Identification of the Ubiquitin-Proteasome Pathway in the Regulation of the Stability of Eukaryotic Elongation Factor-2 Kinase

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Abstract

Eukaryotic elongation factor-2 kinase (eEF-2 kinase) is a highly conserved calcium/calmodulin-dependent enzyme involved in the regulation of protein translation and cell proliferation. Rapid changes in the activity and abundance of eEF-2 kinase have been observed on growth stimulation, and increased enzyme activity is characteristic of malignant cell growth. Yet the mechanism for controlling the turnover of this kinase is unknown. The ubiquitin-proteasome pathway regulates the degradation of many cellular proteins, including transcription factors, cell cycle regulators, and signal transduction proteins. Therefore, we determined whether the ubiquitin-proteasome pathway regulates the turnover of eEF-2 kinase. We found that eEF-2 kinase was a relatively short-lived protein with a half-life of less than 6 hours. eEF-2 kinase was ubiquitinated in vivo as determined by coimmunoprecipitation and polyubiquitin affinity matrix. Incubation of purified eEF-2 kinase with a source of ubiquitination enzymes (rabbit reticulocyte lysate), purified ubiquitin, and ATP revealed the presence of increasing molecular weight species of ubiquitinated eEF-2 kinase. Treatment of cells with MG132, a proteasome inhibitor, inhibited eEF-2 kinase degradation and induced the accumulation of polyubiquitinated forms of the enzyme, resulting in an increase in its half-life. These results suggest involvement of the proteasome in the turnover of the ubiquitinated kinase. Because eEF-2 kinase is chaperoned by heat shock protein 90 (Hsp90), we next determined if disruption of the Hsp90-eEF-2 kinase complex promoted degradation of the kinase. Treatment of cells with geldanamycin, an Hsp90 inhibitor, enhanced ubiquitination of eEF-2 kinase and decreased the half-life of the kinase to less than 2 hours. These results indicate that cellular levels of eEF-2 kinase are maintained by a balance between association with Hsp90 and degradation by the ubiquitin-proteasome pathway. In conclusion, these data show that the turnover of eEF-2 kinase might control the abundance of this enzyme and have implications in the treatment of certain forms of cancer.

Introduction

Eukaryotic elongation factor-2 kinase (eEF-2 kinase; calmodulin-dependent kinase III) is a calcium- and calmodulin-dependent enzyme that catalyzes the phosphorylation of eukaryotic elongation factor-2. Cloning and sequencing of the kinase by our laboratory (1) and others (2) suggest that eEF-2 kinase may represent a new class of protein kinases now known to include myosin heavy chain kinase A, B, and C (1, 3, 4) and several proteins with both ion channel and kinase features (5). The activity of eEF-2 kinase is believed to regulate protein translation (6). For example, phosphorylation of elongation factor-2 terminates peptide elongation by decreasing the affinity of the elongated chain for the ribosome (7). In addition, the activity of this kinase seems to be involved in several cellular processes. For example, increased eEF-2 kinase activity is linked to augmented fibroblast growth (8), myoblast fusion and differentiation (8, 9), fluctuations in oogenesis (10), cell cycle progression of Xenopus eggs and human amnion cells (11), cellular differentiation (12–14), and response of cells to growth factors and serum (8, 15, 16). Growing evidence suggest that the modulation of eEF-2 by eEF-2 kinase may also be important in the development of neuronal functions (17–19). eEF-2 kinase has also been shown to be involved in the generation of synaptic connections and synaptic plasticity (18, 19).

Our laboratory was the first to recognize an increased activity of eEF-2 kinase in malignant cell lines (20) and in human cancers (15, 21). The marked increase in activity in rat glioblastoma led us to investigate the activity of eEF-2 kinase in normal rat glia. These experiments showed that kinase activity was up-regulated in rapidly proliferating normal and malignant glia. In the study of eEF-2 kinase regulation, we observed that the protein content of the kinase, not its regulators such as calcium and calmodulin, changed rapidly on growth stimulation or serum deprivation (16). Furthermore, in recent studies we found that disruption of heat shock protein 90 (Hsp90) chaperoning of eEF-2 kinase also produced rapid disappearance of the enzyme (22). Yet, the mechanisms regulating the stability of eEF-2 kinase remain unknown.

