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GAS41 interacts with transcription factor AP-2β and stimulates AP-2β-mediated transactivation

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ABSTRACT

Transcription factor AP-2 regulates transcription of a number of genes involving mammalian development, differentiation and carcinogenesis. Recent studies have shown that interaction partners can modulate the transcriptional activity of AP-2 over the downstream targets. In this study, we reported the identification of GAS41 as an interaction partner of AP-2β. We documented the interaction both *in vivo* by co-immunoprecipitation as well as *in vitro* through glutathione *S*-transferase (GST) pull-down assays. We also showed that the two proteins are co-localized in the nuclei of mammalian cells. We further mapped the interaction domains between the two proteins to the C-termini of both AP-2β and GAS41, respectively. Furthermore, we have identified three critical residues of GAS41 that are important for the interaction between the two proteins. In addition, by transient co-expression experiments using reporter containing three AP-2 consensus binding sites in the promoter region, we found that GAS41 stimulates the transcriptional activity of AP-2β over the reporter. Finally, electrophoretic mobility shift assay (EMSA) suggested that GAS41 enhances the DNA-binding activity of AP-2β. Our data provide evidence for a novel cellular function of GAS41 as a transcriptional co-activator for AP-2β.

INTRODUCTION

To date, five members of the AP-2 family of transcription factors, AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε, have been identified. The AP-2α, AP-2β, AP-2γ genes are relatively well characterized (1–6). The AP-2 protein forms a unique modular structure consisting of an N-terminal proline- and glutamine-rich transactivational domain and a complex helix-span-helix motif necessary and sufficient for dimerization and site-specific DNA binding (7,8). A number of genes that mediate cell growth, cell shape, cell movement, cell fate and cell communication frequently possess the AP-2 binding site in their *cis*-regulatory sequences (9–15). Several genes related to cancers have also been shown to be regulated by AP-2, such as erbB-2 (3,10,16,17), ERα (12) and IGF IR (16,18) in breast cancer, and MUC18 and c-KIT genes in melanoma (19,20). The AP-2 family of genes also plays important roles in mammalian development. AP-2 genes show overlapping but distinct patterns of expression during vertebrate embryogenesis, and function in the development and differentiation of the neural tube, neural crest derivatives, heart, skin, urogenital tissues and extraembryonic trophoblasts (21–23). The importance of AP-2 genes is highlighted by knockout experiments of AP-2α, AP-2β and AP-2γ. Mice lacking both copies of AP-2α gene die perinatally and exhibit at least six major defects during embryogenesis: morphogenesis of the neural tube, face, eye, body-wall, cardiovascular system and forelimbs (24–27). Mice lacking AP-2β display fewer gross phenotypic defects but die shortly after birth due to the disruption of terminal kidney differentiation (28). The AP-2γ-null mice die around E7.5, shortly after implantation due to the defects within the extraembryonic cell lineages (23,29).

The AP-2 family of transcription factors plays a broad range of roles from cell growth, tissue morphogenesis and cancers. One of mechanisms for the AP-2 family fulfills their roles is to activate or suppress various downstream target genes at transcriptional levels. A number of studies demonstrated that AP-2-interacting proteins can affect the transcription of AP-2 downstream targets by modulating the transcriptional activity of AP-2. In fact, several AP-2α-interacting partners have been identified. For example, the

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transactivation of p21WAF1 by AP-2α was augmented while activation of laminin receptor by AP-2α was reduced through a direct interaction with p53 (30,31). AP-2α represses the transactivation by Myc through associating with Myc and competing the binding site with Myc (11). Other AP-2α-interacting proteins include Yin Yang factor 1 (YY1) (32), retinoblastoma protein (RB) (33,34) and oncogene DEK (35). Wwox tumor suppressor protein was also identified as an AP-2γ interacting partner, and Wwox protein triggers redistribution of nuclear AP-2γ to the cytoplasm, hence suppressing AP-2γ-mediated transactivation (36).

To date, no AP-2β-interacting factor has been reported yet. To search for AP-2β-interacting proteins, we used AP-2β as the bait and screened a HeLa cDNA library in yeast two-hybrid system. We identified GAS41 as a protein partner of AP-2β. The interaction between the two proteins was confirmed *in vivo* by co-immunoprecipitation and co-localization assays, and demonstrated *in vitro* by glutathione *S*-transferase (GST) pull-down assay. The interaction domains between the two proteins were mapped to the C-terminus of AP-2β and C-terminus of GAS41. Furthermore, we demonstrated that GAS41 resulted in enhancement of transcriptional activity of AP-2β over AP-2 response element reporter by, at least in part, enhancing the DNA-binding activity of AP-2β.

