Activation and interaction of ATF2 with the coactivator ASC-2 are responsive for granulocytic differentiation by retinoic acid

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Abbreviations: ATF2, Activating transcription factor 2; C/EBPα, CCAAT/enhancer binding protein α; ASC-2, activating signal co-integrator-2.
ABSTRACT

Terminal differentiation of hematopoietic cells follows a precisely orchestrated program of transcriptional regulatory events at the promoters of both lineage-specific and ubiquitous genes. Here we show that the transcription factor ATF2 is associated with the induction of granulocytic differentiation and the molecular interaction of ATF2 with a tissue-specific coactivator coactivator activating signal cointegrator-2 (ASC-2) potentiates the differentiation procedure. All-trans retinoic acid (RA) induced the phosphorylation and expression of ATF2 in the early and middle phase of granulocyte differentiation, respectively. The activation of granulocyte-specific gene expression is increased with the concerted action of another bZIP factor, C/EBPα, and ASC-2, which function in a cooperative manner. The interaction between ATF2 and C/EBPα in RA-treated cells was enhanced by the ectopic expression of ASC-2. ATF2-mediated transactivation was also increased by cotransfection of ASC-2. This resulted from the direct protein interaction that the N-terminal transactivation domain of ATF2 interacts with the central region of ASC-2. Furthermore, the molecular interaction of ATF2 and ASC-2 was stimulated by RA treatment and inhibited by p38β kinase inhibitor. Taking these results together, these results suggest that the differentiation-dependent expression and phosphorylation of ATF2 protein physically and functionally interacts with C/EBPα and coactivator ASC-2 and synergize to induce target gene transcription during granulocytic differentiation.
INTRODUCTION

The pluripotent blood stem cells mature by transcription factors that activate lineage-specific genes are essential to the commitment and development of specific hematopoietic lineages, erythroid, myeloid or lymphoid cells (1). The vitamin A metabolite, all-trans retinoic acid (AtRA), induces the granulocytic differentiation of the promyelocytic cell line U937, similar to HL60. C/EBPα, C/EBPε and PU.1 etc. have been known to contribute in granulopoiesis (2). Especially C/EBPα is essential to granulocyte differentiation in an early stage and in vitro forms a heterodimeric DNA binding complex with another transcription factors of the bzip family, ATF-2 in liver cells (3).

Among ATF/CREB family, ATF2 (initially called CRE-BP1, 4-5) as been more extensively studied and shown to be ubiquitously expressed with the highest level of expression being observed in the brain. A common characteristic of these factors is the presence of a transcriptional activation domain containing the metal finger structure located in the amino-terminal region and basic region-leucine zipper (bZIP) proteins in the carboxy-terminal region (6). ATF2 is capable of forming homodimers or heterodimers with c-Jun for binding to CRE (5'-TGACGTCA-3') (6). Especially, ATF2 is known to play an important role in inducing cell differentiation including cardiomyocyte (7), adipogenesis in early stage (8) and central nervous system development (9).

Some cofactors, structural modification and phosphorylation were known to influence on transcriptional activation of ATF2. For example, TIP49b was reported as a regulator of ATF2 response to stress and DNA damage (10). ATF2 exhibits intramolecular inhibitory interaction between N-terminal transactivation domain and C-terminal DNA binding domain under normal growth condition (11). Stability, transcriptional activity and histone acetyltransferase (HAT) activity of the ATF2 transcription factor are regulated by phosphorylation and dephosphorylation (12-14). In response to various stress, ATF2 has been shown to be phosphorylated on amino acid residues Thr-69, Thr-71 by stress-activated protein kinases, Jun-N-terminal kinase (JNK, 15-16), p38 mitogen-activated protein kinase (17) and extracellular single-regulated kinases (ERK, 18) and also Thr-73 by Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) (19).
To stimulate transcriptional activity of specific transcription factors, the cooperative association of transcriptional coactivator, CBP/p300, was necessary. Previously, we elicited that differentiation-dependent expressed ASC-2 protein physically and functionally interacts with C/EBPα and increases its transactivation activity in granulocyte differentiation (21). Lee et al. isolated a novel coactivator ASC-2 by using retinoid X receptor (RXR) as a bait (22). ASC-2 was also subsequently identified from several groups as named RAP250, PRIP, and TRBP (23-25) and also identical to AIB-3, which was amplified in breast and other human cancers (26). ASC-2, a typical ligand- and AF2-dependent interactor of nuclear receptors, enhances the receptor transactivation, either alone or in conjunction with SRC-1 and CBP/p300 and functionally interacted with specific transcription factors including PPARγ, TR, NF-κB, AP-1, and serum response factor (SRF) (27).

To understand the process of normal myeloid differentiation, it is important to identify and characterize the transcription factors that specifically activate important genes in the myeloid lineage. Until now, it is wholly lacking that ATF2 is involved in hematopoietic differentiation. But we already reported that retinoic Acid activates the p38β kinase pathway leading to phosphorylation and activation of ATF-2, thereby enhancing PEPCK gene transcription and glucose production (20). Therefore, this reflects the indirect evidence that ATF2 may be related in granulocyte differentiation. In this study, we show two novel points that ATF2 is required for the induction of granulocytic differentiation and associates with granulocyte-specific transcription factor C/EBPα by RA treatment and the ASC-2 functions as a coactivator for ATF2 in the differentiation process. These results support that the granulocytic differentiation requires a specific transcription factor-regulated cascade action including a variety of specific transcription factors and coactivators.
Experimental Procedures

U937 cell culture and induction of differentiation

U937 promyelocytic leukemia cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin 100 U/ml, and streptomycin 100 μg/ml (Gibco BRL). To induce granulocyte differentiation, the cells in logarithmic growth were seeded at 2 × 10^5/ml and grown in the presence of 1 μM of all-trans retinoic acid (RA) for up to 4 days. At the end of differentiation experiment, differentiated cells were confirmed with nitroblue tetrazolium (NBT) assay, harvested and resuspended in an appropriate buffer for each experiments. Reduction of nitroblue tetrazolium by respiratory burst products was assayed with nitroblue tetrazolium tablets (Sigma) in accordance with the manufacturer's protocols. Cells were cytospun and counterstained with safranin.

Western blot analysis

Cells were harvested on ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH7.5, 1% NP-40, 1 mM EDTA, 10% glycerol) containing 1x protease inhibitor at 4°C. The protein content of cell lysates was determined with Bradford reagent (Bio-Rad) using bovine serum albumin (BSA) as standard. After heating at 100°C for 5 min in 1x Laemmli sample buffer (LSB), the samples were separated by 10% SDS-PAGE. The resulting gels were either stained with Commassie-blue or transferred to PVDF (Immobilon-P) membrane (Millipore). For Western blotting, the membrane was incubated with anti-phospho-ATF2 (New England Biolab), anti-ATF2, anti-TBP, anti-C/EBPα (Santa Cruz Biotechnology), Mac-1 (Caltag) in TBS containing 1% non-fat dried milk for 1 h at RT. After washing three times with cold TBS-T (TBS containing 0.04% Tween-20), the blotted membranes incubated with peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 30 min at RT. After washing three times with cold TBS-T, the protein bands were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp.).

Transient and stable transfection
HeLa and U937 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL) and 1% antibiotics. The cells were seeded in 24-well plates with growth medium and co-transfected with pRSV/β-gal vector and expression vectors for ASC-2 and/or ATF-2 by using superfect (Qiagen corp) or electroporation. Total amounts of expression vectors were kept constant by adding pcDNA3.1/His C. Relative luciferase and β-galactosidase activities were determined as described (28). All the transfection results represent the mean of three independent experiments. For establishment of the N-terminal domain ATF2 (ATF2-N)-expressing stable cell line, HeLa cells were transfected with 3 μg of ATF2-N expression plasmid (pcDNA3/HA) using calcium phosphate co-precipitation method with BES. At 48 hr post-transfection, cells were cultured in the presence of 500 μg/ml G418 (GIBCO BRL). After 21 days in selective medium, individual G418-resistant colonies were isolated.

**Glutathione S-transferase (GST) pull-down assay between ASC-2 and ATF2**

GST-fusion proteins were purified as described previously. Equal amounts (approximately 1 mg) of GST and several GST-ATF2 proteins (1-323, 1-352, 323-352, 323-492, 1-492) immobilized on 20 μl of glutathione Sepharose 4B beads were incubated with *in vitro* translated [35S]-ASC-2 in the reaction buffer (25 mM HEPES, pH 7.6, 20% glycerol, 100 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 300 μM PMSF, 1.5% bovine serum albumin) for 4 h at 4°C. After washing three times with PBS, the bound proteins were eluted with 10 mM reduced glutathione and boiled with an equal volume of SDS-PAGE sample buffer at 100°C for 3 min prior to electrophoresis. After electrophoresis, the gel was dried and analyzed with the Molecular Imager Fx (Bio-Rad).

**Mammalian two-hybrid assay between ASC-2 and C/EBP α & ATF2**

CV-1 and HeLa Cells were seeded in 24-well plates with growth medium supplemented with 10% FBS and 1% antibiotics, and co-transfected with expression vectors encoding Gal4-DNA binding domain fusions (pCMX/Gal4N/-, pCMX/Gal4N-ASC-2 series and VP16-activation domain fusions (pCMX/VP16/-, pCMX/VP16-C/EBP α, or pCMX/VP16-ATF-2) as well as the previously described
Gal4-tk-luc reporter plasmid. After 48 h, cells were harvested and the luciferase activity was normalized to the β-galactosidase expression. All the results represent the average of at least three independent experiments.

**Co-immunoprecipitation assay**

Cell lysates (500 μg) were incubated with 1 μg of anti-ATF2 antibody at 4°C for 2 h with gentle agitation. Immune complexes were collected on protein G-Sepharose beads (Gibco BRL). After washing three times with RIPA (-) buffer (1% Triton X-100, 1% deoxycholate in PBS), the precipitates were boiled with an equal volume of 2X LSB at 100°C for 3 min and analyzed by SDS-PAGE.

**Chromatin immunoprecipitation analysis**

Cells were lysed for 5 min in L1 buffer (50 mM Tris pH 8.0, 2 mM EDTA, 0.1% NP-40 and 10% glycerol) supplemented with protease inhibitors. Nuclei were pelleted at 3000 r.p.m. and resuspended in L2 buffer (50 mM Tris pH 8.0, 0.1% SDS and 5 mM EDTA). Chromatin was sheared by sonication, centrifuged and diluted 10 times in dilution buffer (50 mM Tris pH 8.0, 0.5% NP-40, 0.2 M NaCl and 0.5 mM EDTA). Extracts were pre-cleared for 3 h with 60 μl of a 50% suspension of salmon sperm-saturated protein A–agarose. Immunoprecipitations were carried out overnight at 4°C. Immunocomplexes were collected with salmon sperm-saturated protein A for 30 min and washed three times (5 min each) with high-salt buffer (20 mM Tris pH 8.0, 0.1% SDS, 1% NP-40, 2 mM EDTA and 0.5 M NaCl) followed by three washes in no salt buffer (1x TE). Immunocomplexes were extracted in 1x TE containing 2% SDS, and protein–DNA cross-links were reverted by heating at 65°C overnight. After proteinase K digestion, DNA was extracted with phenol–chloroform and precipitated in ethanol. About one-twentieth of the immunoprecipitated DNA was used in each PCR. Quantitative duplex PCR assay was performed to analyze the amount of DNA precipitated by specified antibodies in proportion to input DNA. Two pairs of primers were used: Forward (5'-TTGGGCGGGTTGCAGCAGGCA-3') and Reverse (5'-GTCTGTATTCATGATTCTTC-3') for the G-CSF promoter. The PCR conditions were as follows: 1.25 U of Taq DNA polymerase (Amersham Biosciences), 100 ng of each primer, 200 μM dNTP, 2.5 μl of 10x Taq buffer and
double-distilled water to a final volume of 25 µl: 94°C for 180 s; 34 cycles at 94°C for 45 s, 60°C for 60 s and 72°C for 60 s; final elongation at 72°C for 10 min.

RESULTS

Protein expression of ATF2 are induced by exposure to RA in U937 cells

Human U937 cells undergo differentiation in response to RA and have a commitment of mature granulocytic cells, suggesting that granulocytic differentiation of U937 cells may also require responsiveness against distinct transcription factors by RA signaling. It is known that C/EBPα is expressed during early granulocytic differentiation induced by RA exposure. In addition to this, our previous two different results showed that C/EBPα physically and functionally interacts with ATF2 in vitro and in vivo in liver cells (29). The other is that RA increased ATF2 driven transactivation by inducing the phosphorylation and inhibiting the intramolecular interaction of ATF-2 itself, even though HepG2 cells were used (20). These results prompted us to examine whether ATF2 acts on RA-induced granulocytic differentiation of U937 cells. From the result of Fig. 1, the protein expression level of ATF-2 was examined with Western blot analysis by using antibody against the full-length ATF2 during the granulocytic differentiation of U937 cells by exposure to RA. The protein expression of ATF2 was very low in untreated U937 cells, almost at a background level, but its expression was gradually induced after RA treatment. The time course study showed that the induction of ATF2 was detectable on day 2 and the level was slightly increased until day 4. Therefore it can be considered that ATF2 is one of markers for granulocytic differentiation. The level of C/EBPα protein was increased at early times after RA treatment, although C/EBPα is highly expressed and transcriptionally active in untreated U937 cells. Consistent with the finding of ASC-2 in blood cells (25), the protein expression of ASC-2 here may provide a clue for a functional role in granulocytic differentiation.
ASC-2 functions as a coactivator for ATF2-dependent transactivation of granulocyte specific gene expression

Previously we presented that the coactivator ASC-2 specifically interacts with C/EBPα during granulocytic differentiation and was also known to be induced the protein expression during the granulocytic differentiation. In addition these results, the quantitative changes of ATF2 protein was observed in Fig. 1. Taken together, these results prompted us to examine whether ASC-2 functionally cooperates the transactivation activity with ATF2. First, the transactivation of ASC-2 on ATF2-mediated transactivation was examined. ASC-2 increased the transactivation of ATF2 synergistically in a dose dependent manner (Fig. 2A).

As the stimulatory effects of RA are specific to the onset of the differentiation program, we examined the effect of RA treatment and expression of the granulocyte colony-stimulating factor receptor (G-CSFR) in the differentiation cascade and the ability of the ATF2 to influence transcription from extended regions of G-CSFR promoters was assessed directly in transient transfection assays (Figure 2B). In U937 cells, ATF2 and a combination of ATF2 and ASC-2 induced gene expression from the G-CSFR promoter between 3 and 11 fold (Fig. 2B). By contrast, c-Jun failed completely to activate transcription from the G-CSFR promoter. RA treatment had only a modest stimulatory effect on basal transcription from the G-CSFR promoter but a significant additional effect on expression induced by ATF2. In the presence of ATF2 and a combination of ATF2 and ASC-2, RA treatment enhanced expression from the G-CSFR promoter a 22 fold, while enhancing expression dependent on ATF2 alone a more modest 10-fold.

Identification of the protein-protein interaction of ATF2 and ASC-2 and each interacting region

The association between ATF2 and ASC-2 was characterized by co-immunoprecipitation analysis. ATF2 was immunoprecipitated from freshly prepared RA-treated U937 cells, gel-fractionated, and analyzed for ASC-2 coprecipitation by Western blotting with anti-ASC-2 IgG. As shown in Fig. 3A, endogenous ASC-2 was detected as a coprecipitant in ATF2 immunoprecipitates. A parallel
immunoprecipitated formed with preimmune serum failed to sow ASC-2 immunoreactivity. This result suggests that ASC-2 can stimulate the ATF2 transactivation through direct protein-protein interaction.

To further characterize the interacting region of ATF2 against ASC-2 in vitro, GST pull-down assay was performed. Glutathione S-transferase (GST) fusion proteins encoding the full-length, 1-323aa, and 323-492aa of ATF-2 were expressed in *Escherichia coli*, immobilized on Glutathione Sepharose 4B beads, and incubated with $^{35}$S-labeled full-length ASC-2 produced by in vitro translation system. The N-terminal containing transactivation domain of ATF-2 interacts with ASC-2 protein (Fig 3B). The reciprocal strategy was used to delineate the region of ASC-2 required for interaction with ATF2 using the mammalian two-hybrid assay. For this study, Gal4-ASC-2 and VP16-ATF2 expression vectors were transfected into U937 cells. The 849-1057 region of ASC-2 included LxxLL motif was interacted with ATF-2 (Fig 3C), similarly to region bound by another transcription factor. As a result of mammalian two-hybrid assay and GST pull-down assay, the central domain of ASC-2 specifically interacts with the N-terminal transactivation domain of ATF2.

**The coactivator ASC-2 enforces the interaction between ATF2 and C/EBP α**

From the previous results, ATF2 and C/EBPα associate with ASC-2 in the process of granulocytic differentiation and interact with each other. These results prompted us to examine whether the temporal expression of ASC-2 affects the intermolecular interaction of ATF2 and C/EBPα in U937 cells. For this, in the absence or presence of ASC-2 transfection, the mammalian expression plasmids for ATF2 and C/EBPα were ectopically expressed in U937 cells. As shown in Fig. 4A, ASC-2 expression significantly increased the protein interaction of ATF2 and C/EBPα. In addition to these, we already observed that 849-1057 region of ASC-2 interacts with ATF2 in Figure 3C and 392-930 region of ASC-2 interacts with with C/EBP α (data not shown). Next, we examined the dominant negative function of 849-1057 domain or 392-930 domain of ASC-2 in interaction between ATF2 and C/EBPα. As a result of mammalian two-hybrid assay, ASC-2 increased the interaction of ATF2 and C/EBPα (Fig. 4A, lane 5) and 849-1057 region of ASC-2, the binding region to ATF2, or 392-930 region of ASC-2, the binding region of C/EBP α inhibited the interaction as a
dominant negative mutant (Fig. 4A, lane 6 and 7). These results support that the coactivator ASC-2 specifically enforces the interaction between ATF2 and C/EBPα.

To further confirm that ASC-2 induces a functional transcriptional protein complex with ATF2 and C/EBPα at the granulocytic target gene promoter site, it was addressed whether these factors interact and are assembled on promoters in cells by Chip assays with endogenous G-CSFR promoters as well as endogenous transcription factor proteins. After RA treatment, cells were lysed and solubilized chromatin was immunoprecipitated, initially with antibodies against Myo-D ATF2, C/EBPα or ASC-2, and recovered DNAs were amplified by PCR using promoter-specific primers. It is clear from the data in Fig. 4B that ASC-2 recruitment to the ATF2 and C/EBPα-recognized promoter was confirmed in cells, but not by Myo-D. Collectively, these findings support the notion that RA-induced differentiation controls the recruitment of essential components of the transcriptional activation machinery and consequently the efficiency of ATF2-dependent transcriptional activation of G-CSFR, which is one of granulopoietic gene.

**RA potentiates the physical association of ATF2 and C/EBPα in granulocytic differentiation**

The results shown in Fig. 4 indicate that the temporally increased coactivator increases the association of ATF2 and C/EBPα. The ASC-2 expression was induced in the process of RA-dependent granulocytic differentiation (Fig. 1). To investigate the possibility that differentiation inducer, RA, regulates the similar increased protein association of ATF2 and C/EBPα as shown in ASC-2 expression, the same experimental strategy was applied with RA treatment. The synergistic transactivation effect of RA on ATF2 and C/EBPα transactivation prompted us to examine the direct effect on the protein-protein interaction of ATF2 and C/EBPα by RA. For identifying the possibility, we applied to use the mammalian two-hybrid assay with the cognate ATF2 and C/EBPα constructs (Fig. 5). The physical interaction of ATF2 and C/EBPα was increased by the treatment of 1 μM of RA, but not dimethyl sulfoxide (DMSO) as a vehicle considerably.

**ATF2 and p38β kinase are phosphorylated by RA during granulocytic differentiation**
differentiation

p38β kinase activity is high during the initial stages of differentiation but drastically as the U937 leukemic cells undergo terminal differentiation into granulocytes. To identify the phosphorylation control of ATF2 in differentiation, we applied the appearance of phosphorylated ATF2 protein after induction of granulocyte differentiation. The ability to stimulate ATF2 phosphorylation with differentiation-inducing agents clearly points to a role for ATF2 in granulopoiesis. The degree of phosphorylation of ATF2 by p38β kinase, which displayed earlier than the protein expression of ATF2, increased up to 4-fold (Fig. 6). To confirm these results further, the effect of the specific inhibitor of p38β, the pyridinyl imidazole derivative SB203580 on the granulocyte differentiation-induced phosphorylation of ATF-2 was examined. Since the SB203580 treatment almost blocked the differentiation procedure, it was not detected the phosphorylated ATF2 protein dependent on the differentiation (data not shown).

To further confirm that p38β kinase activation is involved in the RA response, we assayed for the activation of p38β kinase itself by phosphorylation of the kinase following RA treatment. Total cell extracts were prepared at the times indicated and assayed for expression and phosphorylation of p38β kinase. As shown in Fig. 6, although p38β kinase expression levels do not change, its phosphorylation state increased from 1 day after RA treatment. This time course correlates with the phosphorylation of ATF2 as shown in Fig. 6. Hence, these data provide direct evidence that RA stimulation leads to p38β kinase activation and ATF2 phosphorylation, potentiating the transactivation activity of ATF2.

RA treatment and p38β expression increases ATF2 transactivation by ASC-2 and the protein interaction of ATF2 and ASC-2 in vivo

To further characterize the functional meaning of ATF2 phosphorylation by p38β kinase, we examined the effects of p38β on the ASC-2-stimulated transactivation of ATF2-dependent reporter gene. U937 cells were transiently transfected with plasmids encoding ATF2, p38β, p38βm and/or the coactivator ASC-2, along with CRE-reporter plasmid. Ectopic expression of p38β alone did not significantly increase the
ATF2-dependent transactivation (Fig. 7A). Interestingly, additional expression of ATF2 with p38β synergistically enhanced the ATF2-driven transactivation approximately 6-fold relative to transfection of p38β alone (Fig. 7A). Compared to this increased fold effect of co-transfection of ATF2 and ASC-2, p38β contributed a highly enhanced activation of ATF2-driven transcription. RA treatment significantly increased the ATF2-ASC-2-driven transactivation, but the p38βm kinase expression inhibited the enhanced transactivation activity as shown in Fig. 7A. In addition to this, the p38β kinase specific inhibitor, SB203580, also blocked the RA-mediated transactivation by ATF2 and ASC-2 synergistic manner. These results suggest that the granulocytic differentiation by RA activates the p38β kinase pathway followed by increasing ATF2-ASC-2-mediated transactivation.

As previous results, RA treatment and constitutive active p38β expression led to increase the synergistic effect of ASC-2 on the transactivation function of ATF2. This raises the possibility that ATF2 efficiently associates with ASC-2 dependent on extracellular and endogeneous signals. The mammalian two-hybrid assay has been used extremely effectively to study protein-protein interactions in a variety of ways. To verify it, we applied to use mammalian two-hybrid assay using GAL4-ATF2 and VP16-ASC-2 expression plasmids in the presence or absence of RA treatment or p38β overexpression. As shown in Fig. 7B, the molecular interaction of ATF2 and ASC-2 was enhanced by RA treatment. Since we postulated that p38β signaling pathway mediates RA-induced ATF2 transactivation, the effect of p38β expression on the protein interaction was examined by similar experiments. The transient transfection of p38β expression plasmids increased efficiently the molecular interaction of GAL4-ATF2 and VP16-ASC-2 in mammalian two-hybrid assay. As predicted, SB203580, specific inhibitor of p38β kinase, inhibited both RA treatment and p38β expression-induced protein interaction of ATF2 and ASC-2. These results suggest that RA enhances the protein interaction between ATF2 and ASC-2 through p38β kinase-mediated phosphorylation of ASC-2.

**Dominant negative mutant ATF2, ASC-2 and p38β expression inhibits the induction of granulocytic differentiation**
To study the physiological role of ATF2, we generated U937 cell lines that express the wild-type ATF2 and the dominant negative mutant ATF2 (ATF2-Nd), which is deleted with the ASC-2-binding domain and transactivation domain. All genes were expressed under the control of a constitutive promoter. After selection with neomycin, cells were induced to differentiate. Immunoblot analysis revealed that two ATF2 constructs were expressed in transfected U937 cells, but absent in untransfected cells (Fig. 8A). Cells expressing the wild-type ATF2 were able to produce Mac-1 protein (Fig. 8A). ATF2-Nd expression inhibited the induction of late markers of granulocytic differentiation, Mac-1. The differentiation status correlated well with expression levels of ATF2. We also confirmed the interaction of ATF2 and ASC-2 was not detected in the ATF2-Nd expressing U937 cells (Fig. 8A). When clones expressing wild-type ATF2 highly were treated with RA, they likewise underwent granulopoietic development, as assessed by detection of respiratory burst activity with nitroblue tetrazolium (NBT), with approximately 95% of the cells staining positively for NBT (Fig. 7C), suggesting that the ATF2 overexpression is sufficient to mediate granulopoiesis induced by RA. In marked contrast, when the ATF2-Nd expression was unable to induce granulopoiesis (Fig. 7C). In this case, only 22% of the cells were positive upon NBT staining in RA-treated cell fraction. Taken together, these results indicate that ATF2 is a positive regulator of granulocytic differentiation.

In addition to the functional identification of ATF2, we next addressed the effect of ASC-2 and p38\(\text{E}\) kinase on RA-derived granulocytic differentiation by using dominant negative mutants of these two proteins. As shown in Fig. 7B, the Mac-1 protein expression in RA-treated cells was decreased in the mutant cells expressing ASC-2/Co2c and p38\(\text{E}\)m kinase compared to normal cells. ASC-Co2c is an ATF2-binding domain and plays a role as a dominant negative mutant of wild-type ASC-2 shown in Fig. 4A. The expression of ASC-2/Co2c in U937 cells blocked the protein assembly of ATF2 and ASC-2 (Fig. 8B). p38\(\text{E}\)m is an inactive p38\(\text{E}\) kinase mutant (T188A, Y190F), which cannot be phosphorylated by p38\(\text{E}\) kinase kinase. The inhibitory effect of granulocytic differentiation by dominant negative mutants of ASC-2 and p38\(\text{E}\) kinase was weaker than that of ATF2 mutant. This explains that ATF2 can be activated by other kinases than p38\(\text{E}\) kinase and associated with other coactivators when ASC-2 is not responsive. This effect was also confirmed by NBT
reduction assay shown in Fig. 7C. Counting of NBT-positive cells revealed that approximately 22% of the ATF2m expressing cells were granulocytic in response to RA, whereas 53% and 46% of the ASC-2m and p38βm respective expressing cells were granulocytic under these conditions (Fig. 7C). Thus, in the presence of dominant negative mutants of ATF2, ASC-2 and p38β, RA is unable to efficiently induce respiratory activity, as shown by NBT reduction assay (Fig. 7C). These data suggest that the proper expression of ATF2, ASC-2, and p38β kinase play a crucial role in RA-induced granulocytic differentiation of U937 cells.

**DISCUSSION**

Cell differentiation appears to be mediated by an orchestrated series of genetically controlled events. To understand specific lineage commitment and maturation of multipotent hematopoietic progenitors, it is necessary to identify transcription factors required during differentiation. Distinct C/EBPs are specially expressed in myeloid and eosinophil maturation by various differentiating inducers. Promyeloid leukemia cells were differentiated to granulocytic lineage by treatment of retinoic acid. This retinoic acid is also known to promote ubiquitination and proteolysis of cyclin D1 during induced tumor cell (30).

It is considered as one of the difficult answers about poorly understanding on parallel mechanisms between differentiation and proliferation. Moreover, the cyclin D1 gene is a direct target of ATF-2 in chondrocytes because ATF-2 binds as a complex with CREB family to the cAMP response element (CRE) in the cyclin D1 promoter (31). In addition to regulation of cell cycle gene expression, ATF-2 has been known as a regulator in adipogenesis (8) and components of differentiation regulatory factor (DRF) complex, which regulates retinoic acid- and E1A-mediated transcription of the c-jun gene in differentiation of F9 cells (32) and stimulators transcription of genes related oncogenic transformation, apoptosis, and adaptive responses of the cells against a large number of stimuli, including cytokine, viruses and cellular stresses (33-35).
Heterodimerization of ATF-2 appears to be crucial for its functions. We previously observed that ATF-2 and C/EBPα could form a heterodimeric DNA binding complex for transcriptional regulation in vitro (3). Taken together, it is strongly suggested being implication of ATF-2 in RA-mediated differentiation of promyeloid leukemia cells. As a result, the protein expression and phosphorylation pattern of ATF-2 by RA treatment were increased. ASC-2 exhibited as a coactivator in cell specific-lineage differentiation in ATF-2-mediated transactivation such as C/EBPα-mediated transactivation. Through mammalian two-hybrid assay, the direct interaction of ATF-2 and C/EBPα was identified by retinoid signal and enforced by ASC-2. Since the dominant-negative ASC-2 proteins, Co2C and Co-2, were associated with ATF-2 and C/EBPα respectively, those constructs expression inhibited ASC-2-mediated coactivation. This implies that ASC-2 is strong mediator for the interaction between ATF2 and C/EBPα and can promote granulocytic differentiation. Future investigations will decide the functional ordering between C/EBPs and ATF-2 during granulopoiesis.

Our observations demonstrate that the phosphorylation of ATF2 by p38β may facilitate interactions between C/EBPα and coactivators, through inducing a conformational change that unmasks the domains for interaction. Establishment of a stable interaction of ATF2 with C/EBPα or coactivators may be crucial prerequisite for efficient transcription by ATF2. These results suggest that the phosphorylation of ATF2 by p38β may be necessary for recruitment of specific transcription factors and coactivators to ATF2-DNA transcription site.

For active transcription process upon cell differentiation, chromatin modification by several coactivators including CBP/p300, P/CAF, and SRC-1 may be necessary. Although ASC-2 has a homology with the activation domain of CBP/p300, it lacks an inherent histone acetyltransferase activity (30, 31, 40). As CBP/p300 was found to interact with ASC-2, the recruitment of ASC-2 into ATF2 transcription complex may drive active chromatin structure for transcription by histone acetyltransferase proteins, such as CBP/p300. Finally, it is interesting to note that the G-CSFR promoter may be a typical enhancesome, comprised of a series of cis-elements, including binding sites for ATF2 family proteins and several different classes of transcription factors (5, 6). Our findings that ASC-2 interacts with ATF2 protein may
shed some light into how the granulocyte specific target gene enhancesome is regulated upon differentiation inducing stimuli, since ASC-2 functionally associates with other classes of transcription factors including AP-1, SRF, NF-κB, and nuclear receptors. ASC-2 can stabilize the assembly of granulocyte specific target gene enhancesome through association with ATF2 and other transcription factors. In addition, ASC-2 may enhance the function of target gene enhancesome by juxtaposing components of the transcriptional machinery in a more favorable orientation, and may also play a role in recruiting transcriptional coactivators.

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FIGURES AND LEGENDS

Figure 1. Protein expression of ATF2 in granulocyte differentiation of U937 cells. U937 cells were grown to confluency as described under Materials and Methods. The cells were refed with complete growth medium containing 1 μM RA for the times indicated. Approximately 50 μg of cell lysates protein from each sample was separated on 10% SDS-PAGE and transferred to Immobilon-P membranes. Duplicate membranes were subjected to Western blot analysis by using antibodies specific for ATF2, C/EBPα, and ASC-2 as indicated. Western blot detection of TBP estimated protein-loading control for each lane.

Figure 2. The coactivator ASC-2 interacts with ATF-2 increasingly depending on granulocytic differentiation. (A) HeLa cells were transfected with β-galactosidase expression vector and increasing amount ATF-2 expression vector, either in the absence (□) or presence (■) of ASC-2, along with 3 times repeated CRE-luciferase reporter. (B) The mammalian expression plasmids encoding a series of ASC-2, ATF2, and Jun were transfected with G-CSFR reporter plasmid into U937 cells, as indicated. 48 h after transfection in the absence or presence of 1 μM RA, cells were harvested for luciferase activities.

Figure 3. Identification of the protein-protein interaction of ATF2 and ASC-2 and each interacting region. (A) ASC-2 coprecipitates with ATF2 from the RA-treated U937 cells nuclear extracts. ATF2 and ASC-2 were immunoprecipitated (IP) from RA-treated U937 cell nuclear extract by incubation with anti-ATF2 IgG. Antibody complexes were captured on protein A-sepharose. The beads were washed three times with binding buffer and eluted into SDS sample buffer. WB, Western blot. (B) A schematic diagram of ATF2 showing different functional domains and regions encoding ATF2. ASC-2 were labeled with [35S]-methionine by in vitro translation and incubated with glutathione beads containing GST alone, GST-ATF2(1-492), GST-ATF2(1-323), and GST-ATF2(323-492) as indicated. The bound proteins were resolved by SDS-PAGE and autoradiography. Beads were washed and specifically bound material was eluted with reduced glutathione and resolved by SDS-PAGE.
Approximately 10% of the labeled proteins used in binding reactions were loaded as input. HAT; histone acetyltransferase, bZIP; basic leucine zipper region. (C) A schematic representation of ASC-2 polypeptides encoded by expression constructs is shown at the bottom. Domains unique to Q-rich, receptor interacting domain, and S/T rich are shown by different bars. The mammalian expression plasmids encoding GAL4-ASC-2 series and VP16-ATF2 were transfected into U937 cells, as indicated. 48 h after transfection, cells were harvested for luciferase activities. All transfection results were normalized to β-galactosidase activity, and represent the average of three independent experiments, with fold induction over the level observed with the reporter alone.

Figure 4. The ASC-2 enforces the interaction between ATF-2 and C/EBPα. (A) U937 cells were transfected with vectors expressing of Gal4-ATF-2 and VP16-C/EBPα along with 100 ng of a reporter gene Gal4-tk-luc as indicated. The 6 and 7 lanes were added with 393-930 domain and 849-1057 domain and of ASC-2, respectively. All the transfection results were normalized to β-galactosidase activity, and the presented results represented the average of three independent experiments, with fold induction over the level observed with the reporter alone. (B) ChIP analysis of factor occupancy on G-CSFR promoters. Following formaldehyde cross-linking, soluble chromatin was prepared. After IP with antibodies against the indicated proteins (Myo-D, C/EBPα, ATF2 and ASC-2), precipitated DNAs were used in PCR analysis. Input shows the starting chromatin extracts.

Figure 5. RA treatment increases the protein interaction of ATF2 and C/EBPα. The mammalian expression plasmids encoding GAL-ATF2 and VP16-C/EBPα were transfected into U937 cels, as indicated. Then, 48 h after transfection in the absence or presence of 1 μmol/l RA, cells were harvested for luciferase activities. All the transfection results were normalized to β-galactosidase activity, and the presented results represented the average of three independent experiments, with fold induction over the level observed with the report alone.

Figure 6. Phosphorylation of ATF2 and p38β kinase in the early phase of
granulocytic differentiation. U937 Cells were incubated with 1 μM RA for the indicated times. Total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated/activated form of ATF-2 and p38β kinase. The protein amount of ATF-2 and p38β kinase was probed with ATF-2 and p38β kinase specific antibodies as a loading control, respectively.

Figure 7. RA treatment and p38β kinase expression increases ATF2 transactivation by ASC-2 and the protein interaction of ATF2 and ASC-2 in vivo. (A) Synergistic activation of p38β kinase in ATF2-ASC-2-driven transcription. U937 cells were co-transfected with CRE-Luc and β-galactosidase expression vector, along with ATF2-, ASC-2-, p38β-, p38βm-expression vectors in the presence or absence of heat shock treatment and p38β kinase inhibitor, SB203580. All transfections were determined by the β-galactosidase activity assay system in order to normalize results for transfection efficiency. Luciferase activity was determined in cell lysates 48 h later, and the values (± S.E.) from at least two independent experiments performed in triplicate are shown in the form of a bar graph. (B) The mammalian expression plasmids encoding GAL-ATF2 and VP16-ASC-2 were transfected into U937 cells, as indicated. 48h after transfection in the absence or presence of RA treatment, cells were harvested for luciferase activities. All the transfection results were normalized to β-galactosidase activity, and the presented results represented the average of three independent experiments, with fold induction over the level observed with the reporter alone. The transient protein expression of p38β kinase was confirmed by using Western blotting with p38β kinase specific antibody. T; ATF2, A; ASC-2.

Figure 8. Dominant negative mutant ATF2, ASC-2 and p38β expression inhibits the induction of granulocytic differentiation. (A) The wild-type and the C-terminal truncated mutant of ATF2 was constitutively expressed in U937 cells. After harvesting two stable transfectants, the cell extracts were applied Western blot analysis by using antibodies against Mac-1 and TBP (used as loading control). ASC-2 coprecipitates with ATF2 from the RA-treated normal and ATF2-Nd expressing
U937 cells nuclear extracts. ATF2 and ASC-2 were immunoprecipitated (IP) from RA-treated cell nuclear extracts by incubation with anti-ATF2 IgG. Western blot (WB) assay was determined by using anti-ASC-2 IgG. (B) The dominant negative mutants of ASC-2 and p38β kinase were constitutively expressed in U937 cells. After treatment of RA, the cell extracts were applied Western blot as the same as (A). Co-immunoprecipitation assay was done as the same as (A). (C) Wild type and mutant cells were treated with RA and determined the differentiation yield by scoring reduction of NBT, and quantified data are presented.
**Figure 1.**

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- **ATF-2**
- **C/EBPα**
- **ASC-2**
- **TBP**
Figure 2
Figure 3

(A) Nuclear extract (30 μg) was immunoprecipitated (IP) with pre-immune or anti-ATF2 antibodies. Western blot (WB) was performed with anti-ATF2 and anti-ASC-2 antibodies.

(B) Input (10%) and GST/ATF-2 fusions were used for in vitro translation. [35S]ASC-2 was immunoprecipitated with antibodies against ATF2.

(C) Gal4-tk-luc, U937 cells were co-transfected with Gal4N, Gal4N/ASC-2, VP16N/ATF2, and various co-factors. Relative luciferase activity was measured.
**Figure 4**

A

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Gal4-tk-luc, U937 cells

B

- **input**
- Pre-IgG
- Myo-D
- ASC-2
- ATF2
- C/EBPα

G-CSFR promoter

Antibody
Figure 5

Relative Luciferase Activity (fold)

- + - - -
- - + + +
- - - + +

Gal4N
Gal4N/ATF-2
VP16N/C/EBPα

DMSO
AtRA (1 µM)
Figure 6

U937 cells

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Figure 7

A 3xCRE-luc, U937 cells

B Gal4-tk-Luc, U937 cells

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Relative fold activations

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Figure 8

Stable transfectants

HA-ATF2
HA-ATF2-Nd

Mac-1
TBP

With DMSO
With AtRA

NE (30 μg)
normal
ATF2-Nd

IP: α-ATF2
WB: α-ASC-2

HA-p38βm
HA-ASC-2m

Mac-1
TBP

WT
ATF2
ATF2m
ASC-2m
p38βm

NBT positive cells (%)

untreated
RA
Activation and interaction of ATF2 with the coactivator ASC-2 are responsive for granulocytic differentiation by retinoic acid
SunHwa Hong, Hyun Mi Choi, Min Jung Park, Yoon Ha Choi, Hyung Hoi Kim, Young Hyun Choi and JaeHun Cheong

*J. Biol. Chem.* published online January 20, 2004

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