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Activator protein 2 α suppresses intestinal tumorigenesis in the *Apc^{min}* mouse

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Abstract

Activator protein 2 α (AP-2 α) is a putative tumor suppressor, and various reports have described the loss or reduction of AP-2 α expression in cutaneous malignant melanomas, as well as in cancers of the prostate, breast and colon. Previously, AP-2 α was shown to attenuate β -catenin/T-cell factor-4 (TCF-4) nuclear interactions and β -catenin/TCF-4-dependent transcriptional activity in human colorectal cancer cells [Q. Li, R.H. Dashwood, Activator protein 2 α associates with adenomatous polyposis coli/beta-catenin and Inhibits beta-catenin/T-cell factor transcriptional activity in colorectal cancer cells, *J. Biol. Chem.* 279 (2004) 45669–45675]. Here, we show that *in vivo* gene delivery of AP-2 α suppressed intestinal polyp formation in the *Apc^{min}* mouse, and protected against the development of anemia and splenomegaly. Immunoblot analyses and immunohistochemistry following gene delivery revealed an increase in AP-2 α expression in the mouse intestinal mucosa and liver. Co-immunoprecipitation experiments provided evidence for interactions between AP-2 α , β -catenin, and adenomatous polyposis coli (APC) proteins in mouse intestinal mucosa, as well as in a primary human colorectal cancer. Collectively, these studies support a tumor suppressor role for AP-2 α in the gastrointestinal tract, and suggest that AP-2 α represents a novel target for therapeutic intervention in human cancers characterized by dysregulated Wnt signaling.

Keywords

AP-2 α ; APC; β -Catenin; Wnt signaling; Cancer therapeutics

1. Introduction

AP-2 α belongs to the AP-2 family of transcription factors, which are critical regulators of gene expression during vertebrate development, embryogenesis, and transformation [1–3].

AP-2 $\alpha^{-/-}$ mice die perinatally with cranio-abdominoschisis and severe dismorphogenesis of the face, skull, sensory organs, and cranial ganglia [4]. In various human cancer cell lines, AP-2 α over-expression is associated with inhibition of cell growth and induction of cell cycle arrest/apoptosis [5]. Reduction or loss of AP-2 α expression has been observed in cutaneous

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malignant melanoma and cancers of the breast, colon, and prostate [6–9], and AP-2 α expression predicted survival outcomes in ovarian cancer patients [10].

We previously reported the novel finding that AP-2 α forms a complex with adenomatous polyposis coli (APC)/ β -catenin, attenuates β -catenin/T-cell factor-4 (TCF-4) nuclear interactions, and inhibits β -catenin-dependent reporter activity in human colorectal cancer cells [11]. This suggested that AP-2 α has the potential to interfere with β -catenin/TCF transcriptional activity, and that AP-2 α might serve as a novel therapeutic target in cancers with dysregulated Wnt signaling [12]. To test this hypothesis *in vivo*, we used a gene delivery system for AP-2 α in the *Apc*^{min} mouse. This animal model is genetically predisposed to tumor formation in the gastrointestinal tract due to impaired function of the APC protein, resulting from a mutation that introduces a premature translational stop codon at amino acid 850 in one APC allele [13]. The results of the present investigation showed that over-expression of AP-2 α caused a highly significant suppression of spontaneous intestinal polyps, with evidence for AP-2 α /APC/ β -catenin interactions *in vivo*.

2. Materials and methods

2.1. Preparation of plasmids

pcDNA3.1-AP-2 α was generated by subcloning mouse cDNA-encoding full-length AP-2 α into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) between *Hind*III and *Eco*RV. APC fragments were generated from pCMV/APC using standard PCR-based methods, and PCR products were cloned into pcDNA3.1(+) [11]. Plasmids were isolated using QIAGEN QIA filter Plasmid Maxi Kit (QIAGEN, Valencia, CA), with endotoxin removal using the MiraCLEAN® Endotoxin Removal Kit (Mirus, Madison, WI).