Protein modification via covalent attachment of ubiquitin has emerged as one of the most common pathways for targeting protein for degradation by the 26S proteasome (23). Ubiquitination has also been implicated in other regulatory mechanisms, ranging from protein kinase activation to control of protein translation (24). The ubiquitin-proteasome pathway is composed of the ubiquitin conjugating system and the 26S proteasome; the latter contains the multicatalytic protease complex. Coordinated function of the ubiquitin-conjugating system involves several classes of enzymes including ubiquitin-activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). The activity of this pathway may result in mono- or polyubiquitination on the target protein, each of which serves as a different signaling tag (25–27). Whereas polyubiquitin tags the protein for degradation through proteasome pathway, monoubiquitination serves as a signaling marker (23, 28, 29). Because of the role of the ubiquitin-proteasome pathway in regulating the degradation...
of short-lived proteins, and the recent interest in the proteasome as a target for anticancer therapy (30), we investigated whether eEF-2 kinase was subjected to ubiquitin-mediated proteasomal degradation.

Materials and Methods

Cell lines and culture. The human glioblastoma cell line T98G and the N-nitrosomethyleurea–induced rat glioma line C6 were obtained from the American Type Culture Collection (Rockville, MD). The breast carcinoma cell line MCF-7 was kindly supplied by Dr. Kenneth Cowan of the Eppley Institute for Research in Cancer (Omaha, NE). The human ovarian carcinoma cell line A2780 was provided by Dr. Youcef Rustum (Roswell Park Cancer Institute, Buffalo, NY). All cell lines were grown in DMEM. T98G cells were grown in Ham’s F-10/DMEM (10:1; Life Technologies, Inc., Grand Island, NY). Cells were supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2/95% air. All lines were checked routinely and found to be free of contamination by mycoplasma or fungi. All cell lines were discarded after 3 months and new lines were obtained from frozen stocks.

Antibodies and reagents. Polyclonal anti-ubiquitin antiserum was purchased from Sigma-Aldrich, Inc. (St. Louis, MO) and monoclonal anti-ubiquitin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti–eEF-2 kinase antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Ubiquitinated Protein Enrichment Kit was purchased from CalBiochem-Novabiochem (San Diego, CA). Western blot reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All media and cell culture products were purchased from Life Technologies. All other chemicals were purchased from Sigma-Aldrich.

Preparation of cell extracts. Cell monolayers were washed twice in PBS (pH 7.4), scraped into 15-mL conical tubes, and centrifuged at 1,000 × g for 5 minutes. Cell extracts were prepared by lysis in NET buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% NP40, 1 mmol/L EDTA, 0.25% gelatin, 0.025% sodium azide, 1 mmol/L phenylmethylsulfonyl fluoride, and 1% aprotinin]. The lysates were centrifuged at 15,000 × g for 30 minutes at 4°C. The protein concentration of the supernatants was determined according to the method of Bradford using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Western blot analysis. Fifty micrograms of total cell protein extracts or immunoprecipitates were resolved by 7% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in PBS/Tween 20 (0.05%), followed by incubation with an anti–eEF-2 kinase antibody (1:500 dilution in 10% milk/PBS-T) or an anti-ubiquitin antibody. Membranes were washed twice in PBS/Tween 20 and incubated with horseradish peroxidase–labeled secondary antibodies and enhanced chemiluminescence detection reagent.

Immunoprecipitation. Immunoprecipitations were done using 1 μg of protein from total cell lysates and 10 μL of polyclonal anti–eEF-2 kinase or anti-ubiquitin antibody by incubating overnight at 4°C. The immune complexes were precipitated with Protein-A Sepharose CL-4B (Amersham Pharmacia). Immunoprecipitates were resolved on 8% SDS-PAGE followed by Western blotting. Incubation of cell lysates with polyubiquitin-affinity beads comprised of a glutathione-S-transferase (GST) fusion protein containing a ubiquitin-associated sequence conjugated to glutathione-agarose was carried out according to the protocol of the manufacturer followed by immunoblotting with an anti–eEF-2 kinase antibody.

In vitro ubiquitination assay. Ubiquitination reactions were done in a 30-μL total volume containing 50 mmol/L HEPES (pH 7.5), 5 mmol/L MgCl2, 15 mmol/L ZnCl2, 4 mmol/L ATP, 1 μg ubiquitin (Sigma-Aldrich), and 100 ng GST-eEF-2 kinase (31). Rabbit reticulocyte lysate was used as a source of ubiquitinating enzymes E1, E2, and E3. After incubation at 30°C for 60 minutes, the reactions were stopped by addition of 3 × Laemml buffer [190 mmol/L Tris (pH 6.8), 6% SDS, 30% glycerol, 15% 2-mercaptoethanol, and 0.003% bromophenol blue dye]. Samples were boiled for 5 minutes and resolved by 7% SDS-PAGE and processed for Western blotting as described above.

