Characterization of the mUBC9-binding Sites Required for E2A Protein Degradation*

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Mammalian Ubc9 (mUbc9) is required for rapid degradation of the E2A proteins E12 and E47 by the ubiquitin-proteasome system. We have shown elsewhere that mUbc9 interacts with amino acids 477–530 of E12/E47. Here we test the hypothesis that this region, rich in proline, glutamic acid, serine, and threonine (PEST) residues, serves as the E2A protein degradation domain (DD). An E2A protein lacking this region, E47A(478–531), was significantly more stable than wild-type E47 (half-life of more than 6 h versus 55 min). Deletion of the E2A DD had no effect on the E-box-binding and transcriptional activity of E47. We mapped two discreet mUbc9-interacting regions within the E2A DD: amino acids 476–494 and 505–513. E2A(505–513) interacted with mUbc9 but not with human Ubc5, MyoD, Id3, or the polymyositis-scleroderma autoantigen. Substitution of the E2A(505–513) central hydrophobic residues with basic residues abolished interaction with mUbc9. Also, full-length E47 lacking the second mUbc9-interacting region was significantly more stable than wild-type E47. Reintroduction of the E2A DD into the long-lived, naturally occurring chimeric oncoprotein E2A-HLF (hepatic leukemic factor) destabilized it, suggesting that this domain can transfer a degradation signal to a heterologous protein. E2A-HLF-DD chimeric protein was stabilized by the proteasome inhibitor LLNL, indicating the role of the ubiquitin-proteasome system mediating degradation through the E2A degradation domain. Our experiments indicate that the E2A DD mediates E2A protein interactions with the ubiquitin-proteasome system and that the E2A DD is required for metabolism of these widely expressed proteins.

The E2A proteins E12 and E47 are basic helix-loop-helix (bHLH) transcription factors that regulate differentiation and proliferation (1, 2) in many cell types. Although E12 and E47 share the same transcription activation domains (TADs) (3–5), because of alternative splicing their bHLH domains differ (6, 7). Dimerization through the HLH domain coordinates the basic regions for binding to E-box (CANNTG) enhancer elements (8). The E2A proteins regulate lymphopoiesis by activating transcription of the B-lymphocyte heavy chain locus and terminal deoxynucleotidyltransferase. E2A-null mice have a complex immunodeficiency characterized by a complete block in B-cell development (6, 9) and a partial block in T-cell development (10).

The E2A gene is expressed constitutively, in all tissues, with little developmental regulation (11). Consequently, E12 and E47 are regulated mainly by post-translational mechanisms. The Id family of HLH proteins sequester E12 and E47 into non-DNA-binding dimers (1, 2, 12, 13), and phosphorylation of E47 immediately upstream of the basic region inhibits DNA binding (14, 15). Because the transmembrane receptor Notch inhibits full-length E47 by a mechanism independent of its bHLH and TADs, there may be additional E2A regulatory domains or cofactors (16).

Degradation of the E2A proteins through the ubiquitin-proteasome pathway represents another important mechanism of post-translational regulation. Consistent with this mechanism, we found that the E2A proteins are highly unstable, with a half-life of 55 min (17). The three-part mechanism of targeting a protein for degradation begins with the formation of a ubiquitin conjugate with a ubiquitin-activating enzyme (also called E1) (18). Ubiquitin is then transferred to a ubiquitin-conjugating enzyme (also called E2), which transfers ubiquitin to an e-amino group of a lysine residue on a substrate protein (with the assistance of a ubiquitin ligase). There are 12 families of ubiquitin-conjugating enzymes, which in combination with numerous ubiquitin ligases are responsible for all ubiquitin conjugation (18). After the initial ubiquitin conjugate is made, a multi-ubiquitin appendage is produced that serves as a signal for substrate proteolysis by the 26 S proteasome. Beyond the requirement of the 26 S proteasome for E2A protein degradation (17), however, we know little about the mechanisms by which the E2A proteins are targeted for degradation.