2.2. GST pull-down assays

GST pull-down was performed as described previously [11]. GST- β -catenin fusion protein was expressed in the BL21 strain of *Escherichia coli* and purified on glutathione–Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ). [³⁵S]-labeled AP-2 α and fragments of APC were prepared using the TNT® quick coupled transcription/translation system (Promega, Madison, WI).

2.3. In vivo gene delivery

Male C57BL/6 J-*Apc*^{min/+} (*Apc*^{min}) mice and C7BL/6 J^{+/+} (wild type) mice were obtained from the Jackson laboratory (Bar Harbor, Maine). pcDNA3.1-AP-2 α was delivered by tail vein injection using the *TransIT*® *In Vivo* Gene Delivery System (Mirus Bio Corporation, Madison, WI). Immediately before injection, *TransIT In Vivo* Polymer Solution and endotoxin-free sterile water were mixed with pcDNA3.1-AP-2 α , or with empty pcDNA3.1(+) as vector control, according to the manufacturer's protocol. In a pilot immunohistochemistry study, mice were injected with a construct expressing myc-tag-His-AP-2 α . The first injection (10 μ g plasmid) was performed on mice at 7 weeks of age. In a follow-up study with intestinal polyps as the end-point, 3–4 mice per treatment were injected with vector or AP-2 α expression construct, and the injections were repeated every other week for 2, 3, 4, or 5 five injections (i.e., 20, 30, 40, or 50 μ g plasmid total). Twenty-four hours after the last injection, mice were sacrificed using an overdose of CO₂, blood was drawn by cardiac puncture, and the hematocrit was recorded. The intestinal tract was divided into five sections (duodenum, jejunum, upper ileum, lower ileum, and colon), opened longitudinally, and examined by individuals blinded to the treatment status of the animal. A record was kept of the number and location of all polyps. Other tissues were weighed and snap-frozen in liquid nitrogen. All procedures were approved by the Institutional Animal Care and Use Committee.

2.4. Immunoblotting and co-immunoprecipitation

Immunoblotting and co-immunoprecipitation experiments were performed as before [11]. Whole cell lysates were prepared from human and mouse tissues by homogenizing each sample in ice-cold lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerol phosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g/ml}$ leupeptin) and centrifuging at 4 °C, 15,000 rpm, for 15 min. Proteins were separated on 4–12% bis–tris gels (Novex, San Diego, CA) or on 3–8% Tris–acetate gels (for APC) and transferred to nitrocellulose membranes, followed by immunoblotting with primary antibody and horseradish peroxidase-conjugated secondary antibody. Primary antibodies were rabbit polyclonal anti-AP-2 α (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti- β -catenin (Transduction Labs, Lexington, KY), rabbit polyclonal anti-Cyclin D1 (Neomarker, Fremont, CA), and mouse monoclonal anti- β -actin (Sigma–Aldrich, St. Louis, MO). Image analysis and quantitation was performed on an AlphaImager™ 2200 system (AlphaInnotech Corporation, San Leandro, CA).

2.5. Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens of liver and small intestine were sectioned at 4–5 μm and placed on Microprobe slides (Fisher Scientific, Pittsburgh, PA). Following rehydration, slides underwent high temperature antigen retrieval using citrate buffer (pH 6.0; DakoCytomation, Carpinteria, CA) for 10 min in a microwave pressure cooker (Tendercooker) and placed at room temperature for 20 min. Slides were washed in Automation buffer (Biomeda, Foster City, CA) followed by blocking in 3% H_2O_2 in methanol for 10 min. Immunohistochemical staining was performed as reported before [14]. The primary antibody was rabbit polyclonal anti-AP-2 α (Santa Cruz Biotechnology), used at a dilution of 1:1000 and applied for 30 min at room temperature. Universal Negative Control Rabbit (DakoCytomation) was used as a negative control.

