Stability of the ATF2 Transcription Factor Is Regulated by Phosphorylation and Dephosphorylation*

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Trans-activation of the activating transcription factor-2 (ATF2) in response to cellular stress requires the N-terminal phosphorylation of ATF2 by stress-activated protein kinases (SAPK). In this study, we investigated the role of ATF2 phosphorylation in the maintenance of ATF2 stability. Activation of SAPK by forced expression of AMEK1 increased overall ATF2 ubiquitination, presumably because of the enhanced dimerization of ATF2. Treatment of AMEK1-expressing cells with okadaic acid led to the increase in N-terminal phosphorylation, protection from ubiquitination, and accumulation of exogenously expressed ATF2, indicating the role of protein phosphatases in balancing the effects of stress kinases.

Analysis of ubiquitination and degradation of the constitutively dimerized ATF2 mutant (ATF2ab150–248) showed that activation of JNK or p38 kinase renders ATF2 resistant to ubiquitination and degradation. This effect is mediated by JNK/p38-dependent phosphorylation of ATF2 at Thr-69 and Thr-71, because the phosphorylation-deficient mutant (ATF2ab150–248;T69A,T71A) was not protected from ubiquitination and degradation by the activation of SAPK. Treatment of cells with okadaic acid elevated the tumor necrosis factor α-induced ATF2 level and the extent of its specific N-terminal phosphorylation. Cycloheximide, which activates SAPK, while inhibiting protein synthesis, stabilized endogenous ATF2. However, treatment of cells with the high dose of SB203580, which inhibits JNK and p38 kinase, resulted in efficient degradation of ATF2 in cells exposed to cycloheximide. This degradation was abrogated by cotreatment with the proteasome inhibitor MG132. Our findings suggest that N-terminal phosphorylation of ATF2 dimers protect ATF2 from ubiquitination and degradation. We propose the hypothesis that the balance between SAPK and protein phosphatases affects the duration and magnitude of ATF2 transcriptional output because of the effect on substrate recognition for ubiquitination and degradation.

Activating transcription factor 2 (ATF2)† is a member of the ATF/cAMP-response element-binding protein family of basic region-leucine zipper proteins (1, 2), which play an important role in the cellular stress response (3, 4). ATF2 target genes include tumor necrosis factor α (TNFα), transforming growth factor β (5), cyclin A (7), E-selectin (8), DNA polymerase β (9), and c-Jun (10), which are genes that are known to play important roles in the stress response, cell growth and differentiation, and immune response. Although the products of ATF2 target genes have been implicated in the contribution to neoplastic processes and inflammation, the physiological role of ATF2 remains largely uncharacterized.

Under nonstress growth conditions, ATF2 exhibits a low level of transactivation because of an intramolecular inhibitory interaction in which the C-terminal DNA binding domain is bound to the N-terminal transactivating domain (11). Association of ATF2 with viral proteins, such as adenovirus E1A, hepatitis B virus protein X, or human T-cell leukemia virus type I protein Tax, stimulates its transcriptional activity (12–14). N-terminal phosphorylation of ATF2 in response to stress has been demonstrated to relieve intramolecular inhibition (15) and increase transcriptional activation of ATF2 (4, 5). Phosphorylation of amino acid residues Thr-69 and Thr-71 is mediated by stress-activated protein kinases (SAPK) including Jun-N-terminal kinase (JNK, 4, 5, 16) and p38 mitogen-activated protein kinase (17). Transcriptionally active ATF2 recognizes and binds specific ATF/cAMP-response element motifs as a homo- or heterodimer. ATF2 interacts with various heterodimerization partners depending on the cell type.

The ubiquitin proteasome pathway-dependent degradation is an important means of regulating ATF2 activity (18, 19). We have previously demonstrated that in vitro ubiquitination of ATF2, as well as of c-Jun, JunB, and p53, is targeted by association with JNK (20–22). JNK targeting of its associated proteins for ubiquitination is phosphorylation-dependent. In recent studies, we have shown that leucine zipper based heterodimerization with c-Jun, which is one of key ATF2 partners, facilitates ATF2 ubiquitination and degradation (19). Overexpression of c-Jun increased ATF2 ubiquitination and decreased its half-life. Increased ubiquitination and decreased stability were also found in a constitutively active spliced form of ATF2, which lacks amino acids 150–248 (19).

Two possible mechanisms of the effect of ATF2 N-terminal phosphorylation in the regulation of its transcriptional activity can be envisioned. First, phosphorylation-mediated conformational changes leading to disruption of intramolecular inhibition are expected to enhance ATF2 dimerization and DNA binding (15). However, we found that, unlike stable ATF2 engaged in this inhibitory interaction, the homo- and heterodimers of ATF2 are rapidly ubiquitinated and degraded by the proteasome pathway (19). Second, it is also possible that, in addition to favoring dimerization, SAPK-mediated phosphorylation may stabilize ATF2, as in the case with c-Jun (20, 23).

