Involvement of ERKs and mitogen- and stress-activated protein kinase in UVC-induced phosphorylation of ATF2 in JB6 cells

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Activating transcription factor 2 (ATF2) has been shown to regulate gene expression in the cellular response to environmental stresses such as ultraviolet (UV) irradiation. However, the signal transduction mechanism of ATF2 activation by UV is not as yet completely understood. In the present study, we provide evidence showing that UVC-stimulated phosphorylation of ATF2 (Thr71) was to varying degrees prevented by a dominant negative mutant of p38β kinase, c-Jun N-terminal kinase 1 (JNK1) or extracellular signal-regulated kinase 2 (ERK2). The phosphorylation was also suppressed by PD98059, an MEK inhibitor, or H89, a potent inhibitor of mitogen- and stress-activated protein kinase 1 (MSK1), and a C- or N-terminal ‘kinase-dead’ mutant of MSK1 (MSK1-Cd or MSK1-Nd). Furthermore, co-immunoprecipitation experiments revealed a potential intracellular signaling complex consisting of ATF2 and ERKs and/or MSK1. In vitro kinase assays revealed that ERK1, ERK2 and MSK1, like p38 kinase and JNK2, directly phosphorylate ATF2 at Thr71, but addition of RSK2 or Akt1 had almost no effect. Active kinase immunoprecipitated by an MSK1 antibody from an extract of JB6 cells irradiated by UVC can directly phosphorylate ATF2 at Thr71, suggesting UVC induces a direct phosphorylation of ATF2 by ERKs or MSK1. Overall, our results reveal that MSK1 and ERKs, like p38 kinase and JNKs, are required for ATF2 phosphorylation (Thr71) in the UVC response.

Introduction

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). Unlike atf2, c-jun is an immediate-early response gene, and its expression is induced in response to environmental stresses. ATF2 can form stable heterodimers with c-Jun, and binds to the 8 bp sequence motif (TGACNCTCA). In this manner, ATF2 regulates the expression of atf3, cyclin A, cyclin D1, trfa and c-jun itself (3–9).

The activity of the c-Jun/ATF2 heterodimer can be regulated by three possible mechanisms. The first mechanism is by increasing the synthesis of c-jun mRNA and c-Jun protein. ATF2 is constitutively and stably expressed in cells, thus c-Jun plays a key role in this mechanism. The second mechanism is the regulation of the stability of ATF2 and c-Jun. This mechanism is very important for the formation of the c-Jun/ATF2 complex, because both c-Jun and ATF2 can be phosphorylated by c-Jun N-terminal kinases (JNKs) and p38 kinase, which results in an increased stability of the c-Jun/ATF2 complex. For example, under normal, unstressed growth conditions, the ATF2 bZIP domain (C-terminal) and activation domain (N-terminal) are engaged in an inhibitory intramolecular interaction and cannot easily bind with c-Jun. However, under stressed conditions [i.e. ultraviolet (UV) irradiation exposure], the intramolecular interaction is released by phosphorylation of the amino acid residues Thr69 and Thr71 (10,11). In addition, c-Jun also can be phosphorylated at serines 63 and 73 and the c-Jun/ATF2 complex is easily formed, thereby increasing transcriptional activity of the c-Jun/ATF2 complex (8,12–15). However, the formation of the c-Jun/ATF2 heterodimer also results in a shortened half-life of ATF2 caused by an increased basal level of ubiquitination (11). This phenomenon suggests a third mechanism that may be involved in the regulation of c-Jun/ATF2 activation–degradation by the ubiquitin pathway. When ATF2 is phosphorylated at Thr69 and Thr71, it acquires the transcriptional activating ability by binding with c-Jun, which is phosphorylated at Ser63 and Ser73. Phosphorylation of ATF2 can prevent its degradation by ubiquitination, and thus kinases such as p38 kinase and JNKs, which could phosphorylate ATF2, can also increase the half-life of ATF2 (16–18). Thus, phosphorylation of ATF2 at Thr69 and Thr71 can enhance the activity of the c-Jun/ATF2 complex.

Thr69 and Thr71 at the N-terminal of ATF2 can be phosphorylated by mitogen-activated protein (MAP) kinase members. Following UV stimulation, ATF2 is phosphorylated by JNKs and p38 kinases (19–21). After treatment with epidermal growth factor, ATF2 is phosphorylated by extracellular signal-regulated kinases (ERKs) in a two-step mechanism, and Thr69 and Thr71 are phosphorylated at the same time after UV treatment (12,22). However, the signaling pathway related to phosphorylation of ATF2 after UV treatment is not well understood. Not only ERKs, but also the downstream kinases of p38 kinase or ERKs might be involved in the process of ATF2 phosphorylation. Mitogen- and stress-activated protein kinase 1 (MSK1) is one of the known downstream kinases of both ERKs and p38 kinase. MSK1 is located in the nucleus and thus MSK1 may likely be involved in the phosphorylation of ATF2.

