (inactivation) of BP-1 and release of sequestered eIF-4E by early Ad infection was also found to stimulate cap-dependent but not cap-independent mRNA translation. This was shown in cells transfected with plasmids encoding β-galactosidase linked to a cap-dependent or cap-independent 5′ noncoding region. Cells were then superinfected with Ad and labeled 5 h later with [^{35}S]methionine, β-galactosidase levels were determined by immunoprecipitation using specific antisera, and SDS-PAGE and autoradiography were carried out (Fig. 5). Whereas the accumulation of β-galactosidase synthesized from the cap-independent mRNA was unchanged by early Ad infection, cap-dependent translation of the β-galactosidase mRNA was stimulated four- to sixfold. Control studies showed that there was no change in the phosphorylation state of eIF-4E, as determined by in vivo labeling with $^{32}PO_4$, purification by cap affinity chromatography, and SDS-PAGE (Fig. 5B). Northern (RNA) analysis showed no change in reporter (β-galactosidase) mRNA levels during the same period of Ad infection (data not shown). These results therefore demonstrate that phosphorylation of BP-1 during early Ad infection stimulates cap-dependent but not cap-independent mRNA translation, demonstrating that the virus induces authentic phosphorylation and inactivation of BP-1.

BP-1 and eIF-4E phosphorylation pathways are distinct. The signal transduction-phosphorylation pathways that act on both BP-1 and eIF-4E have been only partially characterized. A variety of extracellular inducers, including insulin, epidermal growth factor, platelet-derived growth factor, and phorbol 12-myristate 13-acetate, costimulate BP-1 and eIF-4E phosphorylation. Moreover, eIF-4E can be phosphorylated in vitro by protein kinase C, casein kinase 1, and protamine kinase (1, 16, 27, 37, 38, 45), and other sites of regulatory phosphorylation have not been excluded. Free (uncomplexed) BP-1 was shown to be an excellent substrate for MAP kinase in vitro (22), which inhibits uncomplexed BP-1 from binding to eIF-4E (31, 39). In addition, BP-1 phosphorylation is controlled by a rapamycin-sensitive signalling pathway which involves the p70^{s6k} Rsk protein kinase and, unlike MAP kinases, acts independently of whether BP-1 is complexed to eIF-4E (3, 21, 32). Studies were therefore directed to identifying the protein kinase pathway that is responsible for continuously phosphorylating and inactivating BP-1 during Ad infection. To investigate the possible role of extracellular signal-regulated (ERK-1 and -2) MAP kinases, ERK immunoprecipitates were tested for phosphorylation of the substrate myelin basic protein. The c-Jun N-terminal MAP kinases were tested by using a glutathione *S*-transferase–N-terminal c-Jun polypeptide as the substrate. Neither MAP kinase family was activated during early infection, at a time when BP-1 was phosphorylated (data not shown).

Possible involvement of the rapamycin-sensitive p70^{s6k} Rsk signal cascade in Ad-induced BP-1 phosphorylation was next examined. Ad E1A was previously found to activate the p70^{s6k} pathway in transfected cells, and the activity was localized to CR1, the transformation domain of E1A (6). In Fig. 3C, we showed that E1A is necessary for induction of BP-1 phosphor-

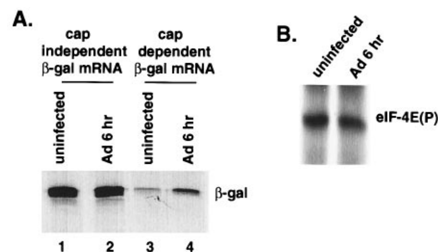

FIG. 5. Effect of Ad early infection on cap-dependent protein synthesis. 293 cells were transfected with 2 μg of plasmid DNA pCR3/βgal or pCMV-Ad/βgal, which encode β-galactosidase (β-gal) reporter mRNA linked to a cap-dependent or cap-independent 5′ noncoding region, respectively. At 24 h after transfection, cells were either left uninfected or infected with Ad for 6 h. (A) Cells were labeled with [^{35}S]methionine for 2 h at 28 h after transfection; β-galactosidase was immunoprecipitated from equal amounts of protein extracts, resolved by SDS–12% PAGE, and autoradiographed. (B) Cells were labeled with 100 μCi of $^{32}PO_4$ as described above; eIF-4E was purified by m^{7}GTP-Sepharose affinity chromatography, resolved by SDS–15% PAGE, and autoradiographed. (P), phosphorylated.