The mammalian (m) homologue of Saccharomyces cerevisiae Ubc9p (previously called UbcE2A and now referred to as mUbc9) was cloned by us (17) and others (19) by using a yeast two-hybrid interaction trap with E12 as bait. mUbc9 is homologous to S. cerevisiae Ubc9p (56% identical and 75% similar) as well as Schizosaccharomyces pombe hus5 (66% identical and 82% similar) (17). Ubc9p is a nuclear protein required for cell viability in yeast. A deficiency in Ubc9p is associated with an arrest of the yeast cell cycle at the G2-M phase and an increase in the stability of the B (20) and the G1 (21) cyclins. The mUbc9 amino acid sequence is completely conserved among the mouse, rat, and human species (22), and numerous mUbc9-interacting partners have been described (22–27). We have demonstrated
Calcium phosphate precipitates and 15 (30) in the absence of competitor and in the presence of a 100-fold excess mM Tris Cl, pH 8.2, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, testinal alkaline phosphatase (New England Biolabs, Beverly MA) in 10 protein G-Sepharose (Calbiochem, La Jolla, CA) and protein A-Sepharose (Pharmingen, San Diego CA) before the immune complexes were precipitated with a mixture of (Pharmingen, San Diego CA) or anti-E2A antibody (Pharmingen) for 90 min. We measured the translation product, as measured by equal amounts of incorporated [35S]methionine (Amersham Pharmacia Biotech) by using the TnT T7 Coupled Reticulocyte System (Promega, Madison WI). E47 and E47 fragments or E47 mutants were cloned in-frame with the LexA gene into the EcoRI restriction site of the pEG202 yeast expression plasmid. The pG4–5 galactose-inducible yeast plasmid was used to express TAD hybrid proteins of full-length mUb9, human (h) Ub5, MnoD, Id3, and rat (r) polyomavirus-sclerodema autoantigen (rPm-Scl) in yeast. pR-C2A-HLF (hepatic leukemic factor) was kindly provided by T. A. Look (28, 29). Using sense (gattcgAGTCGGACTTCGGACTCTCCGGAATC) and antisense (cttcggGATCTTTGTCCGACTCGTGTGGCAG) primers with flanking LexA restriction sites (lowercase), we amplified E47(477–551) by PCR, digested it with BsiAI (Roche Molecular Biochemicals), and cloned it into an internal BseAI site in pRC-E2A-HLF to produce the pRC-E2A-HLF-DD construct. E2A-HLF-DD Control was produced using sense (gattcgAGTCGGACTTCGGACTCTCCGGAATC) and antisense (gattcgAGTCGGACTTCGGACTCTCGTGTGGCAG) primers that amplify the coding region for E47(531–581), which lies outside the destruction domain. All constructs were sequenced with the Thermosequenase Kit (Amersham Pharmacia Biotech).

Pulse-Chase Experiment—NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone, Logan UT), 1-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere at 37 °C with 5% CO2. Calcium phosphate precipitates and 15 µg of plasmid DNA were incubated with NIH 3T3 cells for 6 h. The next day the cells were cultured in methionine-free medium for 1 h, pulsed with medium supplemented with 0.3 µCi/ml [35S]methionine cell labeling mix (NEN Life Science Products) for 1 h and then chased with medium containing cold methionine. The cells were lysed in RIPA buffer (phosphate-buffered saline, 1% Nonident P-40, 0.5% sodium deoxycholate, and Complete protease inhibitor mixture (Roche Molecular Biochemicals)). Clarified cellular extract (200 µg) was incubated with 2.5 µg of anti-E47 antibody (Pharmingen, San Diego CA) or anti-E2A antibody (Pharmingen) for 90 min before the immune complexes were precipitated with a mixture of protein G-Sepharose (Calbiochem, La Jolla, CA) and protein A-Sepharose (Sigma).

Immunoprecipitates were dephosphorylated with 5 units of calf intestinal alkaline phosphatase (New England Biolabs, Beverly MA) in 10 mM Tris Cl, pH 8.2, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 1 µg µl aprotinin (incubated at 50 °C for 1 h). The proteins were eluted with sample buffer, subjected to 10% SDS-polyacrylamide gel electrophoresis, and blotted onto nitrocellulose. The nitrocellulose membrane was then probed with anti-E47 antibody or anti-E2A antibody followed by anti-mouse IgG-HRP. The chemiluminescent image was developed on Kodak BioMax MR film. The relative amount of E47 immunoprecipitated by Western blotting was estimated by using the NIH Image software program. The nitrocellulose membrane was then exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA) to measure the E47 radioactive signal. To correct for differences in loading, each E47 signal measured by the PhosphorImager was normalized against a measurement of total E47 protein by Western blotting. The proteasome was inhibited by culturing transfected cells in medium containing 20 µm N-acetyl-Leu-Leu-norleucinal (LNLN) (Sigma).

