Mutual regulation of c-Jun and ATF2 by transcriptional activation and subcellular localization

Han Liu¹, Xuehong Deng¹, Y John Shyu¹,
Jian Jian Li^{2,4}, Elizabeth J Taparowsky^{3,4}
and Chang-Deng Hu^{1,4,5,*}

¹Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN, USA, ²School of Health Science, Purdue University, West Lafayette, IN, USA, ³Department of Biological Sciences, Purdue University, West Lafayette, IN, USA, ⁴Purdue Cancer Center, Purdue University, West Lafayette, IN, USA and ⁵Walther Cancer Institute, Indianapolis, IN, USA

ATF2 and c-Jun are key components of activating protein-1 and function as homodimers or heterodimers. c-Jun–ATF2 heterodimers activate the expression of many target genes, including *c-jun*, in response to a variety of cellular and environmental signals. Although it has been believed that c-Jun and ATF2 are constitutively localized in the nucleus, where they are phosphorylated and activated by mitogen-activated protein kinases, the molecular mechanisms underlying the regulation of their transcriptional activities remain to be defined. Here we show that ATF2 possesses a nuclear export signal in its leucine zipper region and two nuclear localization signals in its basic region, resulting in continuous shuttling between the cytoplasm and the nucleus. Dimerization with c-Jun in the nucleus prevents the export of ATF2 and is essential for the transcriptional activation of the *c-jun* promoter. Importantly, c-Jun-dependent nuclear localization of ATF2 occurs during retinoic acid-induced differentiation and UV-induced cell death in F9 cells. Together, these findings demonstrate that ATF2 and c-Jun mutually regulate each other by altering the dynamics of subcellular localization and by positively impacting transcriptional activity.

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Introduction

ATF2 belongs to the basic region leucine zipper (bZIP) family of proteins and is an important member of activating protein-1 (AP-1) (Wagner, 2001). ATF2 functions as a homodimer or as a heterodimer with other bZIP proteins to bind specific DNA sequences and activate gene expression. The proto-

oncoprotein c-Jun is a major dimerization partner of ATF2, and c-Jun–ATF2 heterodimers are important for many cellular processes. One major role of ATF2 is to regulate the response of cells to stress signals (Gupta *et al*, 1995; Whitmarsh and Davis, 1996; Karin *et al*, 1997; Hayakawa *et al*, 2004; Bhoumik *et al*, 2005). ATF2 also contributes to cellular transformation induced by several viral proteins, including adenovirus E1A (Liu and Green, 1990, 1994), and, in conjunction with c-Jun, mediates distinct processes of nonviral cellular transformation (van Dam and Castellazzi, 2001; Eferl and Wagner, 2003). ATF2 also plays a role in regulating development of various organs in mice (Reimold *et al*, 1996; Maekawa *et al*, 1999) and cellular differentiation *in vitro* (Monzen *et al*, 2001). For example, treatment of F9 mouse embryonic teratocarcinoma with retinoic acid (RA) induces differentiation (Yang-Yen *et al*, 1990; Alonso *et al*, 1991), which is associated with the binding of ATF2 and the p300 coactivator to a differentiation response element (DRE) (Kawasaki *et al*, 1998). Although it has been well documented that c-Jun–ATF2 heterodimers are responsible for the activation of target genes involved in stress response, it remains unknown whether ATF2 alone, or in cooperation with c-Jun, regulates F9 cell differentiation.

Accumulated evidence suggests that two events are common to the activation of AP-1 proteins. The first is phosphorylation by a mitogen-activated protein kinase (MAPK) and the second is the selective formation of dimers. In mammals, three major MAPKs can phosphorylate and activate ATF2 and c-Jun. These are the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (Davis, 2000; Kyriakis and Avruch, 2001). In response to growth and stress signals c-Jun is phosphorylated on residues S63 and S73, while ATF2 is phosphorylated on residues T69 and T71. Although these MAPK phosphorylation events are critical for the full transcriptional activity of c-Jun and ATF2 (Pulverer *et al*, 1991; Smeal *et al*, 1991; Gupta *et al*, 1995; Livingstone *et al*, 1995; Jiang *et al*, 1996; Stein *et al*, 1997; Ip and Davis, 1998; Ouwers *et al*, 2002), the underlying mechanism of this activation is poorly defined. It has been proposed that phosphorylation by JNK may regulate the intrinsic histone acetylase activity of ATF2 (Kawasaki *et al*, 2000) or the interaction of c-Jun with the coactivator p300 (Arias *et al*, 1994; Bannister *et al*, 1995). It also has been proposed that phosphorylation of c-Jun and ATF2 by JNK/p38 may prohibit their ubiquitination (Fuchs *et al*, 1996, 1997, 1998; Fuchs and Ronai, 1999), leading to increased levels of these bZIP proteins in cells. Given that ATF2 is ubiquitously and abundantly expressed in many tissues while the amount of c-Jun in cells is very limited (Angel *et al*, 1988; Chiu *et al*, 1989; Takeda *et al*, 1991; Stein *et al*, 1992; van Dam *et al*, 1993, 1995; Herdegen and Leah, 1998), it is apparent that additional cellular mechanisms, including events leading to increased transcription of *c-jun*, must be

*Corresponding author. Department of Medicinal Chemistry and Molecular Pharmacology, School of Pharmacy, Purdue University, 575 Stadium Mall Drive, RHPH 224D, West Lafayette, IN 47907, USA. Tel.: +1 765 496 1971; Fax: +1 765 494 1414; E-mail: cdhu@pharmacy.purdue.edu

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operating to control the levels and activities of these bZIP proteins.