Results

Eukaryotic elongation factor-2 kinase is a relatively short-lived protein. Because ubiquitin-mediated protein destruction has been mainly implicated for short-lived proteins, we first sought to determine the half-life of eEF-2 kinase. C6 glioblastoma cells were incubated with cyclohexamide (10 μg/mL) for 16 hours and released in cyclohexamide-free media for different durations of time, followed by immunoblotting using an anti–eEF-2 kinase antibody. As shown in Fig. 1, the amount of eEF-2 kinase protein decreased by 50% after 6 hours.

Eukaryotic elongation factor-2 kinase is subjected to ubiquitination. We next determined whether cellular eEF-2 kinase is ubiquitinated using communoprecipitation techniques. As shown in Fig. 2A, cell extracts were immunoprecipitated with an anti-ubiquitin antibody and then immunoblotted with anti–eEF-2 kinase antibody to detect eEF-2 kinase and other high molecular weight bands indicative of ubiquitin-tagged kinase. Using the reciprocal approach (i.e., immunoprecipitation with anti–eEF-2 kinase antibody and immunoblotting with anti-ubiquitin antibody), we also detected a ladder of high molecular weight species characteristic of polyubiquitinated protein (Fig. 2B). These observations were confirmed using a polyclubiquitin-affinity matrix, which contains a specific peptide ligand for ubiquitinated proteins to probe the presence of eEF-2 kinase. As shown in Fig. 2C, ubiquitinated eEF-2 kinase was detected by immunoblotting the matrix-bound proteins with an anti–eEF-2 kinase antibody.

Eukaryotic elongation factor-2 kinase is ubiquitinated in vitro. We also determined if purified eEF-2 kinase could be conjugated with ubiquitin in a cell-free assay. In this system, rabbit reticulocyte lysates were used as a source of ubiquitinating enzymes (E1, E2, and E3) to which we added purified ubiquitin and purified eEF-2 kinase. In the experiments shown in Fig. 3A, we analyzed whether immunoblotting the reaction mixture with antibodies to eEF-2 kinase would detect both eEF-2 kinase and its ubiquitinated forms. In the experiments shown in Fig. 3B, we tested whether immunoblotting the same reaction mixture with

![Image](325x79 to 486x136)

Figure 1. eEF-2 kinase is a relatively short-lived protein. C6 rat glioblastoma cells were treated with cyclohexamide (10 μg/mL) for 16 hours, followed by release into cyclohexamide-free media for the indicated times. Cell extracts were prepared and run on 7.5% SDS-PAGE, followed by Western blot using anti–eEF-2 kinase (top) and anti–β-actin antibodies (bottom). Representative of three experiments.
antibodies to ubiquitin would detect ubiquitinated protein forms. In both cases, we obtained positive results indicating that eEF-2 kinase could undergo ubiquitination in a cell-free system. The ubiquitinated proteins were not detected in reactions that did not contain either eEF-2 kinase or rabbit reticulocyte lysate (Fig. 3A and B, lanes 1 and 7).

**The proteasome is involved in the degradation of eukaryotic elongation factor-2 kinase.** To determine whether ubiquitination of eEF-2 kinase results in proteasomal degradation of the kinase, the half-life of eEF-2 kinase was determined in the presence of the proteasome inhibitor, MG132. Lysates from cells grown in the presence of MG132 and cyclohexamide were analyzed by Western blot or by immunoprecipitation with an anti–eEF-2 kinase antibody and then by immunoblotting using anti-ubiquitin antibodies. As shown in Fig. 4, MG132 prolonged the half-life of eEF-2 kinase to >24 hours (Fig. 4A) as compared with <6 hours (Fig. 1), and produced an accumulation of ubiquitinated enzyme (Fig. 4B). In each of the three cell lines tested, treatment with MG-132 increased the content of ubiquitinated eEF-2 kinase. C6 cells contained ubiquitinated forms that ran both at higher and lower molecular weights than eEF-2 kinase. In MCF-7 cells the majority of ubiquitinated eEF-2 kinase ran below 100 kDa, and A2780 cells accumulated the ubiquitinated kinase at 100 kDa as well as ubiquitinated forms that ran at higher and lower molecular weights.

**Inhibition of heat shock protein 90 increases ubiquitination and turnover of eukaryotic elongation factor-2 kinase.** eEF-2 kinase is chaperoned by Hsp90 and disruption of this association led to rapid loss of eEF-2 kinase activity (22). To determine whether or not these results were due to changes in degradation of the ubiquitinated proteins, the half-life of eEF-2 kinase was measured.
in the presence or absence of geldanamycin. As shown in Fig. 5, geldanamycin increased the ubiquitination of eEF-2 kinase (Fig. 5A) and decreased the half-life of the protein to less than 1 hour (Fig. 5B).