MATERIALS AND METHODS

Vector construction

For yeast two-hybrid screening, full-length cDNA of AP-2β was ligated in frame with the GAL4 DNA-binding domain of the pDBLeu vector resulting in pDBLeu/AP-2β. For immunoprecipitation and colocalization assays, the full-length cDNA of AP-2β was cloned into the mammalian expression plasmid pCMV-Myc vector (Clontech), forming a Myc tagged AP-2β expression vector pCMV-Myc-AP-2β, while full-length cDNA and the mutations of GAS41 were inserted into pCMV-HA vector (Clontech), forming a HA tagged GAS41 expression vector pCMV-HA-GAS41, and full-length cDNA of GAS41 was also cloned into pCMV-Myc vector. Vector pGEX-4T-2 (Amersham) was used to construct vectors expressing GST-AP-2β fusion proteins. The cDNA fragments encoding full-length and subdomains (Figure 3G) of AP-2β were cloned in frame with respect to GST into pGEX-4T-2 individually. Plasmid pQE-N_3 (Qiagen) was used to generate vectors expressing His-tagged GAS41 fusion proteins. The cDNAs encoding full-length subdomains (Figure 4F) and point mutations of GAS41 were fused in frame to His tag of pQE-N_3 individually. Reporter plasmid A2-Luc was constructed by replacing CAT gene with luciferase gene in pA2BCAT vector (generous gift of T. Williams) which contains three copies of AP-2 binding site in human metallothionein IIa gene in the promoter region (7). Vector pCMV-LacZ was constructed by fusing LacZ gene into pCMV-Myc.

Yeast two-hybrid screens

The pro yeast two-hybrid system was obtained from GIBCO/BRL. A HeLa cDNA library cloned in frame with the GAL4 activation domain in the vector pPC86 was used to screen AP-2β-interacting clones. The MaV203 yeast strain was transformed with the pDBLeu-AP-2β and tested for a basal expression activity, as described in the GIBCO/BRL protocol. The bait-containing MaV203 cells were subsequently transformed with the HeLa cDNA library, and transformants were selected by growing SD-leu$^-$, Trp$^-$, Ura$^-$, His$^-$ medium supplemented with 25 mM 3-amino-1, 2, 4-triazole (3-AT). False positive clones were eliminated by retransforming the prey DNA to the original bait strain and positive clones were further verified using X-gal filter assay. Finally, plasmids from positive clones were sequenced and characterized.

Cell culture and transient transfections

HeLa cells and human HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were cultured at 37°C in a 5% CO_2 incubator. Cells were transfected at 70% confluence using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Immunoprecipitation and western blot analysis

HeLa cells were co-transfected with pCMV-Myc-AP-2β and pCMV-HA-GAS41 or only transfected with pCMV-Myc-AP-2β. Twenty four hours after transfection, HeLa cells were lysed in RIPA buffer [50 mM Tris–HCl (pH 7.2), 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS] with protease inhibitors. Immunoprecipitation using either mouse monoclonal anti-Myc antibody or rabbit polyclonal anti-HA antibody (Santa Cruz Biotech) were performed as described previously (37). Co-precipitated proteins were subjected to electrophoresis on 13% SDS–polyacrylamide gel, and were then analyzed by western blot analysis using rabbit polyclonal anti-HA antibody, monoclonal anti-Myc antibody or rabbit polyclonal anti-GAS41 antibody (Santa Cruz Biotech).

Immunofluorescent staining

HeLa cells were cultured on glass coverslips and transfected with pCMV-Myc-AP-2β and pCMV-HA-GAS41. Twenty four hours after transfection, cells were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, and permeabilized with 0.2% Triton X-100 for 5 min. Cells were then incubated with primary antibodies diluted in PBS with 1% (v/v) normal goat serum for 1 h and with the secondary antibodies under the same conditions. The primary antibodies used were mouse monoclonal anti-Myc and rabbit polyclonal anti-HA antibodies (Clontech) while the secondary antibodies were Alexa 594 goat anti-mouse and Alexa 488 goat anti-Rabbit antibodies (Molecular Probes). Hoechst 33 258 (Sigma) was used to stain the nuclei. Fluorescence on the processed slips was analyzed using a confocal laser microscope (Radiance 2100, BioRad).

GST pull-down assay

GST, GST-AP-2β and other GST fusion proteins, His-GAS41 and other His fusion proteins were expressed and purified according to manufacturer's instructions (Amersham). For the pull-down assay, 1–5 μg of the GST or GST fusion proteins were mixed with 40 μl of 50% suspension of glutathione-Sepharose 4B beads for 2 h in binding buffer

[25 mM HEPES-NaOH (pH 7.5), 12.5 mM $MgCl_2$, 10% Glycerol, 5 mM DTT, 0.1% NP-40, 150 mM KCl and 20 μM $ZnCl_2$]. Then 1–5 μg of His fusion proteins were added followed by incubation for another 2 h. The pellets were washed extensively and boiled. The bound proteins were resolved by 13% SDS–polyacrylamide gel and analyzed by western blot analysis with mouse monoclonal anti-His antibody (Santa Cruz Biotech).

Luciferase assays

Transient transfections of cells with A2-Luc, pCMV-LacZ and the indicated expression vectors were carried out with Lipofectamine 2000 (Invitrogen). Twenty four hours after transfection, the cells were lysed and luciferase assay was performed using the luciferase assay system (Promega). pCMV-LacZ was cotransfected in all experiments, and β-galactosidase activity was used to normalize for different transfection efficiencies.

Electrophoretic mobility shift assay (EMSA)

The consensus AP-2 binding site in human metallothionein IIa promoter was used for EMSA. The oligonucleotide sequences used for EMSA were as follows. Forward, 5′-TGACCGCCCGCGGC CCGTG-3′; reverse, 5′-CACGG-GCCGCGGGCGCTCA-3′. The binding specificity was determined using the AP-2 binding site within the PDIP1 promoter, Sp1 binding site in SV40 promoter and SV40 oligo containing no binding site for transcription factor as specific and non-specific cold competitor DNA, respectively. The upper oligonucleotide sequences were shown, AP-2: 5′-GACGCGGCCCTCGGCCTGGCC-3′, Sp1: 5′-GCGCT-GGGGCGGCTGGTGGACG-3′, SV40: 5′-ATTCGATCGG-GGCGGGGCGAGC-3′ (38). The EMSA assay was performed as described previously (7).

