Translation initiation factor 4E (eIF4E) is a cytoplasmic cap-binding protein that is required for cap-dependent translation initiation. Here, we have shown that eIF4E is ubiquitinated primarily at Lys-159 and incubation of cells with a proteasome inhibitor leads to increased eIF4E levels, suggesting the proteasome-dependent proteolysis of ubiquitinated eIF4E. Ubiquitinated eIF4E retained its cap binding ability, whereas eIF4E phosphorylation and eIF4G binding were reduced by ubiquitination. The W73A mutant of eIF4E exhibited enhanced ubiquitination/degradation, and 4E-BP overexpression protected eIF4E from ubiquitination/degradation. Because heat shock or the expression of the carboxyl terminus of heat shock cognate protein 70-interacting protein (Chip) dramatically increased eIF4E ubiquitination, Chip may be at least one ubiquitin E3 ligase responsible for eIF4E ubiquitination.

The eukaryotic mRNA cap (m7GTP) is a highly conserved structure located at the 5′-end of RNA molecule that plays an essential role in regulating mRNA decay, compartmentalization, maturation, and translation initiation (1). In the cytoplasm, eukaryotic translation initiation factor 4E (eIF4E) specifically binds the mRNA cap structure and regulates cap-dependent translation initiation as a component of the eIF4F complex, which also includes the docking protein eIF4G and the ATP-dependent helicase eIF4A (2–4). The eIF4F complex enhances the assembly of the other initiation factors, such as eIF3, mitogen-activated protein kinase (MAPK) signal-integrating kinase, and the 40S ribosomal subunit. Disruption or overproduction of eIF4E leads to aberrant cell growth or oncogenesis (4), demonstrating the importance of its availability for cellular protein synthesis.

Despite the importance of eIF4E, little is known about the regulation of eIF4E protein expression levels. Transcription of the gene is induced in response to serum, growth factors (5), or immunological activation in T cells (6). Additionally, cellular differentiation state also affects levels of the protein (7, 8). The eIF4E promoter contains two c-Myc-binding sites (9) and a heterogeneous nuclear ribonucleoprotein K-binding site (10), both of which are critical for the transcriptional regulation of eIF4E. However, the rate and mechanism of eIF4E degradation remain unclear, except that Othumpangat et al. (11) reported that eIF4E is ubiquitinated and degraded in a proteasome-dependent manner in response to heavy metal.

Ubiquitin (Ub), a low molecular weight polypeptide composed of 76 amino acids, can be covalently conjugated to Lys residues in target proteins (12, 13). Ub conjugation is a well-coordinated event involving several classes of enzymes, including ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). Protein ubiquitination is a signal for targeted recognition and ATP-dependent proteolysis by the 26S proteasome. The Ub–proteasome pathway is an important factor controlling the expression and activity of regulatory proteins such as transcription factors or oncogenes. Here, we have shown detailed analysis of eIF4E ubiquitination and proteasome-dependent degradation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Antibodies, and Reagents**—Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml of nonessential amino acids (Invitrogen), and penicillin and streptomycin sulfate (Invitrogen). Rabbit anti-eIF4E and mouse anti-phospho-eIF4E antibodies were purchased from Cell Signaling Technology (Beverly, MA), and mouse anti-tubulin antibody was from Oncogene Research Products (San Diego, CA). Mouse anti-Myc antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse and rabbit anti-FLAG antibodies were from Sigma. Mouse and rat anti-HA antibodies were obtained from Roche Applied Science. Horseradish peroxidase-linked goat antibodies to mouse or rabbit IgG were from Amersham Biosciences. Horseradish peroxidase-linked goat antibody to rat IgG was...
acquired from Jackson ImmunoResearch. MG132 was purchased from Peptide Institute (Osaka, Japan).

**Plasmid Construction**—For cloning human cDNAs, total RNA was prepared from HEK293T or Huh-7 cells and amplified by reverse transcription PCR. The coding region of human eIF4E was obtained and was cloned into the vector pcDNA3 or pcDNA3-FLAG (14) to generate pceIF4E or pcFLAGeIF4E, respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively. To prepare pcMycChip and pcMyc4E-BP1–3, the coding regions of human Chip and 4E-BP1–3 were amplified respectively.