**Discussion**

We investigated the possible involvement of the ubiquitin-proteasome pathway in regulating the turnover of eEF-2 kinase because of previous results suggesting that rapid changes in enzyme content might regulate critical cellular process (10, 11, 14). Recent evidence implicates a role for eEF-2 kinase in normal and malignant cell growth as well as cell viability (32, 33). Several studies from our laboratory and others established eEF-2 kinase as a proliferation-dependent and mitogen-activated enzyme that is up-regulated in several forms of cancer (15, 16, 34), rapidly increased in response to growth stimuli (35), and rapidly down-regulated on growth cessation (16). Therefore, we reasoned that studying the degradation of this kinase might reveal an important regulatory mechanism that controls the amounts of cellular eEF-2 kinase. We found that eEF-2 kinase is a short-lived protein (Fig. 1), ubiquitinated both in vitro (Fig. 3) and in vivo (Fig. 2), and degraded via the 26S proteasome (Fig. 4). Furthermore, these data suggest that a balance between the fraction of the kinase complexed with either Hsp90 or ubiquitin regulates the steady-state content and activity of eEF-2 kinase (Fig. 5).

Covalent modification of cellular proteins with ubiquitin regulates diverse cellular processes including response to stress, oncogenesis, transcription, protein turnover, organelle biogenesis, DNA repair, and cell cycle transit (36). Alterations in ubiquitination and deubiquitination reactions have been implicated in the etiology of several malignancies (37). Using a combination of pharmacologic and biochemical approaches, we found that the ubiquitin-proteasome pathway is responsible for the degradation of eEF-2 kinase. First, immunoprecipitation with anti-ubiquitin antibodies revealed the presence of eEF-2 kinase and high molecular weight species recognized by anti–eEF-2 kinase antibodies (Fig. 2A). Next, immunoprecipitation with anti–eEF-2 kinase antibodies followed by Western blotting with anti-ubiquitin antibodies revealed a characteristic polyubiquitination ladder (Fig. 2B). Furthermore, eEF-2 kinase reacts with a ubiquitin affinity matrix consisting of a ubiquitin-associated sequence (Fig. 2C). We further strengthened the observation that eEF-2 kinase is tagged to ubiquitin using a cell-free system where rabbit reticulocyte lysate is a source of ubiquitination enzymes. We found that purified eEF-2 kinase is readily ubiquitinated in the presence of ATP and ubiquitin (Fig. 3).

Degradation of eEF-2 kinase seems to be mediated through the 26S proteasome. This conclusion is derived from the results with the proteasome inhibitor MG132, which blocks proteasomal degradation of proteins and causes an accumulation of ubiquitinated species. MG132 increases the half-life of eEF-2 kinase from less than 6 hours to greater than 24 hours (Fig. 4A). Furthermore,
this increase in half-life by MG132 leads to an accumulation of ubiquitinated eEF-2 kinase (Fig. 4B). In each of the cell lines tested, treatment with MG-132 increased the content of ubiquitinated eEF-2 kinase. However, there were several notable variations between cell lines. For example, C6 cells contained ubiquitinated protein that ran both at higher and lower molecular weights than eEF-2 kinase, suggesting the presence of partially digested kinase. In MCF-7 cells the majority of ubiquitinated kinase ran below 100 kDa, suggesting that MG-132 was less effective in blocking proteasomal degradation of ubiquitinated eEF-2 kinase in this cell line. Finally, the results in A2780 cells showed an accumulation of ubiquitinated eEF-2 kinase around 100 kDa, suggesting oligo-ubiquitinated kinase as well as ubiquitinated forms that ran at both higher and lower molecular weights. We obtained similar results with the second proteasome inhibitor lactacystine (data not shown).

We previously reported that eEF-2 kinase is complexed with Hsp90 (38). The molecular chaperone Hsp90 is involved in the stabilization and conformational maturation of many signaling proteins that are deregulated in cancers, and Hsp90 inhibition results in the proteasomal degradation of these client proteins (39). We previously found that inhibition of Hsp90 by ansamycin antibiotics led to rapid loss of eEF-2 kinase and of cell viability (22). We now find that the Hsp90 inhibitor geldanamycin increases ubiquitin-mediated degradation of eEF-2 kinase. Accordingly, the half-life of eEF-2 kinase is markedly decreased (less than 1 hour) in cells treated with geldanamycin (Fig. 5B), and this decrease in eEF-2 kinase content is associated with an increase in its ubiquitinated species (Fig. 5A). Therefore, we conclude that the complex of Hsp90 with eEF-2 kinase helps maintain the level of the properly folded kinase and disruption of this complex causes its ubiquitination and degradation.

In conclusion, these studies shed significant light on previous observations related to the physiology of eEF-2 kinase. It now seems that the half-life of the kinase can be regulated by its rate of degradation and that turnover is mediated by the ubiquitin-proteasomal pathway.

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