RESULTS

AP-2β interacts with GAS41 in yeast two-hybrid assay

To identify AP-2β−interacting proteins, we first performed yeast two-hybrid screening using full-length AP-2β protein as the bait and the HeLa cell cDNA library as a prey. The transactivational activity of the GAL4-AP-2β fusion protein in yeast was inhibited by 25 mM 3-AT. Approximately 2.0×10^6 transformants were screened and thirty clones were obtained on a SD-Leu^-, Trp^-, $uracil^-$, His^- medium supplemented with 25 mM 3-AT. Two clones were further shown to be positive when analyzed for β-galactosidase activity using the colony lift assay. Sequence analysis revealed that one of the clones was identical to human GAS41 cDNA previously cloned from a glioblastoma cell line (39).

AP-2β and GAS41 are co-immunoprecipitated in HeLa cells

To demonstrate the possible interaction between AP-2β and GAS41 in mammalian cells, we asked whether the two proteins could be co-immunoprecipitated. HeLa cells were transiently transfected with expression vectors pCMV-Myc-AP-2β and pCMV-HA-GAS41. The lysates were immunoprecipitated with either control IgG or anti-HA polyclonal antibody. The co-immunoprecipitated protein was examined for the presence of Myc-AP-2β by immunoblotting assay using anti-Myc monoclonal antibody. As shown in Figure 1A, AP-2β could be co-immunoprecipitated with HA tagged GAS41 (Figure 1A, lane 2) but not by control rabbit IgG (Figure 1A, lane 3). To further confirm this finding, the same lysates were immunoprecipitated with anti-Myc monoclonal antibody, and the bound protein was detected by immunoblotting with anti-HA polyclonal antibody.

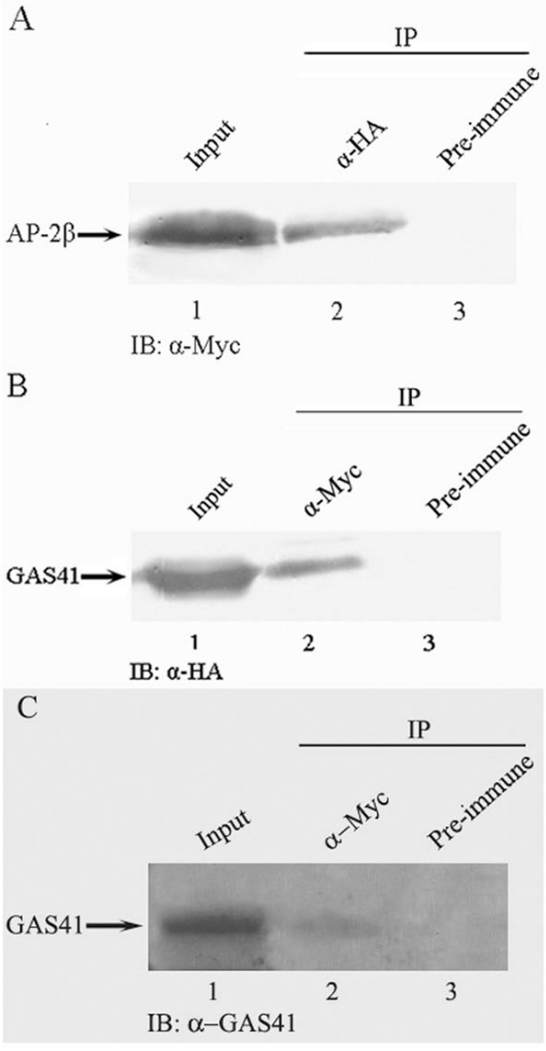

Figure 1. Interaction of AP-2β with GAS41 in HeLa cells. (**A**) Western blot analysis with mouse monoclonal anti-Myc antibody of HA-GAS41 precipitated protein from 300 μg of protein extract of transfected HeLa cells (lane 2). A total of 30 μg of protein extract was used as positive control (lane 1). Immunoprecipitates with rabbit preimmune serum were used as negative control (lane 3). (**B**) Western blot analysis with rabbit polyclonal anti-HA antibody of Myc-AP-2β precipitated protein (lane 2). A total of 30 μg of protein extract was used as positive control (lane 1). Immunoprecipitates with mouse preimmune serum were used as negative control (lane 3). (**C**) Western blot analysis with rabbit polyclonal anti-GAS41 antibody of Myc-AP-2β precipitated protein from 2 mg of nuclear protein extract of HeLa cells (lane 2). A total of 100 μg of nuclear protein extract was used as positive control (lane 1). Immunoprecipitates with mouse preimmune serum were used as negative control (lane 3). IP, Immunoprecipitation. IB, Immunoblot.

As shown in Figure 1B, GAS41 could also be precipitated by Myc tagged AP-2β (Figure 1B, lane 2) but not by control mouse IgG (Figure 1B, lane 3). Furthermore, HeLa cells were only transfected with pCMV-Myc-AP-2β. Endogenous GAS41 in HeLa cells could be co-immunoprecipitated with Myc tagged AP-2β (Figure 1C, lane 2) but not by control mouse IgG (Figure 1C, lane 3). These results indicated that AP-2β and GAS41 could be found in the same complex in mammalian cells, and GAS41 may directly or indirectly interact with AP-2β.