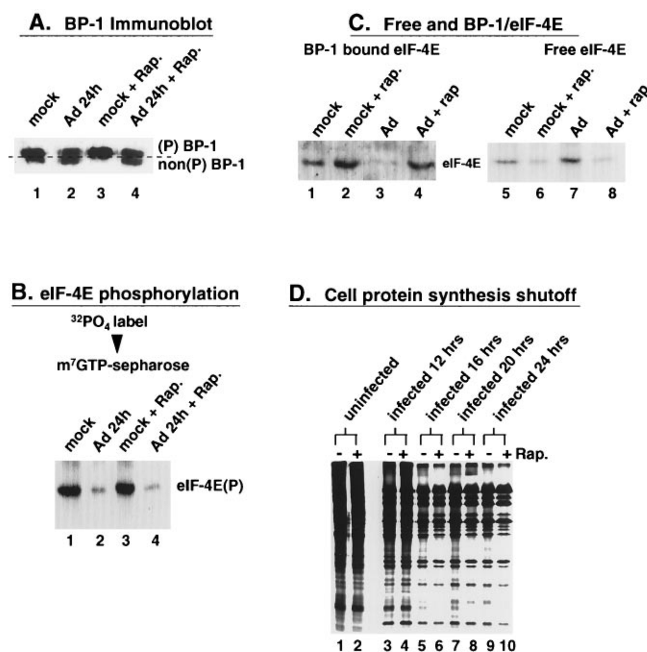

FIG. 6. Effect of rapamycin on Ad-induced dephosphorylation of eIF-4E and release from BP-1 sequestration. Uninfected and Ad-infected 293 cells were treated with rapamycin (Rap.; 20 ng/ml) starting 1 h after Ad infection for 23 h. (A) Whole cell lysates were prepared, equal protein amounts were resolved by SDS–10% PAGE, and BP-1 was detected by immunoblotting with specific antisera to BP-1. (B) Uninfected and 22-h Ad-infected cells (with or without rapamycin treatment) were metabolically labeled with 100 μCi of $^{32}PO_4$ for 2 h, equal amounts of extracts were applied to m^{7}GTP-Sepharose, and eIF-4E was eluted and resolved by SDS–15% PAGE followed by autoradiography. (C) Uninfected and 24-h Ad-infected 293 cells were treated for 23 h with rapamycin, and BP-1 was immunoprecipitated from equal protein amounts. Free eIF-4E not associated with BP-1 was recovered from the soluble fraction by m^{7}GTP-Sepharose chromatography. BP-1 bound (immunoprecipitated) and free eIF-4E were resolved by SDS–15% PAGE and identified by immunoblotting with eIF-4E-specific antisera. (D) Uninfected and Ad-infected cells (with or without rapamycin treatment) at 1 h after infection were labeled with 50 μCi of [^{35}S]methionine per ml for 1 h. Equal protein amounts of extracts were resolved by SDS–15% PAGE and visualized by phosphor image analysis. Quantitation was performed by phosphor image analysis.

ylation. The ability of E1A to stimulate BP-1 phosphorylation through the p70^{s6k} pathway in Ad-infected cells was therefore examined next. Ad-induced phosphorylation of BP-1 was found to be sensitive to inhibition by rapamycin (Fig. 6A). Phosphorylation of BP-1 was measured by the appearance of electrophoretically slower (phosphorylated) bands in uninfected and late Ad-infected cells, with or without rapamycin treatment. In this particular experiment, BP-1 was electrophoresed in a low-resolution gel that separated phosphorylated and nonphosphorylated species of the protein into only two bands. Rapamycin added at 1 h (Fig. 6A) or 7 h (data not shown) after Ad infection prevented BP-1 phosphorylation measured at 22 h after infection. These results indicate that phosphorylation of BP-1 involves a p70^{s6k} Rsk pathway that continuously phosphorylates BP-1.