RESULTS

**Ubiquitination and Proteasome-dependent Degradation of eIF4E**—To confirm ubiquitination of eIF4E in cells, we transfected HEK293T cells with FLAG-tagged eIF4E (pcFLAGeIF4E) and/or HA-tagged Ub (pcHAUb). Cellular proteins were then subjected to immunoprecipitation (IP) with anti-FLAG antibody, followed by immunoblotting (IB) with anti-HA antibody (Fig. 1A, top panel). When both eIF4E and Ub were produced, mono- (eIF4E-Ub) or poly- (eIF4E-Ub) ubiquitinated forms of eIF4E appeared (Fig. 1A, top panel, lane 4). The arrows in Fig. 1 depict the estimated size for non-ubiquitinated eIF4E. Ubiquitination was not detectable when either eIF4E or Ub alone was expressed (Fig. 1A, top panel, lanes 2 and 3), demonstrating the specific linkage of Ub and eIF4E. The membrane was stripped and probed with anti-FLAG antibody to confirm the successful precipititation of eIF4E protein (Fig. 1A, 2nd panel). Additionally, all transfected proteins were expressed and gels were loaded equally as shown in Fig. 1A, 3rd, 4th, and bottom panels. Non-ubiquitinated FLAFlageIF4E protein resolved around 27 kDa and the mono-ubiquitinated form was around 37 kDa. Additionally, we were able to detect mono-ubiquitinated eIF4E in whole cell extracts (WCE) (Fig. 1B, top panel, lane 3).

The ubiquitination of eIF4E may be affected by its phosphorylation, cap binding, and eIF4G binding. We generated mutant forms of eIF4E to further examine this issue (Fig. 1C). We mutated Ser-209, the eIF4E residue phosphorylated by MAPK signal-integrating kinase (16), Trp-56, a residue located within the eIF4E hydrophobic pocket, required for cap binding (17), and Trp-73, a residue essential for the interaction of eIF4E with eIF4G/4E-BP (18). Although the ubiquitination of either S209A or W56A eIF4E was comparable with that seen with WT eIF4E, the ubiquitination of the W73A mutant was dramatically increased (Fig. 1C, top panel, lane 6), suggesting a role of eIF4G/4E-BP association in regulating eIF4E ubiquitination.

Ubiquitinated proteins are typically degraded by the proteasome, and we used MG132, a proteasome inhibitor, to examine whether eIF4E was degraded in this manner (Fig. 1, D and E). When cells co-transfected with pcFLAGeIF4E and pcHAUb were treated with MG132 for 12 h, we observed the accumulation of polyubiquitinated forms of eIF4E (Fig. 1D, top panel, lane 4). Mono-ubiquitinated eIF4E became less prominent with MG132 (Fig. 1D, top panel, lane 4), presumably because of the accumulation of other ubiquitinated target proteins.

Although the levels of eIF4E mutants were almost comparable at 24 h after transfection (e.g. Fig. 1C, middle panel), the band for W73A eIF4E became very faint when incubated for 48 h (Fig. 1E, lane 4) or more (not shown). We assume the shorter half-life of the mutant protein is relevant to the enhanced ubiquitination (Fig. 1C, top panel, lane 6). At 24 h, degradation of the protein is not evident because it is compensated by the massive production from efficient plasmid vector, whereas at 48 h, the production becomes weaker and the degradation becomes apparent (see “Discussion”).

