ERK7 Expression and Kinase Activity Is Regulated by the Ubiquitin-Proteosome Pathway*

Wen-Liang Kuo‡, Crystal J. Duke‡, Mark K. Abe§, Evan L. Kaplan‡, Suzana Gomes‡, and Marsha Rich Rosner¶∥

From the ¶Ben May Institute for Cancer Research and §Department of Pediatrics, University of Chicago, Chicago, Illinois 60615

ERK7 is a unique member of the extracellular signal-regulated kinase (ERK) subfamily of MAP kinases. Although ERK7 shares a TEY motif in the activation loop of the kinase, it displays constitutive activation, nuclear localization, and growth inhibitory properties that are regulated by its C-terminal domain. Because ERK7 is expressed at low levels compared with ERK2 and its activity is dependent upon its expression level, we investigated the mechanism by which ERK7 expression is regulated. We now show that ERK7 expression is regulated by ubiquitination and rapid proteosomal turnover. Furthermore, both the kinase domain and the C-terminal tail are independently degraded at a rate comparable with that of the intact protein. Analysis of a series of chimeras between ERK2 and ERK7 reveal that the N-terminal 20 amino acids of the kinase domain are a primary determinant of ERK7 degradation. Fusion of the N-terminal 20 amino acids is both necessary and sufficient to cause proteolytic degradation of both ERK2 and green fluorescent protein. Finally, ERK7 is stabilized by an N-terminal mutant of Cullin-1 suggesting that ERK7 is ubiquitinated by the Skp1-Cullin-F box complex. These results indicate that ERK7 is a highly regulated enzyme whose cellular expression and kinase activation level is tightly controlled by the ubiquitin-proteosome pathway.

MAP3 kinases are an evolutionarily conserved family that regulate cell growth, differentiation, survival, and a variety of other cellular responses (reviewed in Ref. 1). These kinases, which consist of the extracellular signal-regulated kinases (ERKs), the Jun kinases (JNKs), and the p38 kinases, are classically activated via dual phosphorylation by a MAP kinase kinase. MAP kinase kinases, which phosphorylate an activation loop on MAP kinase consisting of a TXY motif, are in turn phosphorylated by MAP kinase kinase kinases that respond to extracellular stimuli. Although this paradigm applies to the general MAP kinase family, recent evidence suggests that activation of more distant members of this family may be regulated by other mechanisms.

The ERK family of MAP kinases can be divided into at least two classes: 1) the classic MAP kinases that consist primarily of a kinase domain such as ERK1 and ERK2; and 2) the big MAP kinases such as ERK3, ERK5, ERK7, and ERK8 that consist of both a kinase domain and a C-terminal domain and range in size from 60 to over 100 kDa. In contrast to ERK1 and ERK2 that require discrete scaffolding proteins such as KSR for maximal activation, the big MAP kinases have C-terminal regions that can function as protein interaction domains that regulate kinase localization (2), activation (3), and transcriptional activity (4). The C-terminal domain of ERK7 has been shown previously to be required for ERK7 constitutive activation and nuclear localization as well as growth inhibition by ERK7 (2, 3). However, a role for the C-terminal domain in regulating protein expression has not previously been described.

From an evolutionary perspective, ERK7 is a unique member of the MAP kinase family. Unlike ERKs, JNKs, and p38s that share over 90% homology between human and rodents, ERK7, which was originally cloned in rat, shares less than 70% homology with its closest human counterpart identified in the human genome, ERK8. Furthermore, ERK8 differs in its mechanism of activation, localization, and substrate specificity (5). Thus, although ERK8 may be considered the human orthologue of ERK7, its evolutionary divergence that derives in large part from the C-terminal domain raises the possibility that the two enzymes may have unique as well as overlapping functions. The physiological roles of ERK7 and ERK8 are currently under investigation. Initial studies revealed that overexpression of ERK7 can lead to suppression of DNA synthesis that was dependent on the presence of the C-terminal domain of ERK7 but independent of its kinase activity (2). A recent study suggested that exogenously expressed rat ERK7 can regulate the proteolytic turnover of the estrogen receptor in human cells (6). However, in light of the significant sequence and functional differences between ERK7 and ERK8, the physiological relevance of these observations requires further investigation.