2.6. Histopathology and cytology

Formalin-fixed, paraffin-embedded specimens of liver, small intestine, and spleen were sectioned at 4–5 μm and stained with Hematoxylin and Eosin. Cytology (paint-brush) preparations of bone marrow were stained with Wrights-Giemsa. All slides were examined by a board certified anatomic pathologist, blinded to the treatment groups.

2.7. Statistical analysis

Results were expressed as mean $\pm$ SD. Data for test *versus* control was compared using Student's *t*-test. As shown in the figures, results were considered as statistically significant at the * $P < 0.05$ or ** $P < 0.01$ level of significance, respectively.

3. Results

3.1. AP-2 α /APC/ β -catenin complex formation requires full-length APC

We demonstrated previously [11] that in human colorectal cancer cells the Basic region of AP-2 α binds to the heptad and armadillo repeats of APC (Fig. 1A). To confirm that APC is an intermediate between AP-2 α and β -catenin, we first attempted to pull-down ^{35}S -labeled AP-2 α using GST-tagged β -catenin, in the presence and absence of full-length APC or APC fragments. AP-2 α was pulled-down by GST- β -catenin in the presence of full-length APC (Fig. 1B), but not by AP-2 α alone, indicating that APC is essential for AP-2 α /APC/ β -catenin complex formation. We generated APC fragments containing the N-terminal, middle, and C-terminal portions of the APC protein, and designated these as nAPC, mAPC and cAPC, respectively (Fig. 1A). nAPC represents the AP-2 α binding region containing the Heptad and Armadillo (Arm) repeats, mAPC has the 15-amino acid (15-aa) and 20-aa repeats associated

with β -catenin binding, and cAPC contains the Basic region that binds neither AP-2 α nor β -catenin. β -Catenin failed to pull-down AP-2 α in the presence of any of these APC fragments (Fig. 1B). Thus, the ability of AP-2 α to sequester β -catenin away from TCF is APC-dependent, and requires full-length APC for a functional AP-2 α /APC/ β -catenin complex.

3.2. AP-2 α suppresses tumor formation *in vivo*

To test the tumor suppressor function of AP-2 α *in vivo*, we used a gene delivery system in a mouse model of dysregulated Wnt signaling [13]. Thus, AP-2 α was introduced into *Apc*^{min} and wild type mice via tail vein injection, using a nonviral approach for gene delivery. The basic methodology was reported before [15–17], including as means of expressing human APC in *Apc*^{min} mice [18]. The commercial *in vivo* gene delivery system used here was designed primarily for distribution to the liver, but we sought to test whether it might target other organs, such as the small intestine.

We first introduced myc-His-AP-2 α into *Apc*^{min} mice to demonstrate that exogenous AP-2 α can be over-expressed *in vivo*. By design, this short duration pilot study did not include polyps as an end-point. Immunoblotting with anti-myc-tag antibody revealed strong expression levels of myc-His-AP-2 α in the livers of mice injected with pcDNA3.1-myc-His-AP-2 α (Fig. 2A). As expected, no exogenous AP-2 α was observed in the vector controls (Fig. 2A). Immunohistochemical staining revealed that, following gene delivery, AP-2 α was over-expressed in the cytoplasm and/or nuclei of hepatocytes (Fig. 2B). Rather than uniform immunostaining, AP-2 α was strongly expressed in individual cells throughout the liver. No immunostaining was detected in the liver of vector controls (Fig. 2C). Similar observations were made in the small intestine; rather than uniform immunostaining, individual epithelial cells along the villus expressed high levels of AP-2 α after gene delivery (Fig. 2D). This pattern was only obvious at high magnification in the mice injected with AP-2 α expressing construct, and immunostaining was weak to undetectable in the villi of vector controls (Fig. 2E).