Electrophoretic Mobility Shift Assay—Recombinant E47 and E47 proteins were expressed and labeled with [35S]methionine (Amersham Pharmacia Biotech) by using the Trn T T7 Coupled Reticulocyte System (Promega, Madison WI). E47 and E47 proteins have the same number of methionine residues. An equivalent amount of translation product, as measured by equal amounts of incorporated [35S]methionine, was incubated with a [32P] end-labeled E-box oligonucleotide (30) in the absence of competitor and in the presence of a 100-fold excess of unlabeled E-box oligonucleotide or an excess of mutated E-box oligonucleotide. The samples were run on a 5% polyacrylamide gel; the gel was dried and then exposed overnight to Kodak BioMax MR film.

Yeast Two-hybrid Assay—E47(477–550), which is between the second TAD and the bHLH domain (17), E47(478–531) is markedly more stable than wild-type E47. NIH 3T3 cells that had been transfected with plasmids encoding full-length E47 and E47(478–531) were pulsed with [35S]methionine and harvested at time 0 or after a 2-h chase with cold methionine. The E2A-HLF-DD Control was produced using sense (gattcgAGTCGGACTTCGGACTCTCCGGAATC) and antisense (gattcgAGTCGGACTTCGGACTCTCGTGTGGCAG) primers that amplify the coding region for E47(531–581), which lies outside the destruction domain. All constructs were sequenced with the Thermosequenase Kit (Amersham Pharmacia Biotech).

RESULTS

Deletion of the mUb9-interacting Region Produces a Stable E47 Protein—mUb9 interacts with the E2A proteins in a region conserved in E12 and E47(478–531) just upstream of the bHLH domain (17). To test our hypothesis that this region (Fig. 1A) is the E2A degradation domain, we constructed the plasmid E47(478–531), which encodes an E47 mutant lacking this region. After transiently transfecting the
E2A Protein Degradation Domain

E47Δ(478–531) plasmid and an E47 plasmid into NIH 3T3 fibroblasts, we compared the degradation of the two proteins in a pulse-chase experiment. We chose a 2-h chase to evaluate the effect of E47 mutation on degradation because about 25% of the wild-type E47 remains after that time. E47Δ(478–531) was more stable than wild-type E47, with a half-life longer than 2 h (Fig. 1B). Significantly less wild-type E47 remained in comparison with E47Δ(478–531) (Fig. 1C, 26.1% ± 13.5, mean ± S.E., versus 78.1% ± 5.7, p < 0.0001).

E47Δ(478–531) Is Hyperphosphorylated and Retains DNA Binding Activity—We noted a slight but reproducible decrease in the electrophoretic mobility of E47Δ(478–531) after 2 h of chase, consistent with post-translational modification of the protein (Figs. 1B and 2A). Because the E2A proteins have numerous potential serine/threonine phosphorylation sites, we speculated that phosphorylation was responsible for the change in E47Δ(478–531) mobility. Treatment of the E47Δ(478–531) immunoprecipitate with calf intestinal alkaline phosphatase increased E47Δ(478–531) mobility and collapsed a smear of multiple bands to a single band (Fig. 2A) that migrated at a molecular mass similar to that of in vitro transcribed and translated E47Δ(478–531) (data not shown). These results indicate that removal of the mUbc9-interacting domain results in a more stable E47 protein, which is subject to hyperphosphorylation.

To determine whether deletion of the E2A degradation do-

main affected E47 binding to DNA, we tested the ability of E47Δ(478–531) to bind to an E-box probe in an electrophoretic mobility shift assay. The protein-DNA complex formed by the mutant E2A migrated slightly faster, consistent with the reduced size of the mutant protein. Equal amounts of in vitro transcribed and translated E47 and E47Δ(478–531) retained similar amounts of an E-box probe (Fig. 2B, bracket). Therefore, homodimer formation and DNA binding through the HLH domain and the basic region, respectively, were unaffected by removal of the adjacent degradation domain. In addition, E47Δ(478–531) activated transcription of an E-box reporter plasmid in NIH 3T3 cells (data not shown), indicating that the function of the TADs had been preserved. Thus, mUbc9 appears to interact with an E2A domain that is functionally separable from the previously described bHLH domain and TADs.

mUbc9 Interacts with Two Regions in the E2A(478–531) Degradation Domain—The E2A degradation domain is highly conserved across species (Fig. 3A). Almost the entire coding region for E2A(478–531) is contained on a single exon upstream of the alternatively spliced bHLH E12 and E47 exons (exon J of the E2A gene, as described by Sun and Baltimore (31)). The primary amino acid sequence of E2A(478–531) is rich in PEST (proline, glutamic acid, serine, and threonine) residues common to degradation domains (32).