One of the striking features of ERK7 and ERK8 are the extremely low levels of endogenous protein expression in cultured cells and tissues. The present study was undertaken to determine the mechanism by which ERK7 is regulated. Whereas no significant difference in transcript levels were detected, our results indicated that the ERK7 protein is rapidly ubiquitinated and degraded by the proteosome, and this process is regulated in part by the N terminus of the kinase domain.

**EXPERIMENTAL PROCEDURES**

Materials—A rabbit polyclonal antibody against green fluorescent protein (GFP) (GFP FL sc-8334) was purchased from Santa Cruz.
Biotecnology (San Diego, CA). The anti-GFP antibody was used at a 1:1000 dilution in 0.5% milk/TBST buffer (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, and 0.1% Tween 20). Antibody against human Cullin-1 protein (CUL-1 (H-213)) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Proteasome inhibitor I, lactacystin, MG-132, and proteinase inhibitor mixture Set III from Calbiochem was used at a 1:200 dilution in washing buffer (62.5 mM Tris-HCl, pH 7.5, 10% glycerol, 137 mM NaCl, 10 mM NaF, 5 mM EDTA, 1 mM sodium vanadate, 1 mM EGTA, 40 mM p-nitrophenyl phosphate, and protease inhibitor mixture as described above). The cell lysate was centrifuged at 14,000 rpm in an Eppendorf bench top centrifuge for 15 min at 4 °C to clear cell debris. Protein concentration was determined by the Bradford method using a Bio-Rad protein assay reagent (Bio-Rad).

Preparation of Cell Lysates—Cells in culture were washed once with ice-cold phosphate-buffered saline and lysed with RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 20 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM β-glycerophosphate, 137 mM NaCl, 10 mM NaF, 5 mM EDTA, 1 mM sodium vanadate, 1 mM EGTA, 40 mM p-nitrophenyl phosphate, and protease inhibitor mixture as described above). The cell lysate was centrifuged at 14,000 rpm in an Eppendorf bench top centrifuge for 15 min at 4 °C to clear cell debris. Protein concentration was determined by the Bradford method using a Bio-Rad protein assay reagent (Bio-Rad).

Western Blot Analysis—Cell extracts (10–60 µg of protein per lane unless otherwise noted) were resolved by 10%–12% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat dry milk at room temperature for 1 h, incubated with primary antibodies at appropriate concentrations for 1 h, washed for 30 min, and then incubated with secondary antibodies for 1 h. All incubations and washes were done at room temperature. The signal was detected with enhanced chemiluminescence (ECL) reagent (PerkinElmer Life Sciences) and analyzed by autoradiography or digital imaging. To reprobe membranes with a new antibody, membranes were incubated in a stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 0.1% 2-mercaptoethanol) at 50 °C for 30 min and then washed extensively with 1× TBST.
ERK7 Proteolytic Turnover

Fig. 1. Differential expression of HA-ERK7 and HA-ERK2 proteins in COS cells. HA-ERK7, HA-ERK7(K43R), and HA-ERK2 constructs were co-transfected with pCMVβ-galactosidase into COS cells. At 36–48 h after transfection, cell lysates were prepared and 30 μg of protein was loaded into each lane. The proteins were separated by SDS-PAGE and Western blot analyses was performed using antibodies against either HA or β-galactosidase (β-gal). A, HA-ERK2, HA-ERK7, and HA-ERK7(K43R) were transfected separately into COS cells. B, HA-ERK2 was co-transfected with either HA-ERK7 or HA-ERK7(K43R) into COS cells. β-Galactosidase was included as a control for transfection efficiency. HA-ERK7, HA-ERK2, and β-galactosidase proteins are indicated by arrowheads. These results are representative of at least three independent experiments.

To compare the expression levels of ERK2 and ERK7, COS cells were transfected with β-galactosidase and either HA-ERK2 or HA-ERK7 on CMV expression vectors. As shown in Fig. 1, ERK2 protein levels were 10–100 times higher than serum induction. The presence of epitope-tagged proteins in the immunoprecipitates was verified by Western analysis with a 3F10 monoclonal antibody against the HA epitope.

Pulse-Chase Analysis—Cells transfected with HA-ERK7 were pooled and re-plated to normalize for transfection efficiency. Following incubation in medium deficient in methionine and cysteine, cells were incubated with Tran35S-label (MP Biomedicals, Aurora, OH) for a 30-min pulse. Cells were then incubated in growth medium containing an excess of cold methionine and cysteine until harvested at the indicated time points. HA-ERK7 was immunoprecipitated from cleared lysates using an anti-HA antibody and separated by SDS-PAGE. The gel was incubated in an autoradiographic enhancer, dried, and analyzed using the STORM 850 system (Amersham Biosciences).