Treatment with MG132 increased levels of both WT and W73A mutant eIF4E (Fig. 1E). MG132 was added at 24 h after transfection because it is toxic for cells when administered for more than 24 h. These results clearly demonstrate that eIF4E is ubiquitinated and degraded in a proteasome-dependent manner and suggest that the interaction of eIF4E with eIF4G/4E-BP is important for the regulation of eIF4E ubiquitination/degradation.
FIGURE 1. Ubiquitination and proteasome-dependent degradation of eIF4E. A, ubiquitination of eIF4E. HEK293T cells were transfected with pcFLAGeIF4E and/or pcHAUb. 24 h after transfection, cell lysates were immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitates were subjected to SDS-PAGE and analyzed by immunoblotting (IB) with anti-HA antibody (top panel). The membrane was then stripped and reprobed with anti-FLAG antibody (2nd panel). A portion of the whole cell extracts (WCE) was directly subjected to SDS-PAGE, and IB was performed with anti-HA (3rd panel), FLAG (4th panel), or tubulin (bottom panel) antibody. B, detection of eIF4E ubiquitination without IP. Cells transfected with pcFLAGeIF4E and/or pcHAUb were subjected to SDS-PAGE, followed by IB with anti-FLAG (upper) or tubulin (lower) antibody. The upper panel was overexposed to detect mono-ubiquitinated eIF4E. C, effect of mutations on ubiquitination. Cells transfected with pcHAUb and/or wild-type (WT) pcFLAGeIF4E or the mutant forms of pcFLAGeIF4E were lysed for IP with anti-FLAG antibody, followed by IB with anti-HA antibody (top). The membrane was then stripped and reprobed with anti-FLAG antibody (middle). Levels of tubulin in the WCE were examined as a loading control (bottom). D, effect of MG132 on eIF4E ubiquitination. 24 h after transfection, cells were treated with MG132 or Me2SO. At 12 h after the addition of MG132, cells were harvested for IP with anti-FLAG antibody, followed by IB with anti-HA antibody (top). The membrane was then stripped and reprobed with anti-FLAG antibody (middle). Tubulin was used as a loading control (bottom). E, effect of MG132 on eIF4E levels. Cells were transfected with WT or W73A mutant of pcFLAGeIF4E. 24 h after transfection, cells were treated with MG132 (5, 10 μM) and incubated for another 24 h. WCE was subjected to SDS-PAGE and IB with anti-FLAG (upper) or tubulin (bottom) antibody. HC and LC denote bands for IgG heavy chain and light chain. e.v., empty vector. Arrow, estimated size for non-ubiquitinated eIF4E. eIF4E-Ub, mono-ubiquitinated eIF4E; eIF4E-Ubn, polyubiquitinated eIF4E.
Identification of Residues Required for eIF4E Ubiquitination—Target proteins are ubiquitinated on Lys residues, and eIF4E contains 16 Lys residues. To identify the particular residue(s) that become ubiquitinated, we generated a series of Lys to Arg (K/R) mutants (Fig. 2A, lanes 1–5). However, none of these 5 K/R mutants exhibited decreased ubiquitination compared with WT eIF4E, suggesting that multiple Lys residues may be ubiquitinated. Therefore, we next prepared an expression construct, pcFLAgeIF4EKallR, in which all Lys residues were mutated to Arg (Fig. 2A, lane 10), and, as expected, this mutant was hardly ubiquitinated.

We subsequently generated 16 plasmids in which a single Lys residue of the KallR mutant was restored while the other Lys residues remained mutated (Fig. 2B, lanes 4–7, Fig. 2C, lanes 3–15).
Interestingly, restoration of a single Lys, Lys-159, restored the ubiquitination of eIF4E to the same extent as WT (Fig. 2B, lanes 2 and 4). Thus, it is likely that Ub conjugation occurs primarily at Lys-159, although other Lys residues become Ub modified in the absence of this residue (Fig. 2A, lane 3).

**Effect of eIF4E Ubiquitination on m^7GTP Association**—Because cap binding is essential for eIF4E function, we examined eIF4E m^7GTP binding using m^7GTP-Sepharose (Fig. 3). FLAG-tagged eIF4E mutants were expressed in cells, and m^7GTP-binding proteins were precipitated, followed by detection by IB. As expected, a W56A eIF4E mutant only weakly associated with m^7GTP (Fig. 3A, lane 4) (17), but the other eIF4E mutants examined, including KallR and K159R, all bound m^7GTP as efficiently as WT eIF4E (Fig. 3A).

We next wished to examine whether ubiquitinated eIF4E was capable of binding the cap structure. Cells were transfected with pcFLAGeIF4E mutants together with pcHAUb, and m^7GTP-bound proteins were detected using anti-HA (Fig. 3B, upper left panel) and anti-FLAG (upper right panel) antibodies. The anti-HA antibody detected both mono- and polyubiquitinated proteins (Fig. 3B, upper left, lanes 2 and 5), and when precipitated material was blotted with anti-FLAG at least mono-ubiquitinated WT and K159R eIF4E were seen (Fig. 3B, upper right, lanes 2 and 5, arrowhead). Protein expression was...
confirmed in the lower panels of Fig. 3B. Ubiquitinated W56A and KallR eIF4E were not precipitated with m7GTP (Fig. 3B, upper panels, lanes 3 and 4), because the cap binding and ubiquitination of W56A and KallR mutants, respectively, are very low. Thus, these results suggest that ubiquitinated eIF4E remains capable of binding the cap structure.