We used a panel of deletion mutants to more closely map the degradation domain regions necessary for E2A to interact with mUbc9. In a yeast two-hybrid system, E2A protein fragments fused to the DNA-binding domain of LexA were tested for their ability to interact with mUbc9 fused to a TAD. To measure interaction as a function of β-galactosidase production, we used a reporter plasmid encoding the β-galactosidase gene regulated by LxyA. In comparison with LexA-E47(476–651), LexA-E2A(476–520) bound TAD-mUbc9 strongly but LexA-E2A(520–532) did not (Fig. 3B). LexA-E2A(476–494) and LexA-E2A(505–513) interacted strongly with TAD-mUbc9, yet there was no interaction with the intervening region, LexA-E2A(494–504). Hybrid protein LexA-E2A(510–520), which includes a casein kinase II phosphorylation site (14, 15), and hybrid protein LexA-E2A(520–532), which includes a cyclic AMP-dependent protein kinase domain site (15), showed no significant interaction with TAD-mUbc9. These results indicate that mUbc9 interacts with two distinct, nearby regions of the E2A proteins: E2A(476–494) and E2A(505–513). The primary amino acid sequence of E2A(476–494) is rich in nonaromatic hydrophobic and hydroxyl side chains, whereas that of E2A(505–513) has a central threonine-serine pair flanked by glutamic and aspartic acid residues (Fig. 3A).

The Second mUbc9-interacting Region, E2A(505–513), Interacts Selectively with TAD-mUbc9—We focused on E2A(505–513) because it is smaller than E2A(476–494) and more highly conserved. LexA-E2A(505–513) interacted specifically with the mUbc9 portion of TAD-mUbc9 as shown by an approximately 20-fold increase in β-galactosidase activity above that of TAD alone (p < 0.001). Although hUbc5 and mUbc9 are similar in size and structure, the β-galactosidase production of LexA-E2A(505–513) with TAD-hUbc5 was not significantly different from that with TAD alone (Fig. 4). Nor did LexA-E2A(505–513) interact with other known E2A-interacting HLH proteins, including Id3 and MyoD (Fig. 4). Elsewhere we described an interaction between rPM-Scl and E2A(477–530) (33). Because rPM-Scl did not interact with LexA-E2A(505–513), the domain that interacts with rPM-Scl must fall outside the E2A(505–513) region.

Mutation of the Hydrophobic Core of E2A(505–513) Prevents Interaction with mUbc9—We determined the importance to
mUbc9 binding of the central hydrophobic residues of E2A(505–513) by introducing multiple mutations into the coding region of pEG-E2A(505–513). A point and frameshift mutation produced pEG-E2A-mut, which encoded LexA-EE-TRKRLTI instead of LexA-EENTSADH. LexA-E2A-mut did not interact with TAD-mUbc9 (Fig. 5). We also introduced point mutations into the E2A(505–513) coding sequence to determine the effect of individual charge interactions on the interaction with mUbc9. The interaction of TAD-mUbc9 and lysine mutant LexA-E2A-E504K or LexA-E2A-E505K reduced β-galactosidase production slightly (data not shown). Mutation of E2A Ser509, which is conserved from zebra fish to humans, to an aspartic acid residue (S509D) increased β-galactosidase production ($p < 0.05$), whereas an alanine mutation (S509A) had no effect (Fig. 5). In contrast, a lysine mutation (S509K) reduced β-galactosidase production. These results indicate that singular mutations within the second mUbc9-interacting region influence the interaction of mUbc9 with the whole E2A protein but only to a limited degree. A complete interruption of the binding interaction between E2A(505–513) and mUbc9 requires numerous substitutions of hydrophobic residues with charged residues.