Virus Production and Infection of PC12 Cells—The full-length ERK7 and ERK7 tail consisting of the last 193 amino acids were subcloned into the pCLE retroviral vector (9) to generate pCLE-ERK7 and pCLE-ERK7 TAIL, respectively. These constructs were co-transfected into the pCLE retroviral vector (9) to generate pCLE-ERK7 and pCLE-ERK7 TAIL constructs were co-transfected with pCMV β-galactosidase (β-gal) into COS cells, and total RNA was isolated from the cells. Twenty micrograms of total RNA was loaded into each lane, and the RNA was separated in a formaldehyde gel by electrophoresis. Northern blot analyses were performed, and the membranes were probed with 32P-labeled HA antisense oligonucleotides and β-galactosidase antisense DNA fragments. A, analysis of Northern blots by autoradiography. B, plot of transcript levels. Northern blots were scanned with a phosphorimager, and the radioactive bands were quantitated. The levels of HA-ERK7 and HA-ERK7(K43R) transcripts were normalized to the level of HA-ERK2 transcripts. The data plotted represent the average of three independent experiments ± S.D.

RESULTS

To determine whether the regulation of ERK7 expression occurred at the transcriptional level, COS cells transfected with β-galactosidase and HA-ERK2, HA-ERK7, or HA-ERK7(K43R) were lysed and analyzed for mRNA transcripts by Northern blot analysis. As shown in Fig. 2, ERK7 mRNA levels were comparable with those of ERK2. These results indicate that the regulation of ERK7 was likely occurring at a post-transcriptional level.

The previous results suggest that protein rather than mRNA stability might be the source of differential ERK expression. To test this possibility, COS cells transfected with HA-ERK2 or HA-ERK7 were treated with cycloheximide to block new protein synthesis, and the stability of the remaining protein determined by immunoblotting with an anti-HA antibody (Fig. 3A, top). The results indicate that ERK7 is degraded with a t1/2 of about 2 h. In contrast to HA-ERK7, HA-ERK2 is a stable protein and its t1/2 is longer than 6 h (Fig. 3A, top). Pulse-chase 35S-labeled HA-ERK7 confirmed the short t1/2 of ~2 h. (Fig. 3A, middle and bottom panels). To determine whether degradation of ERK7 was mediated by the proteosome, COS cells transfected with HA-ERK7 were pretreated with proteosome inhibitors prior
to cycloheximide treatment. ERK7 degradation was blocked by a number of proteasome inhibitors including lactacystin, proteasome inhibitor 1, or MG132, whereas E64, a cysteine protease inhibitor not specific to the proteosome pathway, had no effect (Fig. 3B). Similar results were obtained when HEK293T cells were used for the experiments (data not shown).

To directly confirm that ERK7 is ubiquitinated, HEK293T cells were transfected with HA-ERK7 and His6-Myc-ubiquitin. Cells were then either untreated or pretreated with proteosome inhibitor 1 to block degradation of ubiquitinated protein. The cell lysates were immunoprecipitated with anti-HA, anti-Myc, or anti-ERK7 antibodies, and the immunoprecipitated complexes were analyzed by immunoblotting with anti-ubiquitin or anti-HA antibodies (Fig. 4). Analysis of immunoprecipitated HA-ERK7 with anti-ubiquitin antibody shows a series of bands corresponding to protein with different amounts of conjugated ubiquitin. Detection of HA-ERK7 in the Myc-ubiquitin immunoprecipitate and the ERK7 immunoprecipitate indicates that most of the ubiquitinated ERK7 is less conjugated. These results clearly demonstrate that ERK7 is ubiquitinated prior to protein degradation.