Studies next examined whether the dephosphorylation of eIF-4E during late Ad infection is blocked by inhibition of the p70^{s6k} Rsk pathway, which would indicate whether eIF-4E complexed to BP-1 is still a target for phosphatase activity. Cells were labeled with $^{32}PO_4$, and the phosphorylation state of eIF-4E was examined by cap affinity chromatography (Fig. 6B). Phosphorylation of eIF-4E was found to be strongly decreased during late Ad infection regardless of rapamycin treatment (Fig. 6B), indicating that the pathways for phosphorylation of BP-1 and eIF-4E are distinct. Although rapamycin treatment did not prevent dephosphorylation of eIF-4E induced by Ad during late infection, it did promote a large increase in the amount of eIF-4E sequestered by BP-1. This was shown by immunoprecipitating BP-1 from late Ad-infected cells (with or without rapamycin treatment) and then detecting the complexed eIF-4E by immunoblotting the immune pellet with 4E-specific antisera (Fig. 6C). The uncomplexed eIF-4E was detected by retrieving it via cap affinity chromatography of the immune pellet wash. It was apparent that the quantitatively dephosphorylated eIF-4E (Fig. 6B, lanes 2 and 4) was complexed with BP-1 in mock- and late Ad-infected cells treated with rapamycin (Fig. 6C, lanes 2 and 4) but not in the untreated Ad-infected cells (lane 3). The level of free eIF-4E (not associated with BP-1 [lanes 5 to 8]) was inversely proportional to the level of eIF-4E associated with BP-1, demonstrating dissociation of eIF-4E from BP-1. The slightly lower total level of eIF-4E detected in the free eIF-4E fractions (lanes 5 to 8) is probably a result of less efficient recovery of eIF-4E by cap affinity chromatography than by specific immunoprecipitation. Ad inhibition of cellular protein synthesis was also not prevented by rapamycin (Fig. 6D), consistent with the inability to block Ad dephosphorylation of eIF-4E. This was shown by infecting cells, treating them with rapamycin at 1 h postinfection, and then analyzing [^{35}S]methionine-labeled polypeptides at various times after infection for the inhibition of host protein synthesis (Fig. 6D). Inhibition of BP-1 phosphorylation by rapamycin actually slightly enhanced Ad shutoff of cell translation, evidenced by the more rapid inhibition of cell protein synthesis during transition into the late phase of infection at 16 h. This result is not surprising in that rapamycin prevents BP-1 phosphorylation, which then sequesters eIF-4E into an inactive complex. Taken collectively, these results indicate that eIF-4E bound to underphosphorylated or dephosphorylated BP-1 is as efficient a target for dephosphorylation as eIF-4E not complexed to BP-1.

DISCUSSION

In this report, we show that cap-dependent mRNA translation is independently regulated by the phosphorylation state of eIF-4E and BP-1 and that BP-1 is not obligatorily involved in all mechanisms for inhibition of cap-dependent translation. Therefore, the activity of eIF-4E regulated by its phosphorylation state and the sequestration of eIF-4E regulated by sequestration with BP-1 do not comprise a coordinated mechanism for controlling eIF-4E-dependent initiation of protein synthesis. Our studies indicate that mechanisms of control of eIF-4E and BP-1 activities by phosphorylation represent distinct, independent mechanisms for controlling cap-dependent protein synthesis that can act either in concert or in opposition to one another. This was an unexpected result in that mitogens and growth factors which stimulate protein synthesis (e.g., epidermal growth factor, platelet-derived growth factor, phorbol 12-myristate 13-acetate, and insulin) also induce activating phosphorylation of eIF-4E and inactivating phosphorylation of BP-1, which blocks BP-1 sequestration of eIF-4E (2, 3, 32, 34, 39).