Deletion of the Second mUbc9-interacting Region Produces a More Stable E2A Protein—To substantiate a role for mUbc9 in the degradation of the E2A proteins, we deleted E2A(505–513) from E47. E47D(505–513) is more selective than E47D(478–531) because it is not missing any potential ubiquitin acceptor sites. After a 2-h chase (Fig. 6A), significantly more E47D(505–513) remained than wild-type E47 (mean ± S.E.: 61.8 ± 5.6%.)

![Graph A](image1.png)

**Fig. 3.** A, the E2A degradation domain is conserved from zebra fish to humans. The mouse, rat, hamster, chicken, and zebra fish E2A sequences were aligned with the human sequence (GenBank). Amino acids from other species conserved in human E2A are shaded; dissimilar residues are on a white background. The aligned sequences end at amino acid 529 because the sequences for E12 and E47 diverge at this point. B, mUbc9 binds to two different sites within the E2A degradation domain. Left, LexA-E2A bait constructs tested for binding to TAD-mUbc9 in a yeast two-hybrid interaction system. An EGY48 culture transformed with pJG4–5-mUbc9 and pSH18–34 was transformed with plasmids encoding LexA-E2A proteins. The β-galactosidase activity for each sample was normalized to that of LexA-E47(476–651), which was assigned a value of 100. Asterisks mark constructs that produced significantly more β-galactosidase ($p < 0.05$) in comparison with LexA alone. The composite mean ± S.E. from three separate experiments is shown. The liquid assays were confirmed with plate assays.

![Graph B](image2.png)

**Fig. 4.** E2A(505–513) interacts selectively with mUbc9. An EGY48 culture transformed with pEG202-E2A(505–513) and a β-galactosidase reporter plasmid was transformed with plasmids encoding a TAD hybrid protein of mUbc9, hUbc5, MyoD, Id3, or rPM-Scl. The mean ± S.E. from one of three liquid β-galactosidase assays is shown. Results of the liquid β-galactosidase assays were confirmed with plate assays.

![Graph C](image3.png)

**Fig. 5.** Many LexA-E2A(505–513) residues must be replaced to inhibit its interaction with mUbc9. Point mutations were introduced into pEG202-E2A(505–513) by using custom-designed mutagenesis primers and PCR techniques. The mutant bait plasmids were then transformed into EGY48 that had been transformed already with pJG4–5-mUbc9 and the β-galactosidase reporter plasmid. β-Galactosidase activity was normalized to the activity of LexA-E2A(505–513). *, E2A-S509D compared with LexA-E2A(505–513), $p < 0.05$. The mean ± S.E. liquid β-galactosidase activity from three experiments is shown.
versus 27 ± 9.4%, p = 0.03). The half-life of E47Δ(505–513) was 3 h (Fig. 6B), which is more than wild-type E47 (half-life 1 h) and less than E47Δ(478–531) (half-life > 6 h).

The E2A Degradation Domain Destabilizes E2A-HLF—The chromosomal translocation t(17;19)(q22;p13) produces a chimeric oncoprotein composed of the E2A TADs and the basic leucine zipper region of HLF (28, 29) (Fig. 7A). E2A-HLF and other E2A chimeric oncoproteins have potent transforming capabilities, and they are responsible for 25% of pre-B-cell acute lymphoblastic leukemias. Because we noted that all E2A oncoproteins contain a translocation site that omits the exon encoding the degradation domain, we hypothesized that E2A-HLF is a stable protein because it lacks the degradation domain and that inclusion of this domain would destabilize E2A-HLF. We cloned a PCR-amplified DNA fragment encoding E2A(477–531) in-frame into a unique restriction site between the two E2A TADs of E2A-HLF (Fig. 7A) to make E2A-HLF-DD. To control for the effect of insertion on degradation of the chimeric protein, the adjacent region encoding E47(531–581) was also cloned into E2A-HLF to produce E2A-HLF-Control. By pulse-chase analysis, we found that E2A-HLF was a stable protein with a half-life of 2 h; E2A-HLF-DD, however, had a half-life of 60 min, similar to that of the E2A proteins (Fig. 7B). After a 2-h chase, significantly more E2A-HLF remained than E2A-HLF-DD (71.0 ± 9.2% versus 13.9 ± 3.6%, p < 0.001), whereas E2A-HLF-Control was stable (74.4 ± 8.5% remaining) (Fig. 7C).