In this report, the role of ERKs and MSK1 in the phosphorylation of ATF2 induced by UVC was studied. We demonstrated that ERKs and MSK1 might play a role in the regulation of the c-Jun/ATF2 complex transcriptional activity.

Abbreviations: ATF2, activating transcription factor 2; DNM, dominant negative mutant; ERKs, extracellular signal-regulated kinases; FBS, fetal bovine serum; JNKs, c-Jun N-terminal kinases; MAPs, mitogen-activated protein kinases; MSK1, mitogen- and stress-activated protein kinase 1; UV, ultraviolet.
Materials and methods

Cell lines and cell culture

Mouse epidermal tumor promotion sensitive JB6 Cl 41 cells and cell lines stably expressing an empty vector (CMV-neo) or a dominant negative mutant (DNM) of ERK2, JNK1 or p38 kinase were cultured as reported previously (23). JB6 Cl 41 cell lines stably expressing pCMV5-Flag vector (CMV), pCMV5-Flag-wild-type MSK1 (MSK1-Wt), pCMV-Flag-MSK1-A195/N-terminal kinase dead (MSK1-Nd) or pCMV-Flag-MSK1-A565/C-terminal kinase dead (MSK1-Cd) from Dr D.R.Alessi (Medical Research Council Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Scotland, UK) (24) were generated and characterized according to previous methods (25). All the JB6 Cl 41 transfectants were cultured in Eagle’s minimum essential medium (MEM; Life Technologies) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. The basal level of protein phosphorylation was reduced by starvation for 24 h in MEM without FBS.

UVC irradiation of cells

The UVC radiation was from germicidal lamps. The doses of UVC irradiation were 15, 30 or 60 J/m² as indicated. The cultured cells were exposed to UVC at various doses as described.

Analysis of phosphorylated proteins by western blotting

Western blotting was performed as described on the website of Santa Cruz Biotechnology (www.scbt.com/support/protocols). In brief, equal numbers of experimental cells were cultured in 100-mm dishes. After the cells grew to 80% confluence, they were starved for 24 h in MEM without FBS. Then UVC (60 J/m²) irradiation was performed, and the cells were harvested and disrupted in 200 μl of RIPA buffer. Equal amounts of samples were diluted with 5× SDS sample buffer and separated by 8% SDS-PAGE followed by western analysis. The phospho-ATF2 (Thr71) antibody was purchased from Cell Signaling (Beverly, MA). H89 was purchased from Alexis Biochemicals (San Diego) and different doses were used to treat the cells for 1 h prior to UVC irradiation.

Immunoprecipitation assay

The cells were starved for 12 h in MEM without FBS, and then treated with UVC. Different samples were collected for determination of protein concentration by the modified Lowry’s method (Sigma, St Louis, MO). Equal amounts of protein were used for immunoprecipitation following the protocol of Santa Cruz Biotechnology (www.scbt.com/support/protocols). Briefly, cell lysates were pre-cleared by centrifugation at 4°C after adding 1.0 μg of the control IgG and 20 μl agrose conjugated Protein A-G beads. The supernatant fractions were transferred to new tubes and 2 μg of selected primary antibodies were added and incubated overnight. Agarose conjugated Protein A-G beads (20 μl) were added and samples were incubated at 4°C for 3 h. Samples were centrifuged and washed three times with RIPA buffer. The immunoprecipitates were collected by centrifugation and supernatant fractions were discarded. Pellets were re-suspended in 40 μl of 2× sample buffer, separated by 8% SDS-PAGE followed by western blotting. Anti-ERKs antibody was purchased from Cell Signaling (Beverly, MA) and anti-MSK1 antibody was purchased from Upstate Biotechnology (Charlottesville, VA).

In vitro kinase assay

Active p38 kinase, ERK1, ERK2, RSK2, MSK1, AKT1, JNK2 and kinase buffer were purchased from Upstate Biotechnology. The ATF2 fusion protein was purchased from Cell Signaling. The ATF2 fusion protein (1.5 μg) was mixed together with 5 mM ATP and 1× kinase buffer and respective kinases, and incubated at 30°C for 15 min. Samples were separated by 12% SDS-PAGE followed by western analysis.