AP-2β colocalizes with GAS41 in HeLa cell nuclei

Because of the tight association found in immunoprecipitation experiments, we next investigated whether these proteins were present in the same region in cells. The immunofluorescent assays were performed as described in Materials and Methods. The images obtained with confocal laser scanning microscope revealed that both Myc-tagged AP-2β (Figure 2B) and HA-tagged GAS41 (Figure 2C) were localized in the nuclei of cells. After overlay, co-localized signals (yellow) were clearly observed (Figure 2D). These results are consistent with the presence of GAS41 and AP-2β in same complex *in vivo* but do not address whether both proteins directly interact.

AP-2β and GAS41 interact directly *in vitro*

It is possible that GAS41-AP-2β interaction may be indirect because other protein factors in the whole cell extract may be involved in mediating the interaction, e.g. acting as 'bridging' factors. Therefore we next decided to examine a possible direct interaction between the two proteins using GST pull-down assays. GST, GST fusion proteins and His fusion proteins were expressed and purified. Figure 3A showed the bacterially expressed and purified proteins. Figure 3B showed that GAS41 could be pulled-down by GST fused AP-2β (Figure 3B, lane 2) but not by GST alone (Figure 3B, lane 3), indicating that GAS41 and AP-2β specifically interact directly *in vitro*. We next decided to map the interaction domains between AP-2β and GAS41 using the same assay. The truncated proteins of AP-2β (Figure 3C and E) were used with His-GAS41 in pull-down experiments (Figure 3D and F). AP-2β P62R with the PY motif mutation that causes Char Syndrome (40) and AP-2βΔN233 efficiently pulled down GAS41 (Figure 3D and F, lanes 4 and 5), whereas other truncations of AP-2β pulled down little or none of GAS41 ((Figure 3D and F). The results obtained indicated that domain of AP-2β interacting with GAS41 is located in the C-terminus.

The domain of GAS41 interacting with AP-2β was also mapped by same assay. As shown in Figure 4A, GST-AP-2β pulled down His-GAS41ΔN162 (Figure 4A, lane 4), but not His-GAS41ΔC96 (Figure 4A, lane 2). The His-GAS41ΔN162 contains C-terminal 65 amino acid residues (amino acids 163–227) of GAS41, indicating that the domain of GAS41 interacting with AP-2β is located in C-terminus. We next examined the ability of different fragments of GAS41 to interact with AP-2β. A further C-terminal deletion that deleted residues 215–227 did not affect the interaction with AP-2β (Figure 4B, lane 7). But deletions of C-terminal residues 203–227 and 193–227 abrogated the interaction (Figure 4B, lanes 8 and 9). And the further deletion of the C-terminal residues 212–227 and 208–227 also affected the interaction (Figure 4C, lanes 6–9). These results suggest lysine at position 212, Asparagine at position 213, Glutamic acid at position 214 or three are possibly critical for the interaction. Moreover, the three point mutations of GAS41, respectively, significantly reduced the interaction with AP-2β confirmed by the GST pull-down assay (Figure 4D) and co-immunoprecipitation (Figure 4E). However, GAS41 containing the three point mutations did not interact with AP-2β, (Figure 4E, lane 2). Therefore, the three residues of GAS41 have played an important role in the interaction, but we haven't found any change in their co-localization (data not shown).

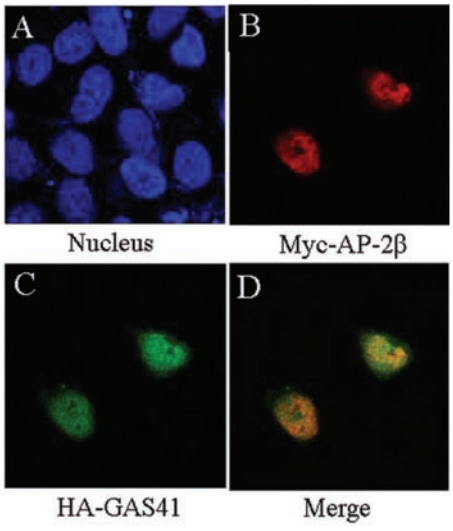

Figure 2. Colocalization of AP-2β and GAS41 in human HeLa cell by immunofluorescence. (**A**) Nuclear staining of HeLa cells by Hoechst 33258. (**B**) Nuclear localization of Myc-AP-2β, detected with mouse monoclonal anti-Myc antibody and Alexa 594 conjugated goat anti-mouse secondary antibody. (**C**) Nuclear localization of HA-GAS41, detected with rabbit polyclonal anti-HA antibody and Alexa 488 goat anti-Rabbit secondary antibody. (**D**) Overlay of images in (B) and (C), showing co-localization (yellow) of two proteins.

GAS41 stimulates activation of transcription by AP-2β

To investigate the physiological relevance of the AP-2β-GAS41 interaction, we asked whether GAS41 could modulate AP-2β transcriptional activity. The A2-luc reporter construct was transfected into HepG2 cells either alone or together with AP-2β expression vector pCMV-Myc-AP-2β and/or GAS41 expression vector pCMV-HA-GAS41 as well as the mutants of GAS41 as indicated in the Figure 5. GAS41 alone hardly stimulated the luciferase expression (Figure 5, lane 2) in HepG2 cells which lack the expression of AP-2 proteins. Transfection of AP-2β significantly stimulated the luciferase activity (Figure 5, lane 3). The addition of GAS41 enhanced AP-2β activity (Figure 5, lane 4). Furthermore, notably, these mutations reduced their ability in stimulating AP-2β activity (Figure 5, lanes 5–8). Taken together, these results suggested that GAS41 can function as co-activator of AP-2β.

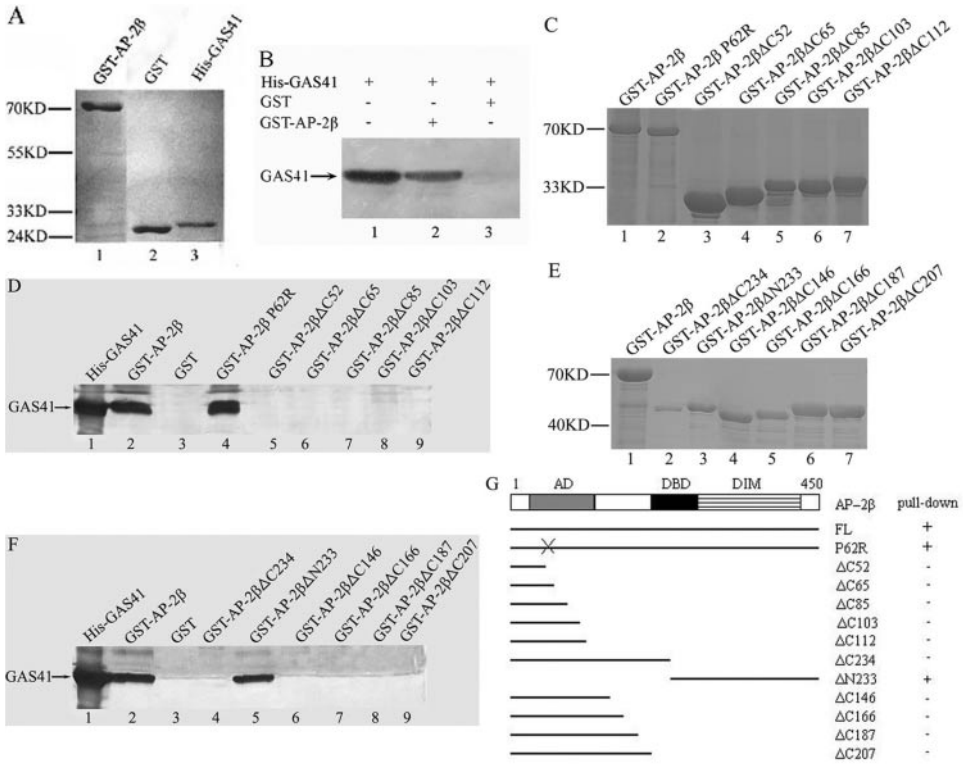

Figure 3. Identification of GAS41 binding domain in AP-2β. (**A**) Expressed and purified AP-2β fusion proteins run on a 10% SDS–polyacrylamide gel. (**B**) Western blot analysis with anti-His antibody of His-GAS41 protein pulled down with GST-AP-2β (lane 2) and GST alone (lane 3). A total of 5 μg of His-GAS41 was used as a positive control (lane 1). (**C**) The truncated proteins of GST-AP-2β were expressed, purified and run on a 10% SDS–polyacrylamide gel. (**D**) The proteins purified above were used in pull-down experiments with His-GAS41. Lane 1 is a positive control. Only lanes 2 and 4 had a pull-down of His-GAS41, whereas other lanes had none. (**E**) Expressed and purified subdomains of GST-AP-2β. (**F**) The proteins expressed from (E) were used to pull down His-GAS41, respectively. Lane 1 is a positive control using His-GAS41. Lanes 2 and 5 contained pull-downs. Other lanes had little or none of pull-downs. (**G**) Linear diagram showing domain structure of GST-AP-2β and summary of the interactions between GST-AP-2β and His-GAS41 detected by pull-down assays. + indicates the interaction between GST-AP-2β and His-GAS41, – indicates the lack of interactions between the two proteins. The AP-2β domains in the schematic cartoon are indicated as follows: AD, activation domain; DBD, DNA binding domain; DIM, dimerization domain.

GAS41 enhances binding of AP-2β to DNA

To elucidate the mechanism for the enhanced transcription activity of AP-2β by GAS41, we then examined whether GAS41 affected the formation of AP-2β–DNA complexes by the EMSA experiment. First, we documented the DNA-binding specificity, 10- and 50-fold amounts of unlabeled AP-2, Sp1 or SV40 oligonucleotide were added in competition. Cold AP-2 oligonucleotide significantly competed away the signal, whereas cold Sp-1 or SV40 oligonucleotide did not reduce the intensity of the band (Figure 6A). The result documented that AP-2β binds the consensus AP-2 site with specificity.