One of the three major classes of ubiquitin ligases that recognize and ubiquitinate substrates are the Cullin-based E3 ligases (reviewed in Ref. 10). Among these E3 ligase complexes are the anaphase promoting complex (APC), the von Hippel-Landau tumor suppressor/Elongin BC complex (CBC), and the SCF (Skp1/Cull/F-box protein) that contains Cullin-1 (Cul-1). To determine whether the degradation of ERK7 occurred via the SCF complex, we expressed ERK7 in HEK293T cells in the presence or absence of a mutant human Cullin-1 (hCul-1) comprising the N-terminal 452 amino acids of Cullin-1 that blocks ubiquitination of substrates (11). Cells were then pretreated with cycloheximide to monitor the rate of protein turnover. The

**Fig. 3.** ERK7 is rapidly degraded and can be stabilized by proteasome inhibitors. A, analysis of HA-ERK7 and HA-ERK2 protein stability in COS cells. HA-ERK7 and HA-ERK2 were transfected into COS cells. The cells were then incubated with CHX (50 μg/ml) for different lengths of time as indicated. Cell lysates were prepared and the protein extracts were analyzed by SDS-PAGE, Western blotting, and chemiluminescent detection as described under “Experimental Procedures.” 10 and 40 μg of protein extracts from HA-ERK2 and HA-ERK7, respectively, were loaded in each lane (top). COS cells were transfected with an HA-ERK7 expression vector, and then metabolically labeled with [35S]methionine for 30 min. The medium was replaced to chase medium containing excess unlabeled methionine and cysteine, and cell lysates were collected at the various time points indicated. [35S]-Labeled HA-ERK7 protein was immunoprecipitated using an anti-HA antibody. The immunoprecipitates were separated by SDS-PAGE and analyzed by autoradiography (middle). The gel was scanned with an imager to quantify the [35S]-labeled protein, and the data were plotted (bottom). B, stabilization of ERK7 with proteasome inhibitors. COS cells were transfected with an HA-ERK7 expression vector, pooled, and divided into smaller dishes. The cells were later treated with 50 μM cycloheximide (CHX), and then harvested at different time points as indicated. For the cells treated with MeSO (DMSO), E64 (50 μM), or proteasome inhibitors (lactacystin, 16.7 μM; MG132, 50 μM; proteasome inhibitor 1, 50 μM), the reagents were added 2 h before the addition of CHX. Following treatment, cells were lysed and the same amount of protein was loaded into each lane for SDS-PAGE analysis. Samples were analyzed by Western blotting with an anti-HA antibody and chemiluminescence. These results are representative of at least three independent experiments.

**Fig. 4.** HA-ERK7 is a ubiquitinated protein. HEK293 cells were cotransfected with expression vectors for HA-ERK7 and His6-Myc-ubiquitin (pCW7). After 36–48 h, cells were either untreated or treated with proteasome inhibitor I (PSI) for 6 h. Following lysis, cells were immunoprecipitated with anti-HA, anti-Myc, or anti-ERK7 antibodies and then probed with anti-ubiquitin or anti-HA antibodies as indicated. Unmodified HA-ERK7 and immunoglobulin heavy chain are indicated with arrows, and ubiquitinated-HA-ERK7 (Ubi-HA-ERK7) is marked with a bracket. These results are representative of at least three independent experiments.
addition of mutant hCul-1 stabilized ERK7 (Fig. 5). The stabilizing effect of the dominant-negative mutant hCul-1 on HA-ERK7 was similar to the stabilizing effect of MG132, a proteasome inhibitor (Fig. 5). These results provide additional evidence that ERK7 is degraded by the proteasome and suggest that ERK7 is degraded by a multisubunit Cullin-based SCF complex.

To identify the region(s) in ERK7 that are responsible for its ubiquitination, we generated chimeras between ERK2 and ERK7. Initially, we analyzed a set of mutants and chimeras in which the kinase domains I–V or I–VII were switched (Fig. 6). The kinase domains were analyzed either alone or with the addition of the ERK7 C-terminal tail. To monitor stability, the mutant and chimeric proteins were expressed in either COS or HEK293 cells along with a GFP expression vector to control for transfection efficiency. As shown in Fig. 6C (right panel), the kinase domain of ERK7 (ERK7/2AT) is highly unstable relative to ERK2. Transfer of the N-terminal kinase domains I–V or I–VII from ERK7 to ERK2 resulted in significantly lower expression of the resultant chimeric proteins ERK7/2(VI) and ERK7/2(VIII) (Fig. 6C, right panel). Conversely, substituting kinase domains I–V or I–VII of ERK2 for the comparable domains in the ERK7 kinase lacking the C-terminal tail (ERK2/7(VI)ΔT and ERK2/7(VIII)ΔT) dramatically stabilized the proteins (Fig. 6C, left panel). These findings indicate that a signal promoting the turnover of ERK7 exists within N-terminal kinase domains I–V of ERK7.