Results

UVC induces phosphorylation of ATF2 at Thr71

ATF2 regulates gene expression in response to environmental stress, and phosphorylation of the N-terminal ATF2 transactivating domain is crucial for this role. After epidermal JB6 Cl41 cells were exposed to UVC, ATF2 (Thr71) phosphorylation was induced in a dose- (Figure 1A) and time-dependent (Figure 1B) manner. Phosphorylation of ATF2 (Thr71) occurred after irradiation with UVC at a dose of 15 J/m², and also increased in a dose-dependent manner up to 60 J/m² (Figure 1A). Phosphorylation of ATF2 (Thr71) occurred 5 min after irradiation with UVC at a dose of 60 J/m² and increased in a time-dependent manner up to 30 min (Figure 1B).

ERKs are involved in UVC stress-induced ATF2 phosphorylation at Thr71

ATF2 can be phosphorylated by JNKs and p38 kinase induced by UV irradiation (19–21), and by ERKs after treatment with growth factors (12,22). To investigate the role of MAP kinases in the phosphorylation of ATF2, DNM of ERK2, p38β kinase and JNK1 were irradiated with UVC and phosphorylation of ATF2 at Thr71 was examined by western blotting. The experiments showed that phosphorylation of ATF2 (Thr71) after UVC (Figure 2) was, to different degrees, prevented by expression of DNM-ERK2, p38β kinase and JNK1 were irradiated with UVC and phosphorylation of ATF2 at Thr71 was examined by western blotting. The experiments showed that phosphorylation of ATF2 (Thr71) after UV-C (Figure 2) was, to different degrees, prevented by expression of DNM-ERK2, p38β kinase and JNK1 compared with control JB6 Cl41 cells expressing the empty CMV-neo vector (CMV-neo). We used the MEK inhibitor, PD98059, to suppress the ERKs pathway, and then tested its effect on UVC-induced phosphorylation of ATF2 (Thr71) to deduce whether ERKs are involved in this process. As shown in Figure 3A, the phosphorylation of ATF2 (Thr71) was inhibited with increasing doses of PD98059, which implies that ERKs are involved...
in the phosphorylation of ATF2. The phosphorylation of ATF2 (Thr71) was markedly blocked by expression of DNM-p38 after UVC stimulation compared with cells expressing DNM-ERK2 or DNM-JNK1. However, the results also indicate that ERK2, like p38 kinase and JNK1, is involved in UVC-induced phosphorylation of ATF2 (Thr71).

MSK1 is required for UVC-induced ATF2 phosphorylation

Because MAP kinases are involved in the phosphorylation of ATF2, their downstream effector kinases might also be involved. One of the common downstream substrates of p38 kinase and ERKs is MSK1. We used the MSK1-specific inhibitor H89 to test its effect on UVC-induced phosphorylation of ATF2 (Thr71) to deduce whether MSK1 is involved in this process. As shown in Figure 3B, the phosphorylation of ATF2 (Thr71) was inhibited with increasing doses of H89, which implies that MSK1 is involved in the phosphorylation of ATF2.

Next, JB6 Cl 41 cell lines stably expressing the pCMV5-Flag vector (CMV), pCMV5-Flag-wild-type MSK1 (MSK1-Wt), pCMV-Flag-MSK1-A195/N-terminal kinase dead (MSK1-Nd), or pCMV-Flag-MSK1-A565/C-terminal kinase dead (MSK1-Cd), were used to investigate whether the phosphorylation of ATF2 (Thr71) was affected by over-expression of wild-type MSK1 or MSK1 kinase-dead mutants. When MSK1 wild-type was over-expressed in JB6 Cl41 cells irradiated by UVC, phosphorylation of ATF2 (Thr71) doubled. On the other hand, over-expression of the C- or N-terminal kinase-dead mutants resulted in a decrease in ATF2 phosphorylation (Figure 4). However, the C-terminal kinase-dead mutant of MSK1 showed a stronger inhibition of ATF2 phosphorylation than that of N-terminal kinase-dead mutant (Figure 4). These results suggest that MSK1, a downstream kinase of p38 kinase and ERKs, is involved in the phosphorylation of ATF2 (Thr71).
after treatment with growth factors, but also with UVC irradiation.

Next we used an in vitro kinase assay to confirm that ERKs and MSK1 not only could bind with ATF2, but could also phosphorylate it. As shown in Figure 6A, ERK1, ERK2 and MSK1 phosphorylated ATF2 (Thr71) in vitro and JNK2 or p38 kinase also phosphorylated ATF2. However, RSK2 and AKT1 had little effect. As shown in Figure 6B, activated protein kinases immunoprecipitated by MSK1, ERKs or p38 antibody from the extract of JB6 cells irradiated by UVC can phosphorylate ATF2 directly, indicating that UVC can induce direct phosphorylation of ATF2 by ERKs or MSK1.