Effect of eIF4E Ubiquitination on Phosphorylation—We next wished to examine the possible relationship between eIF4E ubiquitination and phosphorylation, and we used a phospho-eIF4E antibody for these studies. As expected, this antibody did not react with the phosphorylation mutant S209A, although the WT and W56A mutants were phosphorylated (Fig. 4A, top panel, lanes 2–4). W73A also failed to be phosphorylated (Fig. 4A, top panel, lane 5). This is consistent with published reports indicating that MAPK signal-integrating kinase, the kinase responsible for eIF4E phosphorylation, is recruited by eIF4G (18). The KallR mutant showed little or no phosphorylation, whereas levels of K159R phosphorylation were normal (Fig. 4B, upper panel, lanes 4 and 5).

We next examined whether ubiquitinated eIF4E was phosphorylated. As depicted in the upper right panel of Fig. 4C, despite gross overexposure, mono-ubiquitinated eIF4E was not seen to be phosphorylated (Fig. 4C, upper right, lane 2, arrowhead), whereas ubiquitination of eIF4E in the same samples was evident when blotted with anti-HA or anti-FLAG antibodies (Fig. 4C, upper left and middle panels). Some faint bands were visible above 37 kDa in Fig. 4C (upper right panel), but we assume these are nonspecific. Therefore, we conclude that phosphorylation
and ubiquitination may not occur simultaneously in a single eIF4E molecule. However, we cannot exclude the possibility that levels of eIF4E phosphorylation are very low if the molecule is modified with Ub.

Effect of eIF4E Ubiquitination on eIF4G Binding—Because the association of eIF4E with eIF4G is essential for cap-mediated translation initiation, we analyzed the binding of eIF4E to eIF4G by IP and IB. HA-tagged eIF4G and FLAG-tagged eIF4E mutants were produced in cells, together with Myc-tagged Ub. eIF4G was isolated with anti-HA antibody, and eIF4G-associated eIF4E was detected with anti-FLAG antibody (Fig. 5A, top, 2nd panel). Membranes were stripped and reprobed with anti-HA antibody (Fig. 5A, 3rd panel).

When WT FLAG-tagged eIF4E was co-expressed with eIF4G and Ub, only non-ubiquitinated eIF4E co-precipitated with eIF4G, even upon gross overexposure of the blot (Fig. 5A, 1st and 2nd panels, lanes 4–9). As expected, the W73A mutant of eIF4E did not associate with eIF4G, but the association of KallR mutant with eIF4G was also weak (Fig. 5A, top panel, lanes 7 and 8). As a control, we expressed FLAG-tagged WT eIF4E with Myc-tagged Ub and precipitated total eIF4E with anti-FLAG. As seen in Fig. 5A, 2nd panel, lane 1, at least mono-ubiquitinated eIF4E was detectable under these conditions, but ubiquitinated eIF4E was not found in the eIF4G-associated fractions (Fig. 5A, 2nd panel, lanes 4–9).

As an additional control, we confirmed that the expression of eIF4G did not suppress eIF4E ubiquitination (Fig. 5B, top panel, lane 3). Thus, it is likely that eIF4G does not bind ubiquitinated forms of eIF4E with any appreciable affinity.

4E-BP Binding Suppresses eIF4E Ubiquitination and Degradation—4E-BP proteins bind eIF4E and negatively regulate cap-dependent translation initiation. To examine whether 4E-BP affects the ubiquitination of eIF4E, three 4E-BP isoforms were cloned, Myc tagged, and transfected into cells with Ub and eIF4E. Although mono-ubiquitinated eIF4E was clearly detected in the absence of 4E-BP (Fig. 6A, lanes 2, 5, and 8), 4E-BP production eliminated the observed ubiquitination (lanes 3, 6, and 9). Additionally, only non-ubiquitinated eIF4E was associated with 4E-BP (supplemental Fig. S1).