Using a bimolecular fluorescence complementation (BiFC) assay and fluorescent protein fusions, we present evidence here that ATF2 monomers and ATF2 homodimers are localized predominantly in the cytoplasm. We have identified a nuclear export signal (NES) in the leucine zipper region and two nuclear localization signals (NLS) in the DNA-binding domain of ATF2. These nuclear transport signals contribute to the shuttling of the protein between the cytoplasm and the nucleus. We also demonstrate, for the first time, that heterodimerization with c-Jun prevents nuclear export of ATF2 and is the key event leading to nuclear localization of c-Jun-ATF2 dimers and the transcriptional activity of this complex towards targets such as *c-jun*.

Results

Distinct subcellular localization of AP-1 dimers and AP-1 proteins

We previously developed a BiFC assay using yellow fluorescent protein (YFP) to visualize protein–protein interactions in living cells (Hu *et al*, 2002). Since chromophore maturation and protein folding of YFP are sensitive to higher temperatures (Tsien, 1998), a preincubation at 30°C for a few hours is necessary before visualization of BiFC signals (Hu *et al*, 2005). To circumvent this problem, we have recently identified several new combinations of fluorescent protein fragments that significantly increase the BiFC signal at 37°C culture conditions and display a two-fold increase in specificity (Shyu *et al*, 2006). The combination using N-terminal residues 1–172 (VN173) and C-terminal residues 155–238 (VC155) of Venus fluorescent protein showed higher BiFC signals when bJun–bFos interactions were examined at 37°C (Supplementary Figure 1). To determine the subcellular localization of AP-1 dimers with the newly identified BiFC fragments, we expressed c-Fos, c-Jun and ATF2 as fusion proteins with either VN173 or VC155 in COS-1 cells. Although fluorescent signals derived from JunVN173–FosVC155 and JunVN173–JunVC155 were localized, as predicted, in the nucleus (Figure 1A and B), 90% of fluorescent signals derived from ATF2VN173–ATF2VC155 were located in the cytoplasm. Interestingly, we observed that the BiFC signals derived from JunVN173–ATF2VC155 were located equally in the cytoplasm and the nucleus, whereas the majority of JunYN155–ATF2YC155 was localized in the cytoplasm when YFP fragments were used (Hu *et al*, 2002). This difference may be accounted for by two major differences in experimental approaches: the lack of the quantification of fluorescence intensity and the exposure of cells to lower temperatures in our previous work.

Since subcellular localization of transcription factors is determined by all interacting partners (Hu *et al*, 2002; Hu and Kerppola, 2003; Grinberg *et al*, 2004), we examined the subcellular localization of ATF2 by itself. Using ATF2 fused to full-length Venus, we again observed predominant cytoplasmic localization, whereas Fos-Venus and Jun-Venus were localized in the nucleus (Figure 1A and B). A similar profile of ATF2-Venus localization was also observed in human HEK293 and MCF-7 cells (data not shown).

The distinct cytoplasmic localization of ATF2-Venus was in sharp contrast to the nuclear localization of Fos-Venus and

Figure 1 Subcellular localization of AP-1 dimers and proteins. (A) Plasmids encoding c-Fos, c-Jun and ATF2 fused to N-terminal residues 1–172 (VN), C-terminal residues 155–238 (VC) of Venus, or full-length Venus (Venus) were cotransfected into COS-1 cells. Representative images of fluorescent images of different AP-1 dimers and proteins captured at 12 h post-transfection are shown. Venus alone was included as a control. (B) Quantification of subcellular localization of different AP dimers and proteins from (A). The error bar indicates standard deviation.

Jun-Venus, suggesting that it was unlikely that the cytoplasmic localization of ATF2 was due to saturation of nuclear import machinery by overexpressed proteins. To rule out the possibility that the fusion tag of Venus to the C-terminus of ATF2 may uniquely impede nuclear import of ATF2, we expressed ATF2 as a FLAG fusion protein in COS-1 cells. Similar cytoplasmic localization of the FLAG-ATF2 fusion proteins was detected by immunostaining with anti-FLAG antibody (Supplementary Figure 2). Thus, we conclude that cytoplasmic localization of exogenously expressed ATF2 is not an artifact caused by protein overexpression or by the presence of a fusion tag.

ATF2 shuttles between the cytoplasm and the nucleus

Since subcellular localization of transcription factors can be affected by the relative rates of nuclear import versus export, we examined if the cytoplasmic localization of ATF2-Venus was a result of rapid nuclear export. To this end, we treated cells with leptomycin B (LMB), a specific inhibitor of chromosome region maintenance 1 (CRM1) involved in nuclear export (Nishi *et al*, 1994; Kudo *et al*, 1997, 1998; Ossareh-Nazari *et al*, 1997; Wolff *et al*, 1997; Yashiroda and Yoshida, 2003). After treatment for 12 h, 85% of ATF2-Venus was sequestered in the nucleus (Figure 2A). This demonstrates that ATF2 can be exported in a CRM1-dependent manner and that the ATF2-Venus fusion protein is competent to shuttle between the cytoplasm and the nucleus. Treatment of cells

Figure 2 Identification of NLS and NES motifs in ATF2. (A) Plasmids encoding wild-type ATF2, an NES mutant of ATF2 [ATF2(NES4A)], or ATF2(400–415) fused to Venus were transfected into COS-1 cells. At 12 h post-transfection, cells were treated with or without 20 ng/ml of LMB for an additional 12 h and fluorescent images were captured. The number indicates the percentage of quantified nuclear distribution $\pm$ standard deviation. (B) Alignment of ATF2 NES with several characterized NES motifs. The conserved hydrophobic residues are highlighted. L can be replaced with V, F and I, and X can be any amino acid. (C) Alignment of ATF2 bipartite NLS with several characterized NLS motifs. The conserved basic residues are highlighted. (D) Functional analysis of NLSs. Cells expressing the indicated ATF2 mutants fused to Venus were treated with 20 ng/ml of LMB, or left untreated, for 12 h and the fluorescence intensity localized in the nucleus was quantified. The number indicates the percentage of quantified nuclear distribution $\pm$ standard deviation.

expressing Jun-Venus and Fos-Venus with LMB did not alter their persistent nuclear localization (data not shown).