Previously, we observed that N-terminal phosphorylation of...
ATF2 by JNK precludes ATF2 ubiquitination in vitro (21). In this study, we sought to investigate the role of SAPK in preventing ubiquitination and degradation of ATF2 in vivo. We provide evidence for the role of ATF2 phosphorylation per se, as a consequence of kinase and phosphatase activities, in the regulation of ATF2 stability in vivo. Our results suggest that the maintenance of phosphorylation status of ATF2 may be crucial in determining the duration of ATF2 transactivation.

EXPERIMENTAL PROCEDURES

Reagents—Okadaic acid (OA) and cycloheximide were purchased from Sigma. Anti-ATF2 (phospho-specific and control) polyclonal antibody (New England Biolabs) and anti-HA monoclonal antibody (Couance Research Products) were purchased. Proteasome inhibitor MG132 (Peptide International Co.), human recombinant TNFs (R&D Systems), and p38/JNK inhibitor SB 203580 (Calbiochem) were purchased.

Expression Plasmids—Vectors for expression of HA-tagged ubiquitin (a gift from D. Bohmann (24)) and constitutively active forms of MEKK1 (ΔMEKK1; a gift from A. Minden (25), JNK2 and MKK6 (JNKCA and MKK6ΔN, gifts from M. Karin (26), and ASK1 (ASK1N, a gift from H. Ichijo (27)) were previously described. Constructs encoding N-terminal fusion of hexahistidine with ATF2 (150–248-HA or pCMV-ATF2 150–248-HA) and C-terminal fusion of hemagglutinin epitope (pCMV-ATF2 150–248-HA) were described elsewhere (19). Point mutations to substitute Thr-69 and Thr-71 for alanines were generated by oligonucleotide-directed mutagenesis with the aid of a QuickChange kit (Stratagene). The integrity of each construct was confirmed by partial DNA sequencing, in vitro translation, and immunoblotting.

Cell Culture and Transfections—TIG normal human fibroblasts (a kind gift of H. Tahara) and 293T human embryo kidney cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and antibiotics at 37 °C with 5% CO2. 293T cells were a kind gift of H. Tahara) and 293T human embryo kidney cells were purchased. Cell culture and transfections—TIG normal human fibroblasts (a kind gift of H. Tahara) and 293T human embryo kidney cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and antibiotics at 37 °C with 5% CO2. 293T cells were transfected using the CaPO4 method. The total amount of DNA within the experiments was kept constant by adding the respective empty vector plasmid DNA to the transfection mixtures. The experiments were repeated at least three times, and the representative figures are shown.

Ubiquitination Assay—In vivo ubiquitination was assayed as described by Treier et al. (24). His-ATF2 (4 μg) was co-transfected into 293T cells with ubiquitin-HA vector (3 μg). Twenty-four hours later, cells were lysed with 6 M guanidinium HCl, and his-tagged proteins were purified by nickel resins as described by Treier et al. (24), separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to Hybond N nitrocellulose filter (Amersham Pharmacia Biotech). The filter was cut just above the 71-kDa protein molecular mass marker, and its lower part was analyzed by means of immunoblotting using anti-ATF2 antibody to identify nickel-purified hisATF2.

In Vivo Degradation Assay—293T cells were transfected with pCMV-ATF2 150–248-HA or pCMV-ATF2 150–248 T69A,T71A-HA (5 μg) constructs and pCMV-ASK1ΔN (2 μg) as indicated. Twenty-four hours later the cells were incubated with methionine- and cysteine-free Dulbecco’s modified Eagle’s medium supplemented with 10% dialyzed calf serum for 1 h and then metabolically labeled with 0.5 μCi/ml [35S]methionine/cysteine mix (Amersham Pharmacia Biotech). The labeling was chased with complete medium supplemented with 2 mM cold methionine and cysteine for the times indicated. Cells were lysed, and the ATP2 proteins were immunopurified using HA antibody as described previously (19). Immunoprecipitated material was resolved by SDS-polyacrylamide gel electrophoresis. The gels were fixed, impregnated with Amplify reagent (Amersham Pharmacia Biotech), dried, and subjected to autoradiography.

Quantitation—The experiments were repeated at least three times, and the representative figures are shown. Quantitation was performed using NIH Image 1.61 Software.