FIGURE 5. eIF4G association of eIF4E. A, association between eIF4E mutants and eIF4G. Lysates from cells transfected with WT or mutant forms of pcFLAGeIF4E, pcDNA3HaeIF4G, and pcMycUb were precipitated with anti-HA antibody (lanes 2–9) and subjected to IB with anti-FLAG (top and 2nd panels) or HA (3rd panel) antibody. To assess ubiquitination levels of eIF4G-associated eIF4E, the membrane in the top panel was overexposed in the 2nd panel. For lane 1, cell lysates transfected with pcFLAGeIF4E and pcMycUb were precipitated with anti-FLAG antibody and loaded for the top and 2nd panels to examine the levels of eIF4E ubiquitination. Levels of eIF4G (4th panel), Ub (5th panel), or tubulin (bottom panel) in the WCE were checked using anti-FLAG, Myc, or tubulin antibody, respectively. B, effect of eIF4G overexpression on eIF4E ubiquitination. Lysates from cells transfected with WT pcFLAGeIF4E, pcDNA3HaeIF4G, and/or pcMycUb were precipitated with anti-FLAG antibody and subjected to IB with anti-FLAG (top panel) antibody. Levels of eIF4G (2nd panel), eIF4E (3rd), Ub (4th), or tubulin (bottom) in the WCE were assessed using anti-HA, FLAG, Myc, or tubulin antibody, respectively.
We next determined whether 4E-BP expression affected eIF4E degradation (Fig. 6B). As depicted in Fig. 1E, eIF4E protein levels were lower in the absence of MG132 (Fig. 6B, top panel, lane 1), whereas levels were increased by the addition of MG132 (Fig. 6B, top panel, lane 2). Interestingly, when 4E-BP1, 2, or 3 was co-expressed, eIF4E levels in the absence of MG132 were almost comparable with those with the inhibitor (Fig. 6B, top panel, lanes 3, 5, and 7), suggesting that 4E-BP proteins inhibit the degradation of eIF4E. Taken together with the observation that the W73A mutant shows enhanced ubiquitination, these data strongly suggest that 4E-BP binding negatively regulates eIF4E ubiquitination and degradation.

Ubiquitination—Ubiquitination requires the concerted action of a series of enzymes, and actual Ub addition is performed by E3 ligases. We wished to determine the E3 ligase responsible for eIF4E ubiquitination, and we tested Chip, a well-characterized E3 ligase. When cells were co-transfected with Chip and WT, S209A, W56A, and K159R eIF4E mutants, we observed the enhanced ubiquitination of all constructs (Fig. 7A, top panel). Additionally, KallR was ubiquitinated when Chip was co-expressed in cells (Fig. 7A, lane 5). Because there were no Lys residues in this eIF4E mutant, we speculate that the amino-terminal Met residue of this protein may be ubiquitinated (19) or that the FLAG epitope contains a Lys residue that could be Ub modified. Ubiquitination of the W73A mutant was not clearly increased (Fig. 7A, lane 13), but ubiquitination levels of this mutant are likely saturated. A K30A Chip mutant that is unable to interact with Hsc70 (20) did not enhance eIF4E ubiquitination (Fig. 7B, lane 4), whereas WT Chip did (lane 3).

We next wished to determine whether eIF4E and Chip directly interacted by IP/IB. As shown in Fig. 8A, all forms of eIF4E examined interacted with Chip (Fig. 8A, top panel, lanes 3–8). Interestingly, eIF4E did not associate with the K30A Chip mutant (Fig. 8B, lane 4), suggesting that Chip associates with eIF4E via Hsc70. These data indicate that eIF4E can be ubiquitinated by Chip, possibly in an Hsc70-dependent manner.

Heat Shock-enhanced eIF4E Ubiquitination—Cells respond to certain stimuli through regulating protein synthesis levels (21, 22). Generally, under conditions of cell stress or apoptosis, cap-mediated translation is suppressed, whereas cap-independent, internal ribosome entry site-dependent translation increases. To clarify whether eIF4E ubiquitination plays a role in regulating these changes, we first examined levels of ubiquitination in heat-shocked cells (Fig. 9A). When cells were heat shocked (45 °C, 10 min), Ub conjugation became evident 2 h after heat shock (Fig. 9A, top panel, lane 4), and levels then returned to normal after 4–8 h (Fig. 9A, lanes 6, 8, and 10). Heat shock also induced KallR mutant ubiquitination (Fig. 9B, top panel, lane 7).
Heat shock and Chip expression could additively or synergistically enhance eIF4E ubiquitination. We co-transfected cells with FLAG-tagged eIF4E, HA-Ub, and/or Myc-Chip, and samples were collected 2 h after heat shock (45 °C 10 min) (Fig. 9). Although heat shock or Chip expression independently induced the ubiquitination of eIF4E (Fig. 9C, top panel, lanes 3 and 4), the combination of heat shock and Chip expression did not increase ubiquitination levels (lane 5). It is likely that each condition alone induced maximal eIF4E ubiquitination.

Ubiquitination and Degradation of Endogenous eIF4E—We then wished to see the ubiquitination of endogenous eIF4E (Fig. 10A). It was not visible under normal condition, but mono-ubiquitinated endogenous eIF4E was detected (Fig. 10A, middle panel, lane 3) when cells were heat shocked, eIF4E protein was concentrated by m7GTP-Sepharose precipitation, and highly effective ECL solution was used. The 24-kDa band for endogenous eIF4E whitened out (Fig. 10A, middle panel) because the ECL solution was too strong.

In Fig. 10, B and C, we carried out pulse-chase labeling of endogenous eIF4E. With heat shock, the levels of eIF4E decreased more rapidly (down to 55% at 15 h) and addition of MG132, an inhibitor of proteasome-dependent degradation pathway, restored the levels. These results indicate that endogenous eIF4E protein is also degraded in the Ub/proteasome-dependent manner.

DISCUSSION

Protein synthesis is a tightly controlled process essential for cell survival, and in this study we described the ubiquitination and proteasome-dependent degradation of the cap-binding protein eIF4E. We summarize our working hypothesis in supplemental Fig. S2.

We identified the most probable site for Ub conjugation as Lys-159. The crystal structure of eIF4E has been solved (23–25), and Lys-159 is in the middle of two β-sheets, S5 and S6. The side chain of this residue protrudes outward (25), suggesting it is readily accessible for Ub conjugation by E3 ligases. Interestingly, there is a relationship between Lys-159 and the phosphorlated residue Ser-209. Phosphorylation of Ser-209 causes a retractable salt bridge to form with Lys-159 to clamp the cap moiety and thereby lead to increased binding of capped mRNA (23, 26). In addition, because the K159A mutant exhibited reduced association with cap analogues (27), Lys-159 is involved in the binding of capped mRNA. However, we saw no reduction in m7GTP association with the K159R mutant (Fig. 3). Additionally, we did not observe reduced m7GTP association with ubiquitinated eIF4E. The attachment of a Ub molecule to Lys-159 might stabilize the distance between Ser-209
(S7-S8 loop) and Lys-159 (S5-S6 loop), similar to phosphorylation (23). Alternatively, the Ub molecule may form a bridge between Ser-209 (S7-S8 loop) and Lys-159 (S5-S6 loop) and hold the triphosphate moiety tightly. Additionally, ubiquitination of the S209A mutant of eIF4E was comparable with WT, suggesting that phosphorylation does not affect ubiquitination (Fig. 1C).

The W73A mutant of eIF4E, which only weakly binds eIF4G/4E-BP, showed enhanced ubiquitination and proteasome-dependent degradation (Fig. 1). What is more, expression of 4E-BP clearly reduced the levels of eIF4E ubiquitination and degradation (Fig. 6). Because the only known role for 4E-BP has been as inhibitor of cap-mediated translation initiation, we are suggesting a novel role for 4E-BP as a protector of eIF4E. 4E-BP can inhibit cap-mediated protein synthesis by binding eIF4E, but at the same time may preserve a population of inactive eIF4E-mRNA complexes by preventing eIF4E degradation. Several reports have linked 4E-BP to cellular stress (28–30). 4E-BP binds eIF4E under conditions of stress or reduced growth stimuli. When cells are removed from stress and growth conditions become favorable,
pre-existing eIF4E-mRNA complexes can be used for immediate protein synthesis after 4E-BP release. Thus, 4E-BP buffers stress by suppressing protein synthesis and preparing cells for a swift recovery.