CRM1-dependent nucleocytoplasmic shuttling proteins contain an NES, which is composed of one or more leucine-rich motifs. The predicted consensus motif of an NES is L-X₍₂₋₃₎-L-X₍₂₋₃₎-L-X-L (Mataj and Englmeier, 1998). Sequence analysis of ATF2 revealed that residues 405–413 of ATF2 matched perfectly with several well-characterized NES motifs (Figure 2B), suggesting that this region may act as an NES. To test this, the conserved valine and three leucine residues within the region were replaced with alanines and the subcellular localization of the mutant ATF2-Venus (ATF2(NES4A)-Venus) was examined. Consistent with our prediction, 67% of ATF2(NES4A)-Venus was localized in the nucleus (Figure 2A). Also, the fusion of this region

only to Venus localized 87% of Venus to the cytoplasm (Figure 2A).

The basic region of many bZIP transcription factors has a dual role as a sequence-specific DNA-binding domain and an NLS. To provide evidence that the basic region of ATF2 functions as an NLS, we first identified two potential bipartite NLS motifs in the ATF2 basic region (Figure 2C). Next, we used deletion mutagenesis to test each motif for function. Deletion of either NLS alone did not affect the nuclear sequestration of ATF2-Venus in the presence of LMB, whereas deletion of both NLS motifs completely abolished the nuclear sequestration of ATF2-Venus by LMB (Figure 2D). These results indicate that both NLS motifs are functional and either of them is sufficient to translocate ATF2 to the nucleus. We conclude that ATF2 represents the first AP-1 protein discovered to possess both NES and NLS motifs, and that the coordinated function of these sequences mediates the observed nucleocytoplasmic shuttling.

Nuclear localization of ATF2 depends on physical interactions with c-Jun

The identification of an NES in ATF2 coupled with the cytoplasmic localization of exogenously expressed ATF2 prompted us to examine how the nuclear localization of ATF2 is regulated. As c-Jun can form heterodimers with ATF2 in both the cytoplasm and the nucleus (Figure 1A and B), we reasoned that c-Jun may facilitate the nuclear localization of ATF2. To test this possibility, we examined the effect of c-Jun expression on the subcellular localization of ATF2-Venus. Indeed, increased expression of c-Jun significantly enhanced the nuclear accumulation of ATF2 (Figure 3A). With a 3:1 ratio of plasmids encoding c-Jun to those encoding ATF2-Venus, over 80% of ATF2-Venus was concentrated in the nucleus (Figure 3B). In contrast, c-Jun(Δ L3), a dimerization-deficient c-Jun mutant (Hu *et al*, 2002), failed to localize ATF2-Venus to the nucleus (Figure 3A and B), demonstrating that nuclear localization of ATF2-Venus depends on dimerization with c-Jun. To further confirm this result, we employed subcellular fractionation to prepare cytosolic and nuclear extracts and detected the amount of FLAG-ATF2-Venus localized in both cytosolic and nuclear fractions. Consistent with fluorescence microscopic analysis, we observed that coexpression with increasing amount of c-Jun increased nuclear localization of FLAG-ATF2-Venus from 5 to 70% (Supplementary Figure 3).

To determine whether c-Jun is also required for the nuclear localization of endogenous ATF2, we examined the subcellular localization of ATF2 in F9 cells that lack detectable levels of c-Jun (Yang-Yen *et al*, 1990; van Dam *et al*, 1995). In agreement with c-Jun-dependent nuclear localization of exogenously expressed ATF2, the majority of ATF2 was localized in the cytoplasm of F9 cells (Figure 3C). Consistent with this notion, the majority of ATF2 was localized in the nucleus of c-Jun-expressing cells, such as COS-1 and MCF-7 (Figure 3C). More importantly, knockdown of *c-jun* by siRNA in MCF-7 cells increased the cytoplasmic localization of ATF2 from 20 to 50% (Figure 3D and Supplementary Figure 4). These findings lead us to conclude that c-Jun is required for the nuclear localization of ATF2 at both exogenous and endogenous levels.

Since phosphorylation by the JNK/p38 MAPKs on c-Jun and ATF2 is required for the activation of c-Jun and ATF2

Figure 3 Jun-dependent nuclear localization of ATF2. (A) Nuclear localization of ATF2 is dependent on its heterodimerization. Plasmids encoding wild-type or mutant ATF2 fused to Venus were cotransfected with 10-fold excess of plasmids encoding wild-type c-Jun or the indicated mutants into COS-1 cells. Fluorescent images shown were captured at 12 h post-transfection. The number indicates the percentage of quantified nuclear distribution $\pm$ standard deviation. (B) Dose-dependent nuclear localization of ATF2 induced by c-Jun. Different amounts of plasmid encoding c-Jun (●) or its dimerization-deficient mutant c-JunΔL3 (■) were cotransfected with a fixed amount of plasmid encoding ATF2-Venus into COS-1 cells. The total amount of plasmids was adjusted to 0.5 μ g. At 12 h post-transfection, fluorescent images were captured and the percentage of nuclear-localized ATF2-Venus was determined as described in 'Materials and methods'. The inset shows the levels of wild-type and mutant c-Jun detected with anti-c-Jun antibody in cells transfected with 10-fold excess of plasmids encoding c-Jun or c-JunΔL3 (upper panel). The same blot was used for the detection of β -actin (lower panel). (C) Immunostaining of ATF2 in F9, COS-1 and MCF-7 cells. Endogenous ATF2 was detected with an anti-ATF2 antibody and DNA was stained with DAPI. (D) Immunostaining of ATF2 in control siRNA-expressing MCF-7 cells (upper panel) or c-Jun siRNA-expressing MCF-7 cells (lower panel). The arrow indicates the cell showing even distribution of ATF2 in both the cytoplasm and the nucleus.

(Whitmarsh and Davis, 1996; Karin *et al*, 1997), we examined the impact of phosphorylation on the dimerization and nuclear localization of ATF2. Several experiments were per-

formed and the results indicated that the phosphorylation status of neither c-Jun nor ATF2 regulates their interactions or subcellular localization. First, cells expressing ATF2-Venus

coexpressed with JNK or p38, or treated with the JNK-specific inhibitor, SP600125, or the p38 inhibitor, SB203580, did not alter ATF2 cytoplasmic localization (Supplementary Figure 5A and B). Second, the interactions between c-Jun(63A, 73A) and ATF2(69A, 71A) or ATF2(69D, 71D) as measured by BiFC were essentially the same as their wild-type counterparts (Supplementary Figure 5C). Third, nuclear localization of ATF2-Venus was facilitated by c-Jun(63A, 73A) (Figure 3A) as efficiently as wild-type c-Jun. Fourth, ATF2(69A, 71A)-Venus and ATF2(69D, 71D)-Venus also were localized predominantly in the cytoplasm (Figure 3A) and their nuclear localization can be facilitated by wild-type and mutant c-Jun. Finally, we observed that TAM67, a deletion mutant of c-Jun lacking the N-terminal 123 residues, which is transcriptionally inert (Brown *et al*, 1994), remained capable of facilitating the nuclear localization of ATF2-Venus (Figure 3A). Taken together, these results clearly demonstrate that physical interaction with c-Jun is the major driving force of ATF2 nuclear localization.

Heterodimerization with c-Jun prevents nuclear export of ATF2

Since the increased nuclear localization of ATF2-Venus by heterodimerization with c-Jun could be attributed to either increased nuclear import or decreased export, we compared the import rate of the dimeric versus monomeric form of ATF2. ATF2(L408P) contains a substitution of proline for the fourth leucine (L408) in the leucine zipper region. This mutation abolishes both ATF2 homodimer and c-Jun-ATF2 heterodimer formation (Abdel-Hafiz *et al*, 1993; Fuchs and Ronai, 1999). Although cytoplasmic localization of ATF2-Venus and ATF2(L408P)-Venus was similarly observed 12 h after transfection (Figure 4A), treatment of cells with LMB resulted in a rapid nuclear accumulation of ATF2(L408P)-Venus, but not ATF2-Venus, in less than 2 h (Figure 4B). This result suggests that ATF2 monomers are translocated into the nucleus more efficiently than ATF2 dimers. To provide direct evidence that heterodimerization with c-Jun does not facilitate nuclear import of ATF2, we coexpressed ATF2(Δ NLS1 + 2)-Venus with an excess amount of c-Jun. Again, overexpression of c-Jun failed to localize ATF2(Δ NLS1 + 2)-Venus to the nucleus, although they interacted with each other in the cytoplasm (Figure 4A).

Next, we examined if the nuclear accumulation of ATF2 in the presence of c-Jun was due to impaired nuclear export. The NES of ATF2 is located in the fourth heptad of the leucine zipper region (Vinson *et al*, 2002), suggesting that heterodimerization with c-Jun may mask the NES and prevent the nuclear export. Interestingly, c-Jun, JunB and JunD have identical sequences in their fourth heptads with variable substitutions across the first three heptads (Figure 4C). This implies that JunB and JunD should affect the subcellular localization like c-Jun. Consistent with this prediction, JunB and JunD also sequestered ATF2-Venus in the nucleus (Figure 4D). To further confirm this, we examined if c-Fos sequesters ATF2 in the nucleus. c-Fos has been shown to interact with ATF2 *in vitro* (Kerppola and Curran, 1993) and *in vivo* (unpublished observations). A comparison of the fourth heptad sequences of c-Fos and ATF2 reveals one repulsive force in positions 'g' and 'e', as well as the lack of one hydrophobic interaction in positions 'a' and 'd'. These unfavorable interactions could decrease the stability

of a c-Fos-ATF2 dimer across this critical region (Vinson *et al*, 2002). In support of our hypothesis, overexpression of c-Fos failed to sequester ATF2-Venus in the nucleus (Figure 4D). Additionally, expression of other ATF2 coactivators, such as p65 (Kim and Maniatis, 1997), p300 (Kawasaki *et al*, 1998; Sano *et al*, 1998) and E1A (Liu and Green, 1990, 1994), failed to sequester ATF2-Venus in the nucleus (Figure 4D). Finally, a specific interaction between CRM1 and ATF2 was observed using the BiFC assay (Figure 4E, left two panels). Furthermore, the specific interaction between CRM1 and ATF2 was almost completely abolished by the coexpression with c-Jun, and to a lesser extent by the coexpression with ATF2. These results provide evidence that formation of a coiled-coil structure between the fourth heptads of ATF2 and the Jun proteins masks the NES of ATF2 and prevents the nuclear export of the protein.

Activation of c-jun promoter by ATF2 requires dimerization with c-Jun, but is independent of c-Jun phosphorylation

A major ATF2 target gene is *c-jun*, which has been reported to be activated by c-Jun-ATF2 heterodimers and ATF2 homodimers (Devary *et al*, 1991; Stein *et al*, 1992; van Dam *et al*, 1993, 1995; Herr *et al*, 1994). Interestingly, transient expression of ATF2 in cells barely activates reporter genes (Liu and Green, 1990; Ivashkiv *et al*, 1992; Li and Green, 1996; Sano *et al*, 1998). Based on our findings here, we reasoned that this may be the result of the cytoplasmic localization of ATF2. Since heterodimerization with c-Jun is essential for ATF2 nuclear localization, it is therefore likely that the nuclear anchoring of ATF2 by c-Jun is required for its transcriptional activity. To examine this possibility, we utilized COS-1 cells to monitor expression of a luciferase reporter gene controlled by five tandem copies of the second AP-1-binding site (*jun2*) in the *c-jun* promoter. c-Jun-ATF2 heterodimers and ATF2 homodimers are known to bind the *jun2* site (Devary *et al*, 1991; Stein *et al*, 1992; van Dam *et al*, 1993, 1995; Herr *et al*, 1994). Consistent with previous reports, the *jun2-luc* reporter was not activated by exogenous ATF2, but was activated by exogenous c-Jun in a dose-dependent manner (Figure 5A). Interestingly, coexpression of c-Jun and ATF2 synergistically activated the reporter gene, showing at least two to three times higher activation than c-Jun alone. To test if the transcriptional activity of c-Jun also is required for the activation of the *jun2-luc* reporter, we expressed ATF2 with c-Jun(63A, 73A), a dominant-negative c-Jun known to be a weak transactivator of the collagen promoter and other AP-1 reporter plasmids (Hu *et al*, 2002). Results showed that the *jun2-luc* reporter was activated by the c-Jun(63A, 73A) alone, or in combination with ATF2 (Figure 5B). In contrast, the transcriptionally impaired, dominant-negative ATF2(69A, 71A), produced a 50% reduction in luciferase expression compared to wild-type ATF2 when coexpressed with either wild-type c-Jun or c-Jun(63A, 73A). Equivalent expression of all activator proteins was confirmed by immunoblotting analysis. These results demonstrate that activation of *c-jun* transcription by ATF2 requires c-Jun as a nuclear anchor and dimerization partner and that phosphorylation of ATF2, but not c-Jun, has an impact on the transcriptional activity of ATF2.

Figure 4 Heterodimerization of ATF2 with Jun family of proteins facilitates nuclear localization. (A) Jun-independent nucleocytoplasmic shuttling of ATF2. Subcellular localization of wild-type or ATF2(L408P) fusions with Venus with or without treatment of 20 ng/ml of LMB for 2 h (top two panels), or ATF2(ΔNLS1 + 2) coexpressed without or with excess amount of c-Jun (the third panel). The bottom panel shows the interaction of ATF2(ΔNLS1 + 2) with c-Jun in BiFC assay. (B) Time course of nuclear sequestration of ATF2-Venus and ATF2(L408P)-Venus by LMB. (C) Alignment of leucine zipper regions of AP-1 proteins. The NES in ATF2 is highlighted. (D) Jun-dependent nuclear localization of ATF2. Plasmids encoding ATF2-Venus were cotransfected with 10-fold excess of plasmids encoding the indicated proteins into COS-1 cells. Fluorescent images were captured at 12 h post-transfection and nuclear localization of ATF2-Venus was quantified. The error bar indicates standard deviation. (E) Inhibition of CRM1-ATF2 interaction by c-Jun. Plasmids encoding CRM1(566–720) fused to VN173 (CRM1VN) and plasmids encoding ATF2(342–505) or ATF2(342–505, NES4A) fused to VC155 (ATF2VC or ATF2NES4AVC) were cotransfected into cells in the absence or presence of full-length c-Jun or ATF2(342–505) coexpression. As an internal control, plasmids encoding ECFP were cotransfected. At 12 h after transfection, images were acquired using both CFP and YFP filters. The median of YFP/CFP ratios derived from more than 100 transfected cells for each group was determined as described in 'Materials and methods', and is presented on the right of fluorescent images.

c-Jun induction is associated with nuclear localization of ATF2 in RA-treated and UV-irradiated F9 cells

F9 murine teratocarcinoma cells are widely used as a model system to study the role of AP-1 in regulating cellular differentiation and stress response (Yang-Yen *et al*, 1990; Alonso *et al*, 1991; van Dam *et al*, 1995; Kawasaki *et al*, 1998). Since we observed that the majority of ATF2 in untreated cells is localized in the cytoplasm (Figure 3C), we reasoned that the induction of c-Jun expression following RA

treatment or UV irradiation should induce nuclear localization of ATF2. As shown in Figure 6A, the majority of ATF2 was localized to the nucleus after RA treatment for 72 h. Consistent with a previous report (Yang-Yen *et al*, 1990), we observed an increase in the expression of c-Jun after RA treatment, whereas expression of ATF2 remained unchanged (Figure 6B). Increased activation of the *jun2-luc* reporter also was observed (Figure 6C), indicating that c-Jun-ATF2 heterodimers are functional in RA-treated F9 cells. Morphological