RESULTS

In previous studies, we established the in vivo ubiquitination of ATF2. In these transient transfection-based experiments his-tagged ATF2 is co-expressed with Ub-HA (19). Using nickel beads, ATF2 is purified under denaturing conditions, and the amount of HA-tagged polyubiquitin chains covalently linked to

![Fig. 1. Modulation of stress-activated protein kinase pathway alters ATF2 ubiquitination in vitro.](http://www.jbc.org/cgi/content/fig/12561/8/12561/F1a)

ATF2 is then assessed by immunoblotting. Although the ubiquitination of exogenous ATF2 could not be detected in melanoma or fibroblast cells, even in the presence of proteasome inhibitors, using 293 cells, it was possible to determine the degree of ATF2 ubiquitination in vivo (19). Activation of stress kinases p38 and JNK by the common upstream kinase MEKK1, in its constitutively active form (ΔMEKK1), increased overall ATF2 ubiquitination (Fig. 1). This effect requires the catalytic activity of MEKK1, because co-expression of its kinase dead mutant (MEKK1-TR) did not lead to an increase in ATF2 ubiquitination levels. The lack of effect of MEKK1-TR cannot be explained by the difference in the level of expression of active and inactive kinase (Fig. 1, lower panel). Because in vitro assays revealed that JNK-mediated phosphorylation of ATF2 protects it from ubiquitination (12), the data shown in Fig. 1 suggest that additional factors may affect ATF2 phosphorylation status and its ubiquitination and degradation rates in vivo.

One such factor is the activity of protein phosphatases. To test this possibility, we treated ΔMEKK1-transfected cells with okadaic acid, as a general inhibitor of protein phosphatases (28). Forced expression of ΔMEKK1, but not MEKK1-TR, in OA-treated cells, led to the accumulation of exogenously expressed ATF2 (Fig. 2A). This result indicates that phosphatase activity prevents ATF2 stabilization under conditions when JNK is activated. A 4-fold increase in the level of ATF2 phosphorylation on N-terminal residues, measured by phosphospecific antibodies, was seen in ΔMEKK1-expressing cells treated with okadaic acid (Fig. 2B). This observation suggests that under conditions when stress kinases are activated, ATF2 stability correlates with its phosphorylation level.

Activation of stress kinases by the expression of a constitutively active form of MEKK1 can no longer increase ATF2 ubiquitination when the phosphatase activity is inhibited by okadaic acid (Fig. 2C). Because N-terminal phosphorylation was shown to disrupt the intramolecular inhibitory interaction between the ATF2 leucine zipper and its transactivation domain, thereby leading to ATF2 dimerization, our data suggest that although ATF2 dimers are phosphorylated by stress kinases, they are resistant to ubiquitination and degradation. To test this hypothesis directly, we assessed the ubiquitination and degradation of ATF2 forms whose dimerization status is not affected by N-terminal phosphorylation. To this end, we used the human analog of a spliced form of murine ATF2, which lacks amino acids 150–248. We previously confirmed that this mutant primarily exists as a transcriptionally active
Higher expression of ATF2 under OA treatment (500 nM for 3 h). The loading of nitrilotriacetic acid-purified ATF2 was equilibrated (upper panel, anti-ATF2 antibody), and phosphorylation of ATF2 was analyzed via immunoblotting with anti-phospho-ATF2 antibody (New England Biolabs; lower panel). C, ΔMEKK1-induced in vivo ubiquitination of ATF2 in 293T cells is inhibited by okadaic acid treatment (500 nM for 3 h). In vivo ubiquitination of purified ATF2 proteins was assessed as described above. Immunoblots probed with anti-HA (upper panel) and anti-ATF2 (lower panel) antibodies are depicted.

Okadaic acid sustains endogenous ATF2 phosphorylation and promotes its stabilization in TNFα-treated human fibroblasts. TIG human fibroblasts were harvested at the indicated time after treatment with TNFα (10 ng/ml; R&D Systems) and with or without okadaic acid (50 nM). One hundred μg of whole cell extracts were analyzed by immunoblotting with ATF2 (upper panel) and phospho-ATF2 (lower panel) antibodies. Quantitation data in arbitrary units are shown below.

Activation of JNK or p38 pathways protects ATF2 from ubiquitination and degradation via Thr-69 and Thr-71 phosphorylation. A, 293T cells were transfected as indicated. One μg of JNKKCAA or MKK6D/D cDNAs was used. In vivo ubiquitination of purified ATF2 proteins was assessed as described above. Immunoblots probed with anti-HA (upper panel) and anti-ATF2 (lower panel) antibodies are depicted. B, HA-tagged ATF2 constructs with or without ASK1ΔN were expressed in 293T cells. Metabolic labeling with [35S]methionine/cysteine was chased for the time indicated, and ATF2 proteins were immunopurified with HA monoclonal antibody (Couance Research Products) under stringent conditions (0.5 M LiCl), separated by SDS-polyacrylamide gel, and analyzed by autoradiography.