E2A-HLF-DD Is Stabilized by Proteasome Inhibitor—Previously we have demonstrated that degradation of the E2A proteins is reduced by treatment with a proteasome inhibitor. To analyze the mechanism of degradation of the E2A-HLF-DD protein, transfected cells were treated with the proteasome inhibitor LLNL. E2A-HLF-DD was more abundant following treatment with LLNL compared with E2A-HLF (Fig. 8), indicating that the degradation of E2A-HLF-DD is dependent upon the proteasome.

**DISCUSSION**

We have demonstrated previously that the E2A proteins are degraded by the ubiquitin-proteasome system and that they interact with mUbc9 (17). In the present study we show that removal of the mUbc9 interacting region markedly stabilizes E47. Despite the deletion, E47Δ(478–531) retains the ability to form homodimers that bind to the E-box sequence and activate E-box-dependent transcription. The transcription factor c-Jun also has a degradation domain that is separable from its DNA-binding domain and TADs; the absence of the c-Jun d-domain, as occurs naturally in v-Jun, results in a more stable protein.
with intact DNA binding and transcription activation properties (34).

Domains rich in PEST residues, such as the E2A degradation domain, often serve as phosphoacceptor sites on short-lived proteins (32). Cdc28 kinase phosphorylation of the Cln3 PEST-rich degradation domain decreases the half-life of Cln3 (35), and phosphorylation of the PEST-rich cytoplasmic domain of yeast uracil permease accelerates its degradation (36). Phosphorylation regulates the degradation of many mammalian proteins, including cyclin D (37), IκBo (38), and c-Jun (39). The E2A proteins have numerous potential phosphorylation sites, and deletion of the E2A degradation domain allows multiphosphorylated E47 species to accumulate. Perhaps the hyperphosphorylation of E47 (478–531) (Fig. 2) reflects phosphorylation events designed to initiate E2A protein degradation. Although the relative stability of E47Δ(505–513) (Fig. 6) and the conservation of Ser509 within the PEST-rich E2A degradation domain (Fig. 3A) suggest a link between Ser509 phosphorylation and degradation, we found that a full-length E47 protein with a lysine residue substituted for Ser509 (E47-S509K) had the same half-life as wild-type E47 (data not shown).

We have demonstrated that mUbc9 interacts with the E2A degradation domain (Fig. 1) and that the second mUbc9-interacting region is required for normal E47 degradation (Fig. 6). This association of mUbc9 with E2A degradation is supported by our previous work, which showed that inhibition of mUbc9 expression by full-length antisense overexpression was associated with reduced E12 degradation (17). Our present and former studies stand in contrast to recent work showing that Ubc9 probably forms conjugates with ubiquitin-like proteins (such as yeast Smt3 and mammalian SUMO-1) that are not associated with degradation (40–44). Ubc9-mediated conjugation of SUMO-1 to RanGAP1 was associated with RanGAP1 nuclear pore localization (45), an event separate from degradation, and Ubc9-mediated SUMO-1 conjugation to IκBo was associated with a reduction in degradation (46). We have not found an E2A-SUMO-1 conjugate in NIH-3T3 cells (data not shown), yet E2A protein isolated from mouse thymus does exist in a 66-kDa form, which is the size of the native protein, and an 85-kDa form, which may be an E2A-SUMO-1 conjugate (7).

There are at least three possible explanations for the paradox posed by our results. First, the conventional ubiquitin conjugation system may recognize the same peptide motifs of the E2A proteins as mUbc9. For example, mUbc9 uses the same domain in IκBo for SUMO-1 conjugation that other ubiquitin-conjugating enzymes use for ubiquitin conjugation (46). Although our work suggests at least an indirect association between mUbc9 and degradation, we cannot exclude the possibility that deleting the second mUbc9-interacting region disrupted binding sites for conventional ubiquitin-conjugating enzymes that target E2A proteins for degradation. Second, interaction of mUbc9 with the E2A proteins may be a necessary intermediary step prior to their degradation. This explanation is supported by our observation that both our antisense underexpression studies (17) and deletion experiments shown in this report both demonstrated reduced degradation. Finally, mUbc9 may target the E2A proteins directly for degradation; yet considering that mUbc9 cannot conjugate ubiquitin and that ubiquitin is the preferred signal for the proteasome to initiate degradation, this option is unlikely. Future work will center on identifying additional components of the ubiquitin conjugating system that interact with the E2A degradation domain.