Figure 5 Synergistic activation of *c-jun* transcription by c-Jun and ATF2. (A) The indicated amount of plasmids encoding c-Jun and ATF2 were transfected separately, or cotransfected into serum-starved COS-1 cells along with 0.5 µg of the reporter plasmid *jun2-luc* and 50 ng of pRL-TK using Fugene 6. Fold increase of F/R ratio was calculated when compared with vector control only. (B) Similar experiments were performed as described in (A), except that 1 µg of each plasmid encoding the indicated wild-type or mutant proteins was used for transfection. * $P < 0.05$ when compared with c-Jun + ATF2 (Student's *t*-test).

changes, indicative of differentiation, were observed 3 days after RA treatment. By day 6, greater than 95% of cells were differentiated (data not shown). Likewise, irradiation of cells with UV also induced c-Jun expression (Figure 6D and Supplementary Figure 6), followed by the nuclear accumulation of ATF2 (Figure 6D) and cell death in more than 60% of irradiated cells (Supplementary Figure 6). These observations strongly suggest that induction of c-Jun is a prerequisite of ATF2 nuclear localization and the transcriptional activation of target genes under physiological conditions.

Discussion

Our studies have shown that ATF2 shuttles between the cytoplasm and the nucleus and that heterodimerization with c-Jun is essential for the nuclear localization of ATF2

and for the activation of target gene transcription (Figure 7). These findings resolve, at least partially, the longstanding question of why exogenously expressed ATF2 has little transcriptional activity unless coexpressed with coactivators (Liu and Green, 1990; Ivashkiv *et al*, 1992; Li and Green, 1996; Sano *et al*, 1998). Although these previous studies demonstrated that disruption of intramolecular interaction between the bZIP domain and the N-terminal transcriptional activation domain is essential for ATF2 activation in the nucleus, our present findings clearly suggest that nuclear accumulation of ATF2 induced by c-Jun is essential for the activation of target genes such as *c-jun*. Interestingly, coexpression of several other coactivators such as p300, E1A and p65 did not result in nuclear accumulation of ATF2. It therefore remains to be determined whether additional factors are required for ATF2 activation by these coactivators. Nevertheless, our results clearly show that both c-Jun and ATF2 positively and mutually regulate each other at the level of subcellular localization and transcriptional activity. Given that ATF2 is abundantly and ubiquitously expressed and c-Jun expression is limited, this mutual regulation is undoubtedly critical for the initiation of a well-controlled response to cell signaling, such as differentiation induced by RA and cell death induced by UV in F9 cells (Figure 6 and Supplementary Figure 6).

The cytoplasmic localization of ATF2 homodimers is surprising, since it has been believed that all mammalian AP-1 proteins are constitutively localized in the nucleus where their transcriptional activity is controlled largely by MAPK phosphorylation (Karin *et al*, 1997). Although ATF2 possesses two NLSs, exogenously expressed ATF2 accumulates in the cytoplasm. Several possible mechanisms may account for this. First, the ATF2 NLSs may be partially masked by an intramolecular interaction involving the N-terminus and the bZIP domain (Li and Green, 1996). Indeed, the deletion of N-terminal 341 residues has been shown to increase nuclear localization of c-Jun-ATF2 heterodimers (Hu *et al*, 2002). Second, ATF2 homodimer formation may inhibit the access of nuclear import machinery to the NLS motifs. This mechanism is supported by our observation that a monomeric form of ATF2 has a faster rate of nuclear import than wild-type ATF2 (Figure 4B). These latter results also support our conclusion that the ATF2 monomer is the form of protein that shuttles most effectively between the cytoplasm and the nucleus (Figure 7A and B). Finally, ATF2 homodimers in the nucleus, if formed, may not be stable enough to prevent interaction with CRM1, as only a partial inhibition of ATF2-CRM1 interaction by overexpressed ATF2 was observed (Figure 4E).

The regulation of the subcellular localization of AP-1 proteins in other model systems has been reported. The yeast AP-1 homologous proteins, yAP-1 in budding yeast and Pap1 in fission yeast, possess CRM1-dependent NESs and are localized normally in the cytoplasm (Wilkinson *et al*, 1996; Kuge *et al*, 1997, 1998, 2001; Isoyama *et al*, 2001). In response to oxidative stress, they translocate to the nucleus and activate target gene expression. In mammals, all members of the AP-1 family of bZIP proteins are believed to be located in the nucleus, although newly synthesized c-Fos, for example, has been reported to be localized transiently to the endoplasmic reticulum (Bussolino *et al*, 2001). To our knowledge, ATF2 represents the first AP-1 protein found to possess