Discussion

Ouwens et al. found that growth factors can activate ATF2 in a two-step mechanism: (i) phosphorylation of Thr71 through the Ras–MEK–ERK pathway and (ii) phosphorylation of Thr69 through the RalGDS–Src–p38 pathway. They also found that with UVC stimulation, ATF2 is activated through phosphorylation of Thr71 and Thr69 by the p38 pathway at the same time (22). The signal pathway related to phosphorylation ATF2 after UV treatment is not well understood. In our experiments, we found that ATF2 is phosphorylated at Thr71 and Thr69 at the same time following treatment of UVC (data not shown). Thus, in these experiments we focused only on the phosphorylation site of Thr71 and tried to elucidate the signal pathways involved.

UV irradiation is the most important environmental carcinogen leading to the development of skin cancer. MAP kinase signaling cascades are targets for UV and are important in the regulation of a multitude of UV-induced cellular responses (26). MAP kinases are a family of protein kinases that phosphorylate specific serines and threonines of target proteins, and regulate cellular activities, including proliferation, differentiation, apoptosis, as well as development, growth and inflammation. MAP kinases phosphorylate target molecules and thus control their enzymatic activity, interactions with other proteins, localization and propensity for degradation (27). The MAP kinase family is composed of p38 kinase, ERKs and JNKs. Many stimuli, including growth factors, cytokines, viral infection, transforming agents and carcinogens activate the ERKs pathway, whereas stresses, including UV, activate primarily the JNKs and p38 kinase pathways (26).

In our experiments, we found that ATF2 is phosphorylated at Thr71 after UVC stimulation (Figure 1). JNKs and p38 kinase are known to be involved in this process. However, we found that ERKs were also activated with UV treatment in our previous work (28). We therefore tested whether ERKs are involved in the phosphorylation of ATF2. Our data suggest that ERKs are implicated in the phosphorylation of ATF2 at Thr71 with UVC treatment (Figures 2 and 3A). ERKs can bind together with ATF2 (Figure 5B) and phosphorylate ATF2 directly (Thr71) (Figure 6A and B).

Blocking either ERKs or p38 kinase inhibits the phosphorylation of ATF2. One explanation is that these enzymes are
direct kinases responsible for the phosphorylation of ATF2. Another possibility is that their downstream effectors might also participate in this process. To test whether MSK1 is involved in the phosphorylation of ATF2, we used H89, a specific inhibitor of MSK1, to treat cells prior to UV irradiation. H89 is a member of the H-series of protein kinase inhibitors. It has no effect on ERKs or p38 kinase, but specifically inhibits MSK1 activity (29). H89 inhibited phosphorylation of ATF2 (Thr71) induced by UVC (Figure 3B) suggesting that the phosphorylation is mediated by MSK1. JB6 Cl41 cells expressing DNM-MSK1 could also inhibit phosphorylation of ATF2 (Thr71) induced by UVC (Figure 4) and the immunoprecipitation experiments suggest that MSK1 binds with ATF2 (Figure 5). However, we noticed that the C-terminal kinase-dead mutant of MSK1 exhibits a stronger inhibiting effect than that of N-terminal kinase-dead on the phosphorylation of ATF2 (Thr71) induced by UVC. This suggests that the C-terminal of MSK1 may play a critical role in UVC-induced phosphorylation of ATF2. The in vitro kinase assay confirmed that MSK1 could phosphorylate ATF2 (Thr71) (Figure 6A and B). All the data suggest that MSK1 directly phosphorylates ATF2 (Thr71) following exposure to UVC.

ATF2 is one of many transcription factors implicated in the progression of skin cancer and its resistance to treatment. ATF2 contributes to UV-induced apoptosis through transcriptional silencing of TNF-α (30). Moreover, ATF2 is a histone acetyltransferase (HAT), which specifically acetylates histones H2B and H4 in vitro, and the phosphorylation of ATF2 controls its intrinsic HAT activity (31). Alterations in histone acetylation may lead to changes in chromatin structure and transcriptional dysregulation of genes that are implicated in controlling either cell cycle progression or pathways regulating cell differentiation and/or apoptosis (32). ERKs, JNKs, p38 kinase and MSK1 are involved in the UV-induced phosphorylation of ATF2 (Thr71), suggesting that they are associated with progression of skin cancer through phosphorylation of ATF2. Because MSK1 directly phosphorylates ATF2 and is a downstream kinase of ERKs or p38 kinase, it may also play an important role in skin cancer development.

Taken together, we provide a model (Figure 7) for a better understanding of the mechanism of the phosphorylation pathway of ATF2. Our study suggests that following UV irradiation, ERKs, JNKs and p38 kinase are activated and they can phosphorylate ATF2 (Thr71) directly. Furthermore, MSK1, the downstream kinase of ERKs and p38 kinase, can also phosphorylate ATF2 (Thr71) directly.

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References


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