It is quite interesting to find that the levels of endogenous eIF4E ubiquitination were very low (e.g. Fig. 10A), whereas degradation of the protein was relatively clear (e.g. Fig. 10, B and C). Ubiquitinated fraction was <1% in Fig. 10A, whereas the eIF4E decreased by 45% at 15 h after heat shock in Fig. 10, B and C. This is similar to the ubiquitination/degradation of the W73A mutant in that most of the W73A mutant protein stayed non-ubiquitinated (Fig. 1C, middle panel, lane 6), whereas >90% of eIF4E degraded at 48 h (Fig. 1E, upper panel, lane 4). We thus speculate that the efficiency of Ub conjugation is the bottleneck, and once ubiquitinated, it is proteolyzed rapidly.

The mechanism of eIF4E ubiquitination/degradation is similar to that of the endoplasmic reticulum membrane-tethered dolichol-phosphate-mannose (DPM) synthase (31). DPM1 is tethered by DPM3 to the membrane. When tethering is abolished and DPM1 becomes free, DPM1 is rapidly ubiquitinated by Chip and degraded by the proteasome. Telomeric repeat binding factor 1 and E2F transcription factors are also protected from Ub targeting and degradation by binding their partners, and these proteins are degraded after binding partner release (32, 33). Because these proteins function as complexes, it is likely important to regulate the levels of the free proteins. Thus, this may be a common mechanism of protein expression regulation in cells.

When examining the bottom panel of Fig. 6B, levels of 4E-BP, especially 4E-BP2 and 3, were increased by the addition of MG132. This suggests that 4E-BP is degraded in a Ub/proteasome-dependent manner. As 4E-BP is an important regulator of protein synthesis, the regulation of this process is of great interest.

During the preparation of this report, Othumpangat et al. (11) reported that eIF4E is proteolyzed after ubiquitination when cells are exposed to cadmium chloride. We confirmed this finding (data not shown), and because cadmium presumably acts as a cell stressor, this finding supports our hypothesis that Ub modification of eIF4E is enhanced following cell stress.

**FIGURE 10.** **A**, ubiquitination of endogenous eIF4E. Cells were heat shocked (lane 3) or mock treated (lane 1). As a positive control, cells were transfected with pcelF4E (lane 2) for 24 h. Lysates were precipitated with m7GTP-Sepharose and subjected to SDS-PAGE and IB with anti-eIF4E antibody (top and 2nd panels). As a control, a portion of the WCE was directly subjected to SDS-PAGE and IB was performed with anti-tubulin antibody (bottom panel). To assess ubiquitination levels of endogenous eIF4E, the membrane in the top panel was overexposed in the 2nd panel, using highly efficient ECL solution (see “Experimental Procedures”). **B** and **C**, proteasome-dependent degradation of endogenous eIF4E. Cells were pulse labeled with [35S]Met (36) for 3 h, washed extensively, and then incubated with cold chase medium with or without MG132 for the indicated hours. Cells for lanes 1–4 were heat shocked immediately after the pulse label. The lysates were purified with m7GTP-Sepharose and subjected to SDS-PAGE, and the radioactivity was visualized using BAS2000 system (B, upper panel). As a control, a portion of the WCE was directly subjected to IB with anti-tubulin antibody (lower panel). **C**, radioactivities in panel B (upper) were determined and shown as a line chart.
Cells respond to stress or apoptotic stimuli through regulating protein synthesis levels (21, 22). Nevertheless, the molecular mechanisms regulating cell reactions to such stimuli remain elusive. Chip, an E3 ligase, and stresses, such as heat shock and cadmium, enhanced the Ub conjugation of eIF4E. Because Chip is involved in the quality control of proteins in cells (34, 35), the ubiquitination/degradation of eIF4E may be, at least in part, controlling protein synthesis in response to stress.

eIF4E plays an important role in translation initiation, and it is important to understand the processes regulating eIF4E protein expression levels, including its degradation. Because eIF4E functions in association with many factors, future studies should examine the role of eIF4E ubiquitination within the context of the entire translation initiation complex.

Acknowledgments—We thank Drs. N. Sonenberg and K. Yoshida (McGill University, Canada) for providing the pcDNA3.1HA-eIF4GI vector and for helpful discussions. We thank Drs. T. Ohshima and M. Hijikata (Kyoto University, Japan) for technical suggestions and discussions.

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