To further assess the role of SAPK in the regulation of ATF2 stability, we have followed up the half-life of endogenous ATF2. Cycloheximide treatment allows ATF2 levels to be monitored under conditions where protein synthesis is inhibited and stress kinases are activated (29). Under such conditions we observed that endogenous ATF2 is stable over 8 h of cycloheximide treatment. Treatment of human fibroblasts with the high dose of SB 203580 (100 μM), which is known to inhibit both JNK and p38 kinase (26), resulted in efficient degradation of ATF2 in cells exposed to cycloheximide (Fig. 5). Simultaneous exposure of normal human fibroblasts to cycloheximide, SB...
203580, and the proteasome inhibitor MG132 restored ATF2 stability. These data suggest that phosphorylation of ATF2 by stress kinases protects ATF2 from proteasome-mediated degradation.

**DISCUSSION**

One of the open questions in understanding the regulation of transcriptional activities relates to factors which contribute to the duration and magnitude of these activities. Although ATF2 phosphorylation by stress kinases is required for the transcriptional activities of ATF2, the cellular determinants that limit those activities are not well understood. We previously demonstrated that stress kinases target the ubiquitination of their transcription factor substrates in a phosphorylation-dependent manner. The emerging model, which was proven correct for the regulation of c-Jun, p53, and, presently, ATF2 stability, is the following. Although these transcription factors are not phosphorylated, they serve as good substrates for JNK association and subsequent targeting for ubiquitination, which is the scenario encountered under nonstressed normal growth conditions. Upon stress induction, the activation of the stress kinases results in the respective phosphorylation of their substrates, leading to their protection from JNK targeting. Here we demonstrate that protection of the activated substrates is maintained as long as the protein remains phosphorylated. Thus, the length of time during which ATF2 is phosphorylated directly represents the duration of its protection from ubiquitination. This is the window of time when ATF2 is expected to be transcriptionally active (Fig. 6). The case of ATF2 may represent other transcription factors, which possess the characteristics of intramolecular inhibition, in addition to the ability to mediate transactivation as homodimers.

The finding that ATF2 dimerization with other basic region-leucine zipper proteins increases its basal level of ubiquitination thereby facilitating its degradation denotes an important regulatory aspect of ATF2 stability (19). The latter observation established that through its association with c-Jun, ATF2 has a shortened half-life, thereby establishing one mechanism by which duration of transcriptional output is limited. At the same time, ATF2 is known to acquire its transcriptional activity upon stress kinase-mediated N-terminal phosphorylation, even in the absence of c-Jun (4). Here we propose that the time frame that enables the transcriptional output of ATF2 is limited to the amount of time during which phospho-groups remain on the dimerized ATF2 forms (Fig. 6). Our results point to the active role of protein phosphatases in facilitating the degree of protein ubiquitination and respective degradation, although the phosphatase species responsible for this regulation in vivo are yet to be identified. Our findings provide the explanation as to why the degree of kinase activity does not directly reflect the activity of its transcription factor substrates. Consequently, our studies provide direct evidence for an important mechanism that underlies the stability of the transcriptional complex. Phosphorylation by either p38 or JNK is sufficient to acquire a transcriptionally active component, which is protected from ubiquitination and degradation whereas the protein phosphatases do not remove the phospho groups from the protein. A similar mode of regulation was recently reported for Bcl2, which is protected from ubiquitination and degradation by extracellular signal-regulated kinase 2-mediated phosphorylation in human endothelial cells (30). Inhibition of mitogen-activated protein kinase activity (31) and/or activation of mitochondrial PP2A protein phosphatase (32) in response to treatments with TNFa, ionizing radiation, or ceramide, result in dephosphorylation and proteasome-dependent degradation of Bcl2, which is required for apoptosis.

The case of ATF2 presented here illustrates the ability of multiple stress kinases, activated by alternate stress stimuli, to render the same output. This suggests that although ATF2 phosphorylation by either p38 or JNK will render it equally competent for association with other transcription factors, the nature of specific signal transduction pathways sustaining transcriptional output is likely to depend on the cell type and stimuli given. Another similar type of regulation is expected to occur in the case of transcription factors that are phosphorylated on different residues by multiple kinases, as shown for p53 (reviewed in Ref. 33). Our proposed model should also reflect p53, because it is the type of stress and expressed transcription factors that would define the nature of the complex and its respective output.

The current studies provide evidence to support the hypothesis that protein phosphatases affect the duration and magnitude of ATF2 transcriptional output primarily because of their effect on substrate recognition for ubiquitination and degradation. Thus, one mean, by which the transcriptional output may be regulated, would rely on respective modulation of a specific protein phosphatase activity.

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