Figure 6 c-Jun-dependent nuclear accumulation of ATF2 induced by RA in F9 cells. **(A)** Immunostaining of endogenous ATF2 in F9 cells. F9 cells were treated with 1 μ M RA (RA+) or ethanol (RA–) for 72 h and subjected to immunostaining of ATF2 and DAPI staining of DNA. **(B)** Time course of c-Jun induction by RA. F9 cells grown in 10 cm dishes were treated with 1 μ M RA and harvested at the indicated times. In all, 30 μ g of total proteins were resolved in SDS–PAGE and the expression of c-Jun and ATF2 was detected using anti-c-Jun and anti-ATF2 antibodies. **(C)** Induction of *jun2-luc* reporter activation by RA in F9 cells. F9 cells grown in 12-well plates were transfected with 0.5 μ g of the reporter plasmid *jun2-luc* along with 50 ng of pRL-TK using Lipofectamine 2000. At 24 h post-transfection, cells were treated with 1 μ M RA (RA+) or ethanol (RA–) and harvested at the times indicated. Total protein (5 μ g) was used for the measurement of luciferase activity using the Dual-Luciferase Assay kit. **(D)** F9 cells grown in 12-well plates were irradiated with UVC (40 J/m²), or not irradiated, and were continuously cultured for 24 h before being processed for immunostaining of c-Jun and ATF2 with specific antibodies, followed by Texas Red-conjugated secondary antibody. DNA was stained with DAPI.

both NLS and NES motifs and utilize these sequences in a novel way to modulate its activities. Nuclear import is immediately counteracted by a strong export signal unless

the protein dimerizes with Jun proteins. The Jun–ATF2 dimerization appears to mask or otherwise inhibit the function of the ATF2 NES, and the complex is retained in the

Figure 7 Model of mutual regulation of c-Jun and ATF2. (A) ATF2 possesses an NES and two NLS motifs and continuously shuttles between the cytoplasm and the nucleus. (B) In the absence of c-Jun, ATF2 homodimers are predominantly localized in the cytoplasm and ATF2 monomers continuously shuttle between the cytoplasm and the nucleus. (C) In response to RA treatment or UV irradiation, c-Jun is induced, heterodimerizes with ATF2 and prevents ATF2 nuclear export by masking the NES. The c-Jun–ATF2 heterodimers further activate *c-jun* transcription, forming a positive feedback loop to increase the amount of c-Jun and to facilitate the nuclear localization of ATF2 for their nuclear functions. The activation of target genes by c-Jun and/or ATF2 leads to F9 cell differentiation in response to RA, or cell death in response to UV irradiation. A is for ATF2, J is for c-Jun and X is for an unknown partner of ATF2 or c-Jun.

nucleus. Activation of target gene expression (in the case of *jun2-luc*) by c-Jun–ATF2 heterodimers is enhanced by phosphorylation of ATF2, but not c-Jun. Interestingly, it was observed that c-Jun induction in sympathetic neurons by nerve growth factor withdrawal was not significantly impaired in JunAA mice (Besirli *et al*, 2005). This supports our conclusion that c-Jun may function as a nuclear anchor and a dimerization partner of ATF2 in the activation of *c-jun* expression. Similar observations with regard to the phosphor-

ylation-independent activation of certain target genes involved in c-Jun-mediated G1 progression, liver tumor development and AP-1-mediated retinal photoreceptor apoptosis were also reported (Wisdom *et al*, 1999; Grimm *et al*, 2001; Eferl *et al*, 2003). In addition, although we provided several lines of evidence that phosphorylation of c-Jun or ATF2 by MAPKs is not involved in the dimerization and the nuclear localization of ATF2, it remains to be determined whether phosphorylation on other residues of ATF2 or other post-translational modifications plays a role in the nucleocytoplasmic shuttling of ATF2. It would be particularly important to investigate the regulation of ATF2 shuttling under different physiological and pathological conditions.

The cytoplasmic localization of overexpressed ATF2 is clinically intriguing, since ATF2 is often overexpressed in many cancer cells and its expression is induced by chemotherapeutic agents and radiation (Takeda *et al*, 1991; Fuchs *et al*, 1998; Kyriakis and Avruch, 2001). Furthermore, cytoplasmic localization of ATF2 has been observed in melanoma specimens (Berger *et al*, 2003), neurons of Alzheimer's disease patients (Yamada *et al*, 1997), rat ventricular myocytes (Clerk and Sugden, 1997) and JNK1/JNK2-deficient murine embryo fibroblasts (Ventura *et al*, 2003). Therefore, it will be interesting to examine the relationship between the subcellular localization of ATF2 and these disease states. There is no doubt that defining the molecular mechanisms underlying the regulation of the nucleocytoplasmic shuttling of ATF2, and potentially other transcription factors (Turpin *et al*, 1999; Yashiroda and Yoshida, 2003), will open a new avenue for the development of novel therapeutics.

Materials and methods

Plasmid construction

The cDNAs encoding N-terminal residues 1–172 (VN173) and C-terminal residues 155–238 (VC155) of Venus were subcloned into pFLAG-CMV (Sigma) and pCMV-HA (Clontech) vectors to make the BiFC cloning vectors, pFLAG-VN173 and pHA-VC155, respectively. For BiFC analysis of AP-1 dimers, cDNAs encoding AP-1 proteins and their mutants were subcloned into these BiFC vectors to express as fusions with either VN173 or VC155. The linker sequences between the N-terminal AP-1 proteins and the C-terminal VN173 or VC155 of the fusion proteins were described previously (Hu *et al*, 2002). AP-1 proteins and mutants used in this work include: c-Jun 257–318 (bJun), c-Fos 118–211 (bFos), ATF2 1–505 (WT), ATF2 Δ 342–345 (Δ NLS1), ATF2 Δ 354–357 (Δ NLS2), ATF2 Δ 342–372 (Δ NLS1 + 2), ATF2(V405A, L408A, L411A, L413A) (NES4A), ATF2 L408P (L408P), ATF2(T69A, T71A) (69A71A), ATF2(T69D, T71D) (69D71D), c-Jun 1–334 (WT), c-Jun(S63A, S73A) (63A73A), c-Jun(Δ 3–122) (TAM67) and c-Jun(Δ L297) (c-Jun Δ L3). For AP-1 proteins expressed as Venus fusion proteins, VC155 was replaced with full-length Venus. To determine CRM1–ATF2 interaction using BiFC assay, CRM1(566–720), an NES-binding domain (Ossareh-Nazari and Dargemont, 1999), was fused to VN173, and ATF2(342–505), an N-terminal truncation mutant that is predominantly localized in the nucleus (Hu *et al*, 2002), was fused to VC155.