As shown in Figure 6B, His-GAS41 significantly enhanced the DNA-binding activity of GST-AP-2β (lanes 5–7), comparing with BSA (lanes 2 and 3). The stimulatory effect of His-GAS41 was dose dependent. GAS41 alone incubated with the probe did not produce a band (lane 4). Furthermore, the point mutants of GAS41 markedly reduced the enhancement for DNA-binding activity of GST-AP-2β (lanes 9–11) compared to the same amount of wild-type GAS41 (lane 7), whereas the mutant GAS41 that is not capable of interacting with AP-2β did not stimulate the DNA-binding activity of GST-AP-2β (lane 8). Taken together, our result suggests GAS41 enhances the transcriptional activity by a mechanism that appears to involve an enhancement in the formation of AP-2β–DNA complex.

DISCUSSION

We reported here the interaction between transcription factor AP-2β and GAS41, which resulted in the enhancement of transcriptional activity of AP-2β. The stimulating effect of GAS41 to AP-2β was, at least in part, due to the enhancement of AP-2β to bind to its specific DNA-binding site. In the EMSA assay, GAS41 appears to enhance the binding of AP-2β to DNA without affecting the rate of migration of this complex. There may be a transient interaction between GAS41 and AP-2β in which GAS41 induces conformational change of AP-2β, favoring its DNA-binding. After AP-2β bound to its DNA, GAS41 leaves without forming a ternary complex. Such 'hit and run' mechanism has been demonstrated for the effects of Miz 1 over the DNA-binding of transcription factor Msx2 (41) and oncogene DEK over AP-2α (35). In both transient transfection and EMSA assays, the GAS41 protein with mutation of three critical amino acid residues that does not interact with AP-2β lost its stimulating

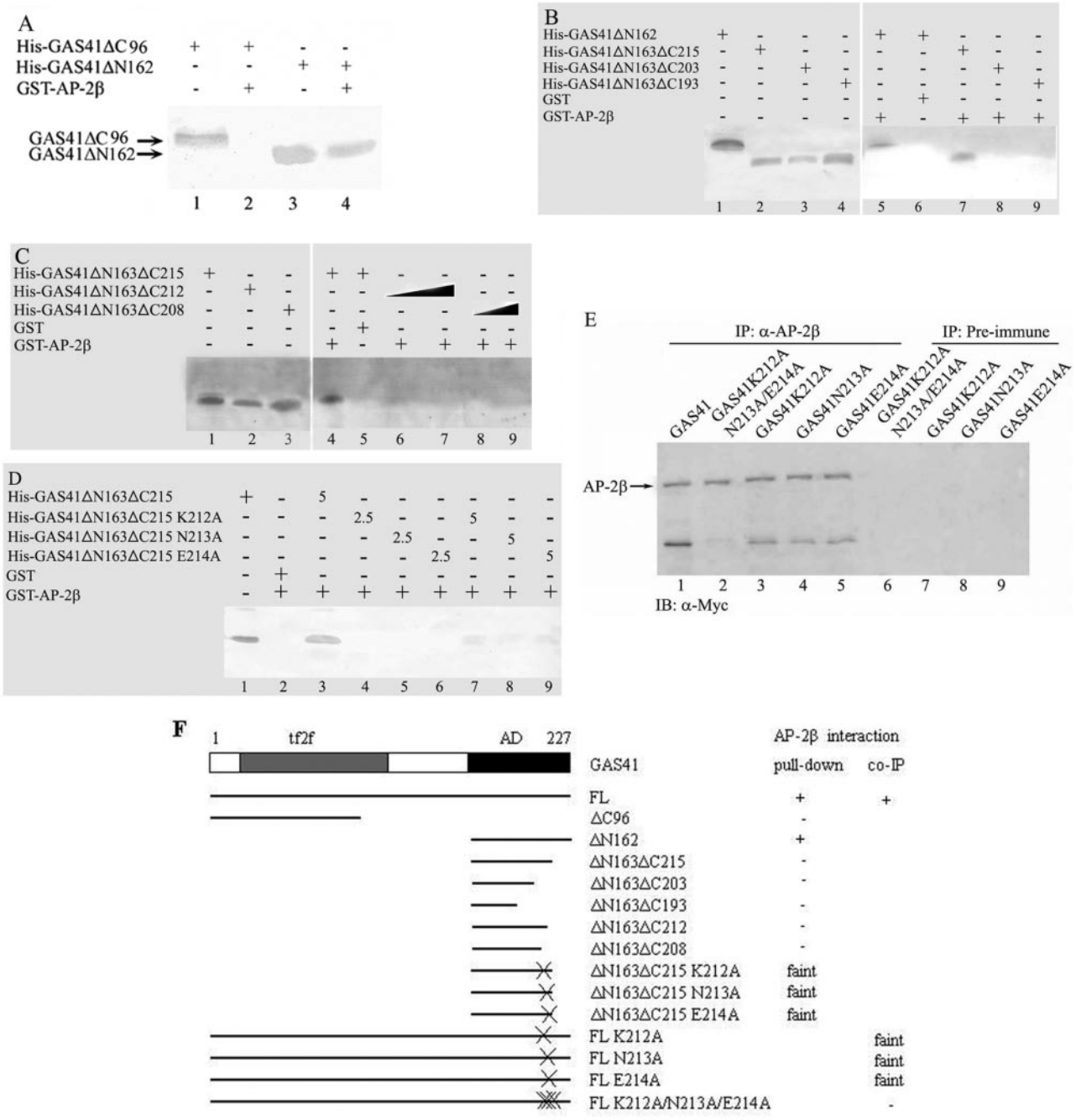

Figure 4. Identification of AP-2β binding domain in GAS41. (**A**) His-GAS41ΔC96 (lane 2) and His-GAS41ΔN162 (lane 4) pulled down by GST-AP-2β were analyzed by anti-His antibody immunoblot. His-GAS41ΔC96 (lane 1) or His-GAS41ΔN162 (lane 3) was used as positive controls. (**B**) Pull-down experiments were performed with AP-2β and purified truncated proteins of GAS41 from lanes 1–4. (**C**) The same pull-down experiments were performed as Figure 4B. Lane 5 is a control experiment in which GST alone was used. Increasing the input amount of GAS41 deletions didn't lead to any pull-downs (lanes 6–9). (**D**) Three point mutants of GAS41 were used in pull-down assays with AP-2β. A total of 2.5 μg of point mutants of GAS41 used were not pulled down by AP-2β (lanes 4–6), while 5 μg only detected weak bands (lanes 7–9). (**E**) Western blot was performed using cell extracts with transfected plasmids expressing the proteins indicated and reprobed with anti-Myc antibody to detect AP-2β and mutants of GAS41. Lanes 2, 3, 4 and 5 contained proteins immunoprecipitated by AP-2β antibodies, whereas other lanes were immunoprecipitated by preimmune IgG. (**F**) Schematic representation of GAS41 constructs used for pull-down and co-immunoprecipitation analysis. Faint indicates the weak interaction. AD, activation domain.

effect on AP-2β activity, whereas those with mutation of single amino acid residue that significantly reduced their abilities to interact with AP-2β also significantly reduced their stimulating activity over AP-2β (Figures 4–6), suggesting that the interaction between GAS41 and AP-2β is essential for the physiological relevance of these two proteins. To the best of our knowledge, this is the first report that suggests a role for GAS41 as a co-activator of a sequence-specific transcription factor by directly interacting with the transcription factor AP-2β. In addition, GAS41

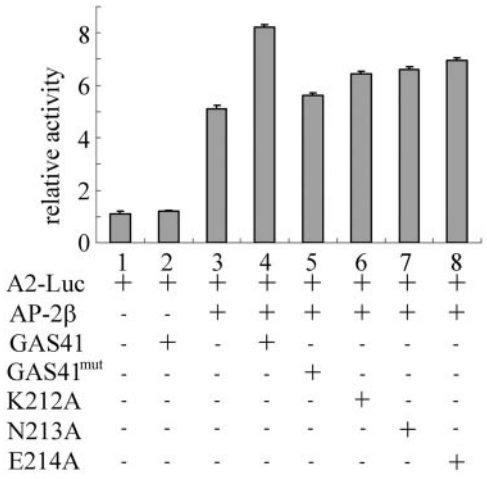

Figure 5. Effects of GAS41 on transcriptional activity of AP-2β in HepG2 cells. HepG2 cells were transfected with 0.5 μg of luciferase reporter A2 Luc vector alone (lane 1) or with 0.3 μg of GAS41 expression vector pCMV-HA-GAS41 (lane 2) or with 0.3 μg of AP-2β expression vector pCMV-Myc-AP-2β (lane 3) or with both AP-2β expression vector and 0.3 μg of pCMV-HA-GAS41 (lane 4) or the mutations GAS41 (lanes 5–8). Relative activity of luciferase was presented as the mean ± SD of three independent transfection experiments performed in triplicate each treatment.

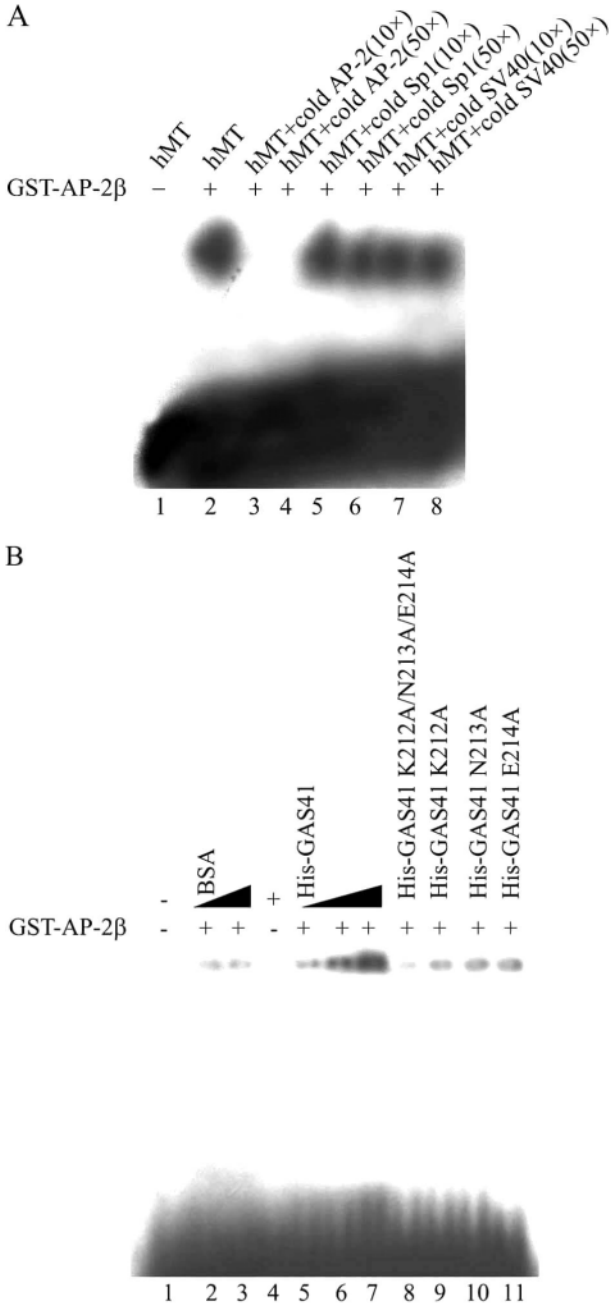

Figure 6. Effect of GAS41 on binding of AP-2β to DNA *in vitro*. (**A**) The fusion protein GST-AP-2β was incubated with ^{32}P-labeled hMT sequences, including competition assay with 10- or 50-fold unlabeled oligos containing AP-2 or Sp1 binding site, or SV40 oligo. (**B**) The probe was radioactively labeled and incubated with 30 ng of AP-2β in the present of 30 (lane 5), 100 (lane 6) or 200 ng of GAS41 (lane 7) and the GAS41mutants (lanes 8–11). As negative controls, the probe was incubated with AP-2β in the presence of 30 (lane 2) or 200 ng (lane 3) of BSA. GAS41 alone (lane 4) were also incubated with the probe in the absence of AP-2β. The intense bands at the bottom were unbound oligonucleotides.

also stimulated the transcriptional activity of endogenous AP-2 family members in COS7, MCF-7 and NIH3T3 cells (data not shown).

GAS41 gene was originally identified as an amplified sequence in the chromosome region 12q13–15, a region known to be involved in gene amplification in human gliomas (39). GAS41 amplification was detected in 23% of glioblastomas and 80% of grade I astrocytomas, suggesting gene amplification can occur not only in late tumor progression but also in early tumor development (39,42). Sequence analysis and comparison of GAS41 and known protein sequences revealed a high similarity between GAS41 and human AF-9 and ENL proteins (43). This finding is intriguing since AF-9 and ENL genes are frequently involved in translocation events in leukemia, in particular, AF-9 being found fused to the ALL-gene with t(9:11) translocations and ENL being found fused to the ALL-1 gene with t(11:19) translocations (44), leaving the question open whether GAS41 also plays roles in leukemia.

GAS41 is a highly conserved protein with homologous found in invertebrates, vertebrates, plants and fungi (43). It is probably one of most highly conserved proteins during evolution with the degree of homology between human and *Drosophila* proteins of 61% identity and 70% overall similarity (43), suggesting GAS41 may play an essential role during biological evolution. A number of proteins involved in nuclear matrix formation, chromatin remodeling, nuclear scaffolding or mitotic spindle assembly have been shown to interact with GAS41, those including NuMA (43), TACC1 (37) and AF10 (45). It is possible that by associating with those factors, GAS41 can produce a change in chromatin conformation in such a way that enhances the DNA-binding of transcription factor, such as AP-2β *in vivo*. Recent studies have also been shown that targeted disruption of GAS41 in chicken pre-lymphoid cells results in cell death, indicating that it is essential for cell viability. It has been further demonstrated that depletion of GAS41 causes a significant decrease in RNA synthesis and subsequently cell death, suggesting a role of GAS41 in gene transcription (46). The gene transcription in eukaryotes is a quite complicated process which has not been fully understood. However, the

transcription of a specific gene is generally processed by: (i) remodeling of chromatin to facilitate the binding of subsequent factor to nucleosomal DNA, involving the participation of SWI–SNF complex or other homologous multiprotein complexes with similar chromatin-remodeling activities, (ii) formation of transcription initiation complex involving recruiting of general transcription factors (TFIIA, TFIIB, TFIID, TFIIF, etc.) and RNA polymerase II to the transcription initiation sites of specific gene, (iii) the interaction of sequence-specific transcription factors with the basal transcriptional initiation complex to enhance or repress the transcription rate. GAS41 seems to be a mediator among these three steps. GAS41 appears to be a human homologue of yeast ANC1, a protein known to be an integral member of two basal transcription factor complexes, TFIID and TFIIF. ANCI binds to the SWI–SNF chromatin-remodeling complex through its interaction with SNF5, a component of SWI–SNF complex (47). Homologues of SNF5 have been isolated in both human and *Drosophila*, named INT1 and Snr1, respectively. They have been shown to be component in large complexes equivalent to the yeast SWI–SNF5 complex (48,49). It has been shown that GAS41 can interact with INI1 (45), which bridges the chromatin-remodeling complex and the basal transcription complex. As a component of basal transcription complex, GAS41 has been proposed to regulate gene transcription by directly associating with the sequence-specific transcription factor. However, previous to this report, GAS41 has not been shown to bind directly to any known transcription factor. So, it is proposed that an additional protein may be required for GAS41 to bind to sequence-specific transcription factor. Our finding fulfils such gap, indicating that GAS41 can bind to DNA-sequence-specific transcription factor, such as AP-2β. It is possible that, as a component of basal transcription complex, one of the roles of GAS41 is to act as a recruiting protein for certain sequence-specific transcription factors *in vivo*. Taken together, it is attemptable to speculate that the general role of GAS41 is to function as a mediator which bridges the chromatin-remodeling complex, basal transcription complex and DNA-sequence-specific transcription factor to facilitate the efficient gene transcription, and deregulation of GAS41 transcription would result in diseases, such as gliomas and leukemia.

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