In addition, it appears that a region in the C terminus of ERK7 can confer instability. Thus, the ERK2/7(VI) chimera that contains kinase domain I–V of ERK2 is expressed at a lower level than its counterpart lacking the C-terminal region of ERK7, ERK2/7(VI)ΔT (Fig. 6C, left panel). However, the ERK2/7(VI) chimera is less stable than the ERK2/7(VIII) chimera that also has ERK2 domains VI and VII (see chimera ERK2/7(VIII) in Fig. 6C, left panel), suggesting that domains VI and VII in the ERK7 kinase may also contribute to the instability conferred by the tail. This possibility is supported by the observation that addition of only the C-terminal region of ERK7 to ERK2 causes some degradation but little loss in overall protein expression (Fig. 6C, right panel). Surprisingly, the isolated C-terminal tail of ERK7 has a turnover rate that is at least as high as that of ERK7 (Fig. 7), suggesting that the tail is more stable when associated with the kinase domain. In this figure, the band with the slower mobility in the gel probably represents phosphorylated ERK7/7 protein. Neither serum stimulation, nor serum starvation made a difference in the relative expression levels (data not shown). Taken together, these results indicate that the N terminus of ERK7 is a major site of instability in the protein and regions(s) within the C-terminal tail also contribute.

To narrow the region of instability in the kinase domain further, additional chimeras on the N terminus consisting of domains I, I–II, I–IV, and I–V from ERK7 fused to the remaining domains of ERK2. As shown in Fig. 8B, all of the ERK7/2 chimeric proteins were expressed at a level much lower than that of ERK2 and similar to the level of ERK7 expression (Fig. 8B). These results indicate that domain I of ERK7 is sufficient to impart instability to ERK2. ERK7/2(VIa) and ERK7/2(Vb) are two chimeric constructs that differ by one residue. Both constructs contain domains I–V from ERK7, but ERK7/2(Va) ends with residues IYLV and ERK7/2(Vb) with residues IYL. The expression levels of ERK2/7(Vla) and ERK7/2(Vb) were comparable, confirming that the different levels of protein expression were not because of the process of subcloning or subtle differences in amino acids at the sites of fusion. Conversely, the addition of the N-terminal domains I or I–II of ERK2 to the kinase domain of ERK7 enhanced the stability of the ERK7 kinase chimeras ERK2/7(VII)ΔT and ERK2/7(VIII)ΔT (Fig. 8C).

To determine whether the instability of the ERK7/2 chimera was regulated by the proteasome pathway, one of these constructs, ERK7/2(II), was co-transfected with the dominant negative hCul1-N452 plasmid into HEK293 cells. Reduced expression of both the wild-type ERK7 and ERK7/2(II) chimera was seen following treatment with cycloheximide. However, the levels of both were stabilized following treatment with cycloheximide when co-expressed with the dominant negative hCul1-N452 (Fig. 9). The results showed that the hCul1-N452 mutant stabilized the ERK7/2(II) chimera containing Domain I from the N terminus of ERK7 (Fig. 9). Taken together, these results indicate that the N-terminal 20 amino acids of ERK7 are both necessary and sufficient for the SCF-mediated ubiquitination and rapid proteolytic turnover of the ERK7 kinase domain.

One possible explanation for the results obtained is that the ERK7/ERK2 chimeric proteins are misfolded. To ensure that these chimeric proteins are not structurally altered, we determined whether the ERK7/2 chimeric protein that contains domains I and II of ERK7 is an active kinase. As shown in Fig. 10, the TEY motif in the activation loop of the chimeric protein is phosphorylated in response to serum stimulation similar to that of the wild type ERK2. These results indicate that the chimeric protein is functionally active and therefore not denatured.

To determine whether the 20-amino acid N-terminal domain is sufficient to confer instability on proteins unrelated to ERK7, we fused these N-terminal amino acids to GFP. As shown in Fig. 11, the addition of this N-terminal ERK7 domain significantly decreased the stability of GFP. Furthermore, the addition of proteasome inhibitor I increased the stability of ERK7/1/GFP proteins (Fig. 11B), suggesting that the regulation of ERK7/1/GFP was through the proteasome pathway.
These results confirm that the N-terminal 20 amino acids of ERK7 are necessary and sufficient as a signal for ubiquitination and proteosomal degradation of ERK7.