BiFC analysis

Quantification of BiFC efficiency with fragments from enhanced YFP and Venus was performed in essentially the same way as reported previously (Hu *et al*, 2002; Shyu *et al*, 2006), except that the median of the YFP/CFP ratios was used to calculate fold increase of BiFC efficiency as a better measure for highly skewed distributions.

Analysis of subcellular localization of AP-1 dimers and proteins

COS-1 were subcultured in 12-well plates to grow overnight, and then cotransfected with the expression vectors indicated in each experiment (0.5 µg/each) using Eugene 6 (Roche). At 12 h post-transfection, cells were examined under a Nikon TSE2000 inverted fluorescence microscope, and fluorescent images were captured using YFP filters. Fluorescence intensity in the nucleus and the whole cell of more than 100 individual fluorescent cells was measured using an automated intensity recognition feature of Metamorph II (Universal Imaging Corp) and the nuclear localization of fluorescent signal was calculated as a percentage of the total fluorescence intensity.

For the analysis of subcellular localization of FLAG-ATF2 using immunostaining, cells were coexpressed with or without c-Jun and similarly fixed and permeabilized as described for immunostaining. Subcellular localization of FLAG-ATF2 was detected with anti-FLAG antibody (Sigma), followed by Texas Red conjugated secondary antibody. Fluorescent images were similarly quantified as described above.

For subcellular fractionation analysis, COS-1 cells were transfected with plasmid encoding FLAG-ATF2-Venus without or with different amounts of plasmid encoding c-Jun. Cells were harvested at 16 h after transfection and cytosolic and nuclear fractions were prepared according to the manufacturer's instruction (Sigma). Cytosolic and nuclear fractions were verified with β -actin (Sigma) and histone 3 (Abcam) antibodies, respectively, and the amount of FLAG-ATF2-Venus localized in each fraction was detected with anti-FLAG antibody and quantified using NIH Image software.

Immunostaining

Subconfluent COS-1, MCF-7 and F9 cells were fixed in ice-cold 3.7% formaldehyde for 20 min, followed by permeabilization in ice-cold 0.2% Triton X-100 for 5 min. Cells then were incubated with rabbit polyclonal anti-ATF2 (c-19) antibody (Santa Cruz) for 1 h, followed by three washes, and the incubation with the secondary antibody conjugated with Texas Red (Jackson ImmunoResearch Laboratories) for 1 h.

Immunoblotting

COS-1 cells cotransfected with indicated plasmids were harvested from 12-well plates and approximately 1/10 of the total lysate was resolved in 10% SDS-PAGE and transferred to nitrocellulose filters for immunoblotting. Monoclonal anti-FLAG, anti-HA and anti- β -actin antibodies were purchased from Sigma. Polyclonal anti-c-Jun(H79) and anti-ATF2 (c-19) antibodies were purchased from Santa Cruz (Santa Cruz, CA). For the detection of endogenous c-Jun and ATF2 in F9 cells, cells were cultured in 10 cm dishes and harvested for immunoblotting. Approximately 30 µg of total protein was used.

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siRNA analysis

MCF-7 cells were maintained in DMEM supplemented with 10% FCS, insulin (10 µg/ml) and antibiotics. Cells at 80% confluency were transfected with control siRNA or c-Jun SMARTpool siRNA (100 pmol/ml) using the transfection reagents provided according to the manufacturer's instruction (Upstate). At 3 days after transfection, subcellular localization of ATF2 was examined using immunostaining and the subcellular localization of ATF2 was quantified as described above. The effect of c-Jun knockdown was determined by immunoblotting using anti-c-Jun antibody (Santa Cruz).

Luciferase reporter assay

COS-1 cells were subcultured in 12-well plates and starved for 18 h before transfection with the indicated amount of plasmids encoding c-Jun or ATF2, along with 0.5 µg of *jun2-luc* reporter plasmid (driven under five tandem copies of *jun2*) (Le *et al*, 2004) and 50 ng of pRL-TK (Promega). At 24 h post-transfection, cells were lysed and assayed for luciferase activity using the Dual Luciferase Assay kit according to the instructions from the manufacturer (Promega). For F9 cell transfection, cells were maintained in serum-containing DMEM since serum starvation caused cell death. Lipofectamine 2000 was used to transfect plasmids into F9 cells and Eugene 6 was used for COS-1 cell transfection.

RA treatment and UV irradiation

F9 cells were maintained in gelatin-coated dishes in DMEM supplemented with 10% FCS and antibiotics. For the treatment with RA, cells were grown to 60% confluence and treated with 1 µM RA. After treatment for different times, cells were either fixed and permeabilized for immunostaining, or harvested for immunoblotting of c-Jun and ATF2. For UV irradiation, cells were irradiated with UVC (40 J/m²) in a Spectrolinker XL-1000 UV crosslinker (Spectronic Corporation) and cultured for different times before being processed for immunostaining and immunoblotting of c-Jun and ATF2. Cell viability was determined using Vibrant MTT Cell Proliferation Assay Kit (Molecular Probes).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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