In vivo gene delivery had no obvious deleterious effect 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. Collectively, these findings provided initial confirmation that the methodology was a quick, simple approach for *in vivo* delivery of AP-2 α .

In subsequent experiments, *Apc*^{min} or wild type mice were injected with pcDNA3.1-AP-2 α expression construct, or with pcDNA3.1(+) as the vector control, and intestinal polyps were assessed as in our prior studies [19–21]. For both wild type and *Apc*^{min} mice, there were no deleterious effects of the treatment regimen on organ weights or final body weights, and no macroscopically visible tumors were observed in any of the wild type mice (data not presented).

In *Apc*^{min} mice, however, there was an inverse association between final tumor multiplicity and AP-2 α dose administered (Fig. 3A). Maximal tumor suppression was attained after three injections (30 μ g plasmid), and one or two additional injections had no further inhibitory effect. Given the similar outcomes for mice injected with 30, 40, or 50 μ g plasmid (Fig. 3A), the data were combined and compared with controls given vector alone. As shown in Fig. 3B, AP-2 α treatment resulted in a highly significant suppression of tumor multiplicity; vector controls had an average of 50.2 ± 11.2 polyps, whereas animals injected with the AP-2 α expressing construct had 23.0 ± 2.0 polyps ($P < 0.01$). The distribution of polyps along the gastrointestinal tract was as reported before [19–22], with the majority of tumors occurring in the ileum (Fig. 3C). AP-2 α treatment suppressed polyps most effectively in the upper and lower ileum ($P = 0.005$ and 0.046 , respectively). Polyps also appeared to be slightly smaller in size for mice injected with the AP-2 α expressing construct compared with mice given vector alone (not shown).

3.3. AP-2 α protects against anemia and splenomegaly

In addition to spontaneous polyps throughout the gastrointestinal tract, the *Apc*^{min} mouse has a phenotype that includes development of anemia and splenomegaly. Previously, we observed an inverse association between the hematocrit (packed cell volume) and the multiplicity of polyps in *Apc*^{min} mice [19]. In the present study, hematocrits were 22.7 ± 3.07 and 53.6 ± 2.56 in vector-treated *Apc*^{min} and vector-treated wild type mice, respectively (open bars, Fig. 3D). AP-2 α treatment had no effect on hematocrit in wild type mice, but there was a significant improvement in *Apc*^{min} mice (37.8 ± 2.05 , $P = 0.001$ compared with *Apc*^{min} mice treated with vector). Spleens in *Apc*^{min} mice were grossly enlarged, with an average organ weight of 0.25 ± 0.045 g versus 0.063 ± 0.006 g for vector controls (open bars, Fig. 3E). AP-2 α treatment decreased the average spleen size in *Apc*^{min} mice to 0.13 ± 0.017 g ($P = 0.02$), and this reduction was evident by gross observation of spleens (Fig. 3F). Thus, AP-2 α protected against splenomegaly and anemia in the *Apc*^{min} mouse.

3.4. AP-2 α forms a complex with Apc/ β -catenin in vivo

Western blotting revealed low levels of AP-2 α in the ileum of vector-treated *Apc*^{min} mice, but high levels of AP-2 α were detected following *in vivo* gene delivery (Fig. 4A).

Immunohistochemistry studies of polyps revealed generalized, low-level expression of AP-2 α throughout the tumor tissue (data not presented). By immunoblotting, tumors typically had lower expression of AP-2 α than the adjacent normal-looking tissue, but this was not always the case (Fig. 4B). As reported before in *Apc*^{min} mice [19], β -catenin and the β -catenin/Tcf downstream target Cyclin D1 were highly over-expressed in tumors compared with adjacent normal-looking tissue (Fig. 4B).

Co-immunoprecipitation experiments with tissue lysates from the intestines of *Apc*^{min} mice established that full-length Apc was pulled-down by anti-AP-2 α and anti- β -catenin antibodies, whereas anti-APC and anti- β -catenin antibodies immunoprecