Published in final edited form as:

Hepatol Res. 2011 August ; 41(8): 776–783. doi:10.1111/j.1872-034X.2011.00827.x.

Activator protein-2 α functions as a master regulator of multiple transcription factors in the mouse liver

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Abstract

Aim: Activator protein 2 α (AP-2 α) belongs to the AP-2 family of transcription factors that are involved in the regulation of cell proliferation, differentiation, apoptosis and carcinogenesis and has been suggested to function as a tumor suppressor in many cancers. However, the physiological role of AP-2 α in hepatocytes is unknown. The present study is to characterize the expression and function of AP-2 α in the liver of conscience mouse.

Methods: Exogenous AP-2 α was overexpressed in the mouse liver by *in vivo* gene delivery and changes in transcription factor expression were identified by using protein-DNA arrays and immunoblotting.

Results: Western blotting and protein/DNA arrays showed that AP-2 α is expressed in the nuclei of mouse hepatocytes. Overexpression of AP-2 α *in vivo* significantly suppressed transcription factors AP-1, CREB and c-Myc, and markedly increased CBF, c-Myb, NF-1, Pax-5, RXR, Smad3/4, TR(DR-4), USF-1 and GATA. Among all GATA proteins, only GATA-4 level was dramatically elevated and there was a concomitant loss of phospho-GATA-4. Corresponding changes were detected in upstream kinases Akt, GSK-3 β and PKA, which regulates the phosphorylation status and stability of GATA-4 protein.

Conclusions: AP-2 α is expressed in mouse hepatocytes and it acts as a master regulator of numerous transcription factors in the liver.

Keywords

activator protein-1; activator protein AP-2; GATA4 transcription factor; liver; protein kinase A

INTRODUCTION

ACTIVATOR PROTEIN-2 α (AP-2 α) belongs to the AP-2 family of transcription factors comprising five members (α β γ ϵ δ) encoded by separate genes.¹ AP-2 proteins are viewed as gatekeepers controlling the balance between proliferation and differentiation during embryogenesis² and the AP-2 α transcription factor is required for multiple aspects of vertebrate development as AP-2 $\alpha^{-/-}$ mice die perinatally with severe dysmorphogenesis of the face, skull, sensory organs and cranial ganglia.^{3,4} AP-2 proteins are localized predominantly in the nucleus, where they bind to target sequences and regulate transcription of target genes. Depending on the cellular context, AP-2 α can function either as a transcriptional activator or a repressor.^{5,6} It regulates specific genes involved in cell cycle, cell death, adhesion, and migration.⁷ There is growing evidence for a tumor suppressor function of AP-2 α in cutaneous melanomas,^{8,9} colon tumors,^{10,11} prostate cancers,¹² pancreatic cancer cells¹ and breast cancers.^{13,14}

In mice, AP-2 α is co-expressed with AP-2 β and AP-2 γ in neural-crest cells, the peripheral nervous system, facial and limb mesenchyme, various epithelia of the developing embryo and the extra-embryonic trophoblast.¹⁵⁻¹⁷ Currently, little is known about AP-2 α in the liver and its effects on the expression of other transcription factors. In AP-2 α deficient HepG2 cells, expression of AP-2 α markedly increases basal promoter activity of serine : pyruvate aminotransferase (SPT), a liver-specific enzyme.¹⁸ AP-2 α also inhibits the promoter activity of serum amyloid A1, an acute-phase protein restricted to liver hepatocytes.^{6,19} These reports suggest an important role of AP-2 α in the liver.

Transcription factors such as AP-1, CREB and GATA play critical roles in energy combustion, hepatic steatosis, lipoprotein synthesis, inflammation and hepatocarcinogenesis.²⁰ The GATA family of transcription factors consists of six members that are implicated as critical regulators of gene expression during eukaryote development.²¹ They contain one or two distinctive zinc-finger domains and bind to the consensus sequence WGATAR. GATA-1 plays an important role in hematopoiesis,²² whereas GATA-4 is critical for heart formation and endoderm/mesoderm development.^{23,24} GATA-4 and HNF3 (FoxA) are the earliest known transcription factors associated with the albumin gene enhancer in embryonic liver precursor cells.²⁵ Hepatic development was arrested in GATA-4 null embryos, which were unable to generate a normal liver bud.²⁶ Despite their importance in hepatogenesis and early developmental processes, little is known about the regulation of GATA proteins *in vivo*.

Our previous work has shown that AP-2 α forms a complex with adenomatous polyposis coli (APC) and β -catenin to disrupt β -catenin/TCF-4 interactions in the nucleus and suppress intestinal tumorigenesis in *Apc*^{min} mice.^{10,27} Here, we provide evidence, for the first time, that AP-2 α is expressed in the mouse liver and *in vivo* overexpression of AP-2 α significantly alters numerous transcription factors in the liver of transgenic mouse.

METHODS

Plasmid preparation

PCDNA3.1/MYC-HIS-AP-2 α WAS GENERATED by subcloning mouse cDNA-encoding full length AP-2 α into pcdNA3.1(+)/myc-His B (Invitrogen, Carlsbad, CA, USA) between *Hind*III and *Xba* I. Plasmids were isolated and purified using the Qiagen (Valencia, CA, USA) QIAfilter Plasmid Maxi Kit followed by the Mirus Bio LLC (Madison, WI, USA) MiraCLEAN Endotoxin Removal Kit.

In vivo delivery of AP-2 α

Male C7BL/6J^{+/+} mice and *Apc*^{min} mice were obtained from the Jackson Laboratory at 5 weeks of age. The Mirus *TransIT In Vivo* Gene Delivery System was used to deliver pcdNA3.1/myc-His-AP-2 α or empty pcdNA3.1/myc-His B (vector control) by tail vein injection. Delivery complexes were prepared by combining 20 μ L of a 1 μ g/ μ L plasmid solution, 170 μ L of endotoxin-free sterile water, and 10 μ L of *TransIT In Vivo* Polymer Solution, according to the manufacturer's protocol. The first of three weekly injections was performed on mice at 6 weeks of age, and 24 h after the last injection mice were killed by CO₂ inhalation. Portions of liver were placed in formalin or snap-frozen in liquid nitrogen and stored at -80°C. All procedures with mice were approved by the Institutional Animal Care and Use Committee.

Immunohistochemistry

Immunohistochemical procedures were as described by Yu *et al.*²⁸ In brief, formalin-fixed, paraffin-embedded tissues sectioned at 4–5 μ m were rehydrated, and antigen retrieval was performed in citrate buffer, pH 6.0 for 10 min in a microwave pressure cooker. After 20 min at room temperature, slides were washed in Biomed Automation buffer, followed by blocking in 3% H₂O₂ in methanol for 10 min. Rabbit polyclonal AP-2 α primary antibody (C-18, Santa Cruz, CA, USA) was applied at a dilution of 1:1000.

Immunoblotting

Immunoblotting was performed according to the methodology described before,¹⁰ on mouse liver whole cell lysates or nuclear and cytoplasmic extracts isolated using NE-PER Reagents (Pierce). Proteins were separated on Novex 4–12% bis-tris gels and transferred to nitrocellulose membranes, followed by immunoblotting with the specified primary antibody and horseradish peroxidase-conjugated secondary antibody. Antibodies are as follows: anti-Myc-tag rabbit polyclonal (ab9106), anti-phospho-GATA-4 (S105) rabbit polyclonal (ab5245), anti-PKA alpha + beta (catalytic subunits) antibody (abcam); anti- β -catenin mouse monoclonal (610154, Transduction Labs); anti-histone H1 goat polyclonal (N-16), anti-AP-2 α mouse monoclonal (SC-12726), anti-GATA-4 goat polyclonal (C-20), anti- α -tubulin mouse monoclonal (6A204) (Santa Cruz); anti-Akt rabbit polyclonal, anti-phospho-Akt (S473) rabbit polyclonal (Cell Signaling, Danvers, MA, USA); anti-c-Jun rabbit polyclonal (MBL, Woburn, MA, USA); anti-CBF rabbit polyclonal (PA5-12304) (Thermo Scientific, Waltham, MA, USA); anti-phospho-GSK-3 α/β (S21/S9) rabbit polyclonal (R&D Systems, Minneapolis, MN, USA).

Protein/DNA arrays

TranSignal™ Spin Protein/DNA Array I (Panomics, Santa Clara, CA, USA) was used to identify transcription factors regulated by AP-2α in mouse liver, as described before.²⁹ Nuclear extracts (15 μg of nuclear protein) were incubated with TranSignal Probe Mix provided in the kit. After isolation of protein-bound probes, hybridization to the array membranes, and detection, images were acquired using X-ray film. Image analysis and quantification was performed on an AlphaInnotech AlphaImager 2200 system (Santa Clara, CA, USA).

Quantitative real-time RT-PCR

Total RNA was extracted from mouse liver using the Qiagen RNeasy Mini Kit. Purified RNA (2 μg) was reverse transcribed with the Qiagen Omniscript RT Kit. Quantification of *GATA4* gene expression by real-time polymerase chain reaction (PCR) was performed with a Peltier Thermal Cycler PTC-200 (Bio-Rad), and *GATA-4* was amplified in the presence of Master Mix SYBR Green. *GAPDH* expression was quantified as an internal control. Primers were as follows: GATA4-RT-F, 5'-TCT CAC TAT GGG CAC AGC AG-3'; GATA4-RT-R, 5'-GCG ATG TCT GAG TGA CAG GA-3'; GAPDH-F, 5'-TCC CAC TCT TCC ACC TTC GA-3'; GAPDH-R, 5'-AGT TGG GAT AGG GCC TCT CTT G-3'. All samples were assayed in triplicate in an Optical 96-well reaction plate with Optical Adhesive Covers (Applied Biosystems) in a 20-μL volume containing 5 μL (2 μL for GAPDH) diluted cDNA (1:3 dilution with MQ water), 10 μL SYBR Green Master Mix, and 1 μL of each primer (10 μM).

Kinase assays

Kinase activities were assessed using the Omnia Lysate PKA or Akt/PKB Assay Kit (BioSource, Dacula, GA, USA), with 5 μL of 5 μg/μL liver extract for each reaction. Fluorescence intensity readings ($\lambda_{\text{ex}}360/\lambda_{\text{em}}485$) were recorded for up to 50 min after the addition of the Master Mix. Omnia Cell Extraction Buffer was used as negative control.

Statistical analysis

Results (mean ± SD, $n = 6$, unless stated otherwise) for AP-2α were compared with vector control using the two-tailed Student's *t*-test, taking $P < 0.05$ as statistically significant.

RESULTS

Overexpression of AP-2α in the mouse liver

IMMUNOBLOTTING OF HEPATIC whole cell lysates using anti-myc-tag antibody revealed strong expression of myc-His-AP-2α in wild type (Fig. 1a, Top panel) and *Apc^{min}* (same as wild type, data not shown) mice given exogenous AP-2α, but not in animals given empty vector. β-actin was used as loading control. Western blot with anti-AP-2α antibody showed high levels of exogenous myc-His-AP-2α (upper band) and endogenous AP-2α (lower band) in the nuclear extracts isolated from livers of AP-2α-treated mice. Endogenous AP-2α protein was also observed in that of vehicle-treated mice, indicating AP-2α expression in the nuclei of hepatocytes (Fig. 1a, bottom panel). Histone H1 was probed for

loading control. Immunohistochemical staining confirmed that there was high nuclear and cytoplasmic myc-His-AP-2 α in the hepatocytes of mice given AP-2 α (Fig. 1b, upper panel, inset), but not in the liver of vector controls (Fig. 1b, lower panel). Rather than uniform immunostaining, myc-His-AP-2 α was strongly expressed in individual hepatocytes throughout the liver, indicating relatively high transfection efficiency (Fig. 1b). No obvious deleterious effect was observed on animal behavior, or on food and water consumption. Histopathological examination of spleens, livers, and small intestines, and cytological examination of bone marrows, revealed no differences between the treatment groups, suggesting low toxicity of AP-2 α .

AP-2 α alters transcription factors in the mouse liver

To establish functional significance of AP-2 α in the liver, protein-DNA arrays using mouse liver nuclear extracts were performed to examine whether AP-2 α modulates DNA bindings of various transcription factors. Consistent with the immunoblotting data, protein-DNA arrays revealed a strong signal for AP-2 α in the liver of control animals (Fig. 2b, arrows), suggesting a role of AP-2 α in the mouse liver. Significantly stronger signal for AP-2 α was shown in AP-2 α -treated mice compared with vector controls that expressed only the endogenous levels of AP-2 α (Fig. 2a,d, arrows), indicating successful over-expression of myc-His-AP-2 α . Importantly, markedly decreased protein/DNA interaction was seen for transcription factors AP-1, CREB, Myc-Max and MRE in response to exogenous AP-2 α (Fig. 2a–c), whereas significant increases were seen for CBF, c-Myb, NF-1, Pax-5, RXR, Smad3/4, TR(DR-4), USF-1 and GATA (Fig. 2a,b,d), suggesting that AP-2 α functions as a master regulator of numerous transcription factors in the mouse liver.

AP-2 α elevates GATA-4 protein but not mRNA level

Since GATA plays an important role in the liver and it was among the most strongly increased signals in the protein-DNA arrays (Fig. 2); thus, we next examined the response of selected GATA family members to AP-2 α . Compared with vector controls, nuclear GATA-4 protein was highly overexpressed in mice treated with AP-2 α (Fig. 3a), but nuclear GATA-1, 2, 5, and 6 were not significantly altered (data not shown). Western blots also showed that c-Jun was dramatically suppressed, whereas CCAAT-binding factor (CBF) was markedly elevated (Fig. 3a), which agree with protein/DNA array results (Fig. 2). β -catenin was unchanged, consistent with previous findings in human colon cancer cells showing that AP-2 α attenuated β -catenin/TCF transcriptional activity without decreasing nuclear β -catenin expression.¹⁰ Interestingly, quantitative real-time RT-PCR showed that GATA-4 mRNA levels remained unchanged in the presence and absence of exogenous AP-2 α (Fig. 3b), suggesting that AP-2 α did not affect *GATA-4* gene transcription.

AP-2 α suppresses the phosphorylation status of GATA-4

We speculated that AP-2 α might increase hepatic GATA-4 protein level by modulating its phosphorylation status and hence its proteasomal degradation. Using phospho-specific antibody, there was evidence for a decrease in nuclear pGATA-4 (S105) in mice treated with AP-2 α , compared with vector controls (Fig. 4a). To identify upstream kinases that might be responsible for suppressed pGATA-4, cytoplasmic protein levels of PKA, Akt, pAkt(S473),

pGSK-3 β (S9) and PKC were detected by immunoblotting. Immunoblot analyses showed that cytoplasmic PKA protein level was markedly suppressed by AP-2 α , which suggested a role for PKA in GATA-4 phosphorylation (Fig. 4a). AP-2 α also increased PKC, but did not alter Akt in the cytoplasmic fraction (Fig. 4b). However, pAkt(S473) was dramatically augmented, suggesting highly elevated Akt kinase activity. Accordingly, pGSK-3 β (S9), a downstream target of Akt, was significantly upregulated, indicating suppressed GSK-3 β activity, which may contribute to decreased GATA-4 phosphorylation (Fig. 4b). Kinase assays confirmed that AP-2 α inhibited PKA activity and enhanced Akt activity significantly compared with vector controls (Fig. 4c,d), suggesting that AP-2 α inhibited PKA and GSK-3 β activities, and hence suppressed GATA-4 phosphorylation and proteasomal degradation.

DISCUSSION

AP-2 PROTEINS PARTICIPATE in the regulation of various different signaling pathways,^{10,30} and are known to be essential biological factors during development, cell growth, differentiation and apoptosis.^{2,8} AP-2 α is receiving increasing interest as an important gene regulator and a possible tumor suppressor, and the list of AP-2 α target genes is growing.^{8,10} We identified several new transcription factors as being activated or repressed by AP-2 α in mouse liver, and confirmed that myc-max is under negative regulation of AP-2 α .⁸ The Myc oncoprotein is a transcription factor that is both necessary and in many ways sufficient to promote the growth of diverse cell types.³¹ It is noteworthy that the repressed transcription factors by AP-2 α are all anti-apoptotic proteins, which strongly suggest that AP-2 α functions as a tumor suppressor in the liver. AP-1 plays important roles in oxidative stress and signal transduction pathways associated with alcoholic liver disease and other pathologies.^{32,33} AP-1 is often portrayed as a general, nuclear decision-maker that determines life or death cell fates in response to extracellular stimuli. However, it is increasingly clear that the cellular context is critical for determining the contribution of AP-1 to cellular fates, and the role of AP-1 in apoptosis should be considered within the context of a complex network of nuclear factors that respond simultaneously to a wide range of signal transduction pathways.³⁴ Different lines of existing evidence support a direct role of CREB in oncogenesis. Constitutive activation of CREB in tumor cells by various mechanisms, including gene amplification, chromosome translocation, interaction with viral oncoproteins, and inactivation of tumor suppressor genes, overturns the cellular control of cellular growth, proliferation and survival.³⁵ By enhancing expression of certain cell-cycle genes, CREB appears to promote growth-factor-independent proliferation and survival of myeloid cells.³⁶

Transcription factors that were activated in mouse liver by AP-2 α included CBF, NF-1, Pax-5, RXR, Smad3/4, TR(DR-4), USF-1 and GATA. To our knowledge, this is the first report to identify these transcription factors as AP-2 α targets, some of which have established roles in vertebrate development, embryogenesis, and cell transformation. Although each is worthy of further study, we elected to focus on GATA proteins implicated in hepatogenesis and malignant hematopoiesis.^{21,24,37}

Among all GATA proteins detected, there was dramatic increase only in GATA-4 protein expression following AP-2 α treatment, with no alterations in *GATA-4* mRNA level (Fig. 3), suggesting mechanisms involved with protein turnover or stability. The degradation of GATA proteins is poorly understood at present, but phosphorylation and the ubiquitin-proteasome pathway were implicated in prior studies.^{38,39} In support of the notion, phospho-GATA-4 was decreased by AP-2 α , and subsequent experiments suggested a role for PKA and/or GSK-3 β in GATA-4 phosphorylation (Fig. 4). In the presence of AP-2 α , degradation of GATA-4 might have been inhibited due to less phosphorylation caused by significant suppression of PKA and GSK-3 β . To our knowledge, this is the first report to describe the regulation of GATA family members in mouse liver by AP-2 α , and to identify the possible upstream kinases that might be involved *in vivo*, namely PKA and GSK-3 β . These findings should provide new avenues to studying the diverse functions of AP-2 α , and the possibility that its tumor suppressor activities are mediated, at least in part, by changes in GATA family members.

In conclusion, we successfully overexpressed AP-2 α using an *in vivo* gene delivery system and identified numerous transcription factors as novel AP-2 α targets in the mouse liver. The results suggested that AP-2 α is expressed in the nuclei of hepatocytes and acts as a master regulator of transcription factors. In addition, AP-2 α inhibited PKA and GSK-3 β , thereby attenuating the phosphorylation of GATA-4 and stabilizing GATA-4.

Acknowledgments

WE THANK BARBARA Delage, W. Mohaiza Dashwood, Valerie Elias, Mandy Louderback and Kate Cleveland for their assistance during necropsy examinations. This work was supported by NIH grant CA80176 (Dashwood).

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Figure 1.

Activator protein 2 α (AP-2 α) overexpression in the mouse liver. Male C7BL/6J^{+/+} mice and *Apc*^{min} were injected with an AP-2 α -expressing plasmid or with the vector control. Immunoblotting (a) and immunohistochemical staining (b) were performed to detect endogenous or exogenous AP-2 α . Inset: cell magnified to show high nuclear and cytoplasmic expression of exogenous myc-His-AP-2 α . Data are representative of four independent experiments.

Figure 2. Transcription factors altered by activator protein 2α (AP-2α) *in vivo*. Nuclear extracts from mouse liver were evaluated in protein-DNA arrays to identify transcription factors in animals treated with AP-2α (a) or vector (b). Top panels, array images; lower panels, transcription factor identities and densities. Arrows, AP-2α. As stated in Appendix B of the manufacturer's instruction booklet, genes on the array are spotted as normal and 1:10 diluted DNA for each alternating row (each spotted in duplicate). Signals were quantified using an AlphaImager 2200 system in order to define transcription factors suppressed (c) or activated (d) by AP-2α. Data, mean ± SD (*n* = 4). *P < 0.05; **P < 0.01.

Figure 3.

Activator protein 2α (AP-2α) enhances hepatic GATA-4 protein expression without altering GATA-4 mRNA level. (a) GATA-4, c-Jun, CBF, β-catenin and Histone H1 (loading control) were detected in immunoblots of mouse liver nuclear extracts. (b) Expression of GATA-4 mRNA in mouse liver was determined by real-time reverse transcription-polymerase chain reaction (RT-PCR), using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control. Data, mean ± SD ($n = 6$).

Figure 4.

Activator protein 2α (AP-2α) inhibits GATA-4 phosphorylation and kinase activities of PKA and GSK-3β in the mouse liver. (a) Western blot analyses were performed for the proteins indicated in whole cell lysates of mouse liver. (b) Protein expressions of kinases in cytoplasmic fractions of mouse liver. All immunoblots are representative of four independent experiments. (c) Kinase assay for hepatic PKA. (d) Kinase activity of Akt in mouse liver; mean $\pm$ SD ($n = 6$). * $P < 0.05$.