**DISCUSSION**

ERK7 is expressed at extremely low levels in tissues and cultured cells. The results presented here demonstrate that ERK7 is rapidly degraded by the ubiquitin-proteosome pathway. Whereas the ERK7 mRNA levels are comparable with those of ERK2, the stability of the ERK7 and ERK2 proteins are significantly different. Although the C-terminal tail also contributes to instability, the main determinant of ERK7 protein turnover is the first 20-amino acid region (Domain I) at the N terminus. Because ERK7 is constitutively activated, its kinase activity is dependent upon the level of ERK7 expression. We have also previously shown that the C-terminal domain of ERK7 can confer a number of properties including inhibition of DNA synthesis. The results shown here now demonstrate that...
the N terminus of the kinase domain and the C-terminal domain control ERK7 stability and provide a mechanism for the regulation of both ERK7 kinase activity as well as the kinase-independent activity of the ERK7 C terminus.

Interestingly, the members of the ERK subfamily that are “big” MAP kinases characterized by extended C-terminal domains are expressed at significantly lower levels than ERK 1 and 2. It has recently been shown that ERK3, which has a different activation loop motif (SEG instead of TEY) but shares homology with the ERK subfamily, is also regulated by ubiquitination and proteosome turnover. In the case of ERK3, two regions within its N terminus were identified as responsible for its turnover with a t<sub>1/2</sub> of 30 min (12). In contrast, ERK7 is regulated by one 20-amino acid region at the N terminus of ERK7 as well as a region within the C-terminal tail, and the t<sub>1/2</sub> for turnover occurs between 1 and 2 h. Analysis of ERK2/ERK7 chimeras shows that the N-terminal amino acids are both necessary and sufficient to regulate protein turnover but an interaction between the C terminus and domains V–VII chimeras can also confer instability to the full-length enzyme. It is possible that, in the three-dimensional structure of ERK7, there is an interaction between the N terminus and C-terminal tail with the same E3 ligase. However, resolution of this question must await ERK7 crystallization and identification of the regions of instability within the C-terminal tail as well as the nature of the E3 ligase. Taken together, these results indicate that there is a subset of MAP kinases that have an alternative mechanism for regulation consisting of protein turnover. Given the low level of ERK7 expression, the tight regulation and rapid turnover suggests that ERK7 expression rapidly responds to cellular signals.

Other kinases are also regulated by a similar mechanism. One in particular, SGK, is a serine/threonine protein kinase related to Akt (13). Interestingly, the amino acid residues that are responsible for SGK turnover are also located at the N terminus. However, analysis of the sequences reveals no obvious homology. It has been suggested that this region might regulate SGK localization, bringing the kinase in proximity to the ubiquitination complex. However, although the kinase domain of ERK7 is localized in the cytoplasm and the intact ERK7 is nuclear, both proteins are turned over at similar rates. Similarly, the ERK7 C-terminal domain when expressed alone is localized in the nucleus but also degraded at a comparable rate.

Fig. 7. Turnover of ERK7 and the ERK7 tail in stably transfected PC12 cells. Both ERK7 and the ERK7 C-terminal tail domain (ERK7/T) were subcloned into a pCLE retroviral vector, and then transduced into PC12 cells. Clones stably expressing the ERK7 genes were selected, and two of these stable lines were used for assaying ERK7 protein turnover. One million cells were seeded onto each 60-mm dish, and, 24 h later, the cells were treated with CHX (50 μg/ml). Cells were harvested at different time points, lysed, and analyzed for ERK7 and ERK7/T protein by SDS-PAGE and Western blotting as described under “Experimental Procedures.” An ERK7 antibody raised against a C-terminal peptide was used to probe ERK7 and ERK7/T proteins. Membranes were stripped and reprobed with an anti-tubulin antibody as a control for protein loading. These results are representative of three independent experiments.

Fig. 8. The N-terminal domain I is responsible for rapid turnover of the ERK7 kinase. A, schematic of domains I to V from both ERK7 and ERK2. B, the N terminus of ERK7 contributes to rapid protein turnover of the kinase. Chimeric constructs were made as described under “Experimental Procedures.” ERK7/2(II) represents a chimera containing domain I from ERK7 fused to the remaining domains of ERK2. The ERK7/2(II):H9004 chimera contains domains I–II from ERK7, the ERK7/2(V) contains domain I–IV from ERK7, and both ERK7/2(V1a) and ERK7/2(V1b) contain domains I–V from ERK7. The chimeric constructs were co-transfected with GFP into HEK293T cells, and cell lysates were analyzed by SDS-PAGE, Western blotting with anti-HA and anti-GFP antibodies, and chemiluminescence as described under “Experimental Procedures.” The protein amount in the first ERK2 lane (B) was only one-tenth of the amount loaded in each of other lanes. HA-ERK7, HA-ERK2, and GFP proteins are marked by arrows. The GFP protein is a control for transfection efficiency. C, ERK7 protein is stabilized by swapping the N-terminal domain I of ERK7 with the N-terminal domain I from ERK2. Construction of ERK2/7(I):H9004 and ERK3/7(I):H9004 was described under “Experimental Procedures.” ERK2/7(I):H9004 represents the fusion of domain I from ERK2 to the remaining domains of ERK7 without the C-terminal tail region. The constructs were transfected into HEK293T cells, and cycloheximide (CHX) was added and incubated with cells as indicated. Cell lysates were analyzed by SDS-PAGE, Western blotting, and chemiluminescence as described under “Experimental Procedures.” These results are representative of at least three independent experiments.
ERK7 Proteolytic Turnover

The stabilization of ERK7 by expression of dominant-negative Cullin-1 suggests that the proteolytic recognition complex is an SCF complex. SCF is one of the best characterized ubiquitin ligase complexes and consists of a number of subunits including Cullin-1. Functioning as a scaffold protein, Cullin family proteins bind both to the ubiquitin E3 ligase complex and Roc 1, a RING finger protein that binds to the ubiquitin-conjugating E2 enzyme (14). The N terminus of Cullin-1 interacts with the adapter protein Skp1, which complexes to a number of different F-box proteins such as Skp2 to assemble different SCF-Roc1 E3 ligases. The E3 ligase complexes then recruit and ubiquitinate specific substrates including many nuclear cell cycle regulatory proteins such as p27 (15) and p21 (16, 17). The dominant-negative Cullin-1 that we used to stabilize ERK7 expression lacks the C terminus that interacts with the Roc1 protein, and thus does not ubiquitinate its substrates (11). The result we obtained suggests that ERK7 is ubiquitinated by an SCF E3 ligase, but does not identify a specific recognition protein. Because ERK7 is localized in the nucleus and cannot inhibit DNA synthesis (2), proteolytic regulation similar to that of cell cycle proteins would not be surprising.

Proteolytic turnover of ERK7 was demonstrated using exogenously expressed ERK7 that was either transiently or stably expressed in cells. Similar approaches have been used to show proteolytic turnover of other unstable kinases such as SGK (13). Endogenous levels of ERK7 are extremely low and difficult to detect above nonspecific background levels, even with the addition of proteosome inhibitors, preventing accurate quantitation of turnover rates. Thus, we cannot rule out the possibility that regulation of ERK7 also occurs at a transcriptional as well as a post-transcriptional level. Expression of ERK7/ERK2 chimeras enabled us to localize one main site of proteolytic recognition to a region at the N terminus of ERK7. The ability to destabilize other proteins such as ERK2 and GFP after transferring this region to them confirmed the identity of this domain. Two types of experiments were included to ensure that the proteolytic turnover observed was not a result of misfolding or overexpression of a cytotoxic protein. First, we demonstrated that the ERK7/ERK2 chimera containing only the N terminus of ERK7 was activated by serum indicating that the basic kinase structure was not altered. Second, we showed that an unrelated and benign protein such as GFP was also rapidly degraded by the addition of the N-terminal ERK7 sequence. Taken together, these results clearly identify a region in ERK7 that is recognized by a Cullin-1-dependent, proteosome degradation complex.

The tight regulation of ERK7 expression raises the possibility that ERK7 is cytotastic or cytotoxic to the cell. The previous observation that overexpression of ERK7 leads to an inhibition of DNA synthesis in CV-1 cells is consistent with this interpretation (2). Similarly, stabilization and the resulting higher expression of ERK3 results in G1 arrest (12). In both cases,
inhibition of proteolytic turnover would enable rapid suppression of cell cycle progression similar to other regulators of the cell cycle such as p53. Although we have identified a major N-terminal domain that promotes turnover of ERK7, other regions also contribute to ERK7 instability. Once all the regions of E3 ligase recognition have been identified and mutated, it should be possible to determine the effect of stable ERK7 expression on cell growth and integrity.

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