The states of eIF-4E and BP-1 phosphorylation were examined during heat shock and late Ad infection, two systems in which cap-dependent mRNA translation is blocked by inhibiting eIF-4E phosphorylation. Heat shock blocked cap-dependent translation by inducing both eIF-4E dephosphorylation and sequestration of dephosphorylated eIF-4E by BP-1 (Fig. 2). Thus, these results could implicate coordinated dual control

in the cell over eIF-4E and BP-1 phosphorylation. Nevertheless, rapamycin inhibited BP-1 sequestration of eIF-4E during heat shock without blocking heat shock-induced dephosphorylation of eIF-4E (Fig. 2B and C). In this regard, rapamycin only partially blocked heat shock-induced shutoff of cell protein synthesis (Table 1). These findings support a dual role for eIF-4E dephosphorylation and sequestration by BP-1 in blocking protein synthesis during heat shock. These results are also consistent with the fact that heat shock induced less severe dephosphorylation of eIF-4E than did late Ad infection in the cell lines investigated (compare Fig. 2B and 6B). It is conceivable that different cell types differentially balance the extent to which eIF-4E dephosphorylation or sequestration by BP-1 occurs during heat shock.

Further evidence that BP-1 and eIF-4E are not coregulated was derived from studies using Ad-infected cells. We showed that early during infection, the virus induces phosphorylation of BP-1 and release of sequestered eIF-4E (Fig. 3 and 4), which stimulated cap-dependent protein synthesis (Fig. 5 and Table 2). The early Ad-induced phosphorylation of BP-1 persisted into the late phase of infection, at a time when Ad also blocks eIF-4E phosphorylation. In addition, rapamycin was found to block Ad-induced phosphorylation of BP-1 without influencing the dephosphorylation of eIF-4E, and eIF-4E complexed with BP-1 was shown to be as efficient a target for dephosphorylation as free eIF-4E (Fig. 6). The results for heat-shocked and Ad-infected cells therefore indicate that the regulation of eIF-4E by cycles of phosphorylation and dephosphorylation and the regulation of BP-1 by phosphate turnover are not always linked. All reported mechanisms for the induced phosphorylation of BP-1, including that of Ad infection presented here, apparently converge on the activation of the same signal transduction pathway, that involving the $p70^{s6k}$ Rsk protein kinase. Our results demonstrating that E1A induces BP-1 phosphorylation, and those reported previously for S6 phosphorylation (6), also indicate that the increased protein synthetic rate during early Ad infection is attributable to E1A activity. E1A activation of the $p70^{s6k}$ pathway appears to reside in the CR1 transforming domain (6), implicating a possible role for stimulation of protein synthesis in the wide variety of cell physiological effects associated with E1A transformation.

The fact that the phosphorylation state of eIF-4E is controlled independently of the availability (sequestration) of eIF-4E by BP-1 perhaps suggests two possible purposes in the cell. (i) Independent regulation of eIF-4E and BP-1 might permit the two regulatory systems to act in concert or in opposition, as found in heat-shocked and late Ad-infected cells, respectively. It is conceivable that this provides the cell with a fine-tuning mechanism for translational selection of mRNAs based on their dependence for eIF-4F. (ii) Independent regulation of eIF-4E and BP-1 could instead (or in addition) indicate that these two mechanisms enact fundamentally different types of control over cap-dependent translation, perhaps for different purposes. For instance, the in vivo rate of eIF-4E dephosphorylation, which correlates with reduced translation activity (44), is rapid and averages ~20 min in fibroblasts (48). The rate of BP-1 dephosphorylation in response to protein synthesis inhibitors (heat shock or serum withdrawal) has not been rigorously investigated. In preliminary studies, we have found that growing cells must be treated with rapamycin for more than 3 h to sequester eIF-4E, suggesting that phosphorylation of BP-1 might rapidly activate translation in mitogen-treated quiescent cells, but dephosphorylation of BP-1 might inactivate cap-dependent translation more slowly than dephosphorylation of eIF-4E. Future studies need to address whether BP-1 and eIF-4E represent strategies for maintaining, respectively, long-term and short-term translational control.

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ADDENDUM IN PROOF

Similar findings on control of BP-1 during Ad infection have been obtained by N. Sonenberg and colleagues (personal communication).

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