We searched for a consensus sequence within the E2A degradation domain that could be used by conventional ubiquitin-conjugating enzymes. The second mUbc9-interacting region resembles a class II degradation signal (47), which typically features a hydrophobic center flanked by charged residues. Ubc6/5 and Ubc6/7 both target proteins for degradation through a class II degradation signal; however, the E2A degradation domain lacks the amino acid sequence SWNKLYVM, which resembles a proposed degradation signal for these enzymes. We did not find an interaction between the second mUbc9-interacting region and hUbc5, which is similar to mUbc9 in size but structurally distinct from it (48). An alternate Ubc6/7 degradation signal has been proposed, F(T/S)(T/V)S/I, that features a central pair of serine or threonine residues (49). The second mUbc9-interacting region, EENTTSADH, resembles this sequence, even though the mUbc9-binding site is not flanked by bulky hydrophobic residues.

The biological importance of the E2A degradation domain is underscored by our observation that every translocation that produces a chimeric E2A-oncoprotein excludes the exon that encodes the degradation domain. The prolonged half-life of E2A-HLF (Fig. 7), like that of v-Jun (34), probably potentiates its transforming capabilities by allowing more protein to accumulate and activate transcription or sequester an important interacting partner (52). The long half-life of a stable protein can be shortened by the addition of a degradation domain (35, 50, 51). When we added an E2A degradation domain to E2A-HLF, the new protein was degraded more quickly than was E2A-HLF (half-life of 60 min versus more than 2 h, Fig. 7). By comparison, addition of the 51-amino acid region of E47 adjacent to the degradation domain did not destabilize E2A-HLF. Finally, E2A-HLF-DD was stabilized by a proteasome inhibitor, demonstrating that the E2A-DD targets protein for degradation through the proteasome. Our findings that removal of E2A(478–531) produces a stable E47 protein and that inclusion of E2A(477–531) produces an unstable protein support the conclusion that this mUbc9-interacting domain is the E2A degradation domain.

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REFERENCES

1. Peverali, F. A., Ramqvist, T., Saffrich, R., Peppercok, R., Barone, M. V., and Phillipson, L. (1994) EMBO J. 13, 4291–4300
2. Prabhu, S., Ignatova, A., Park, S. T., and Sun, X.-H. (1997) Mol. Cell. Biol. 17, 5888–5896
3. Aronheim, A., Shiran, R., Rosen, A., and Walker, M. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8063–8067
4. Massari, M. E., Jennings, P. A., and Murre, C. (1996) Mol. Cell. Biol. 16, 121–129
5. Quong, M. W., Massari, M. E., Zwart, R., and Murre, C. (1993) Mol. Cell. Biol. 13, 792–800
6. Zhuang, Y., Soriano, P., and Weintraub, H. (1994) Cell 79, 875–884
7. Zhuang, Y., Barnitz, R. J., Pan, L., Kelley, R., and Dai, M. (1998) Mol. Cell. Biol. 18, 3340–3349
8. Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Baskin, J. N. Hauschka, S. D., Lassar, A. B., Weintraub, H., and Baltimore, D. (1989) Cell 58, 537–544
9. Bain, G., Robanus Maandag, E. C., te Riele, H. P. J., Berns, A., and Murre, C. (1994) Cell 79, 885–892
10. Roberts, V. J., Steenbergen, R., and Murre, C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7583–7587
11. Bennerza, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990) Cell 61, 49–59
12. Sun, X.-H. (1994) Cell 79, 893–900
13. Johnson, S. E., Wang, X., Hardy, S., Taparowsky, E. J., and Konieczny, S. F. (1996) Mol. Cell. Biol. 16, 1604–1613
14. Sloan, R. S., Chen, C.-P., McCarrick-Walmsley, R., and Kadesch, T. (1996) Mol. Cell. Biol. 16, 6900–6908
15. Ordentlich, P., Lin, A., Shen, C.-P., Blaumueller, C., Matsumo, K., Artavanis-Tsakonas, S., and Kadesch, T. (1998) Mol. Cell. Biol. 18, 2230–2239
16. Kho, C.-J., Huggins, G. S., Endege, W. O., Hsieh, C.-M., Lee, M.-E., and Haber, E. (1997) J. Biol. Chem. 272, 3845–3851
17. Haas, A. L., and Steppmann, T. J. (1997) FASEB J. 11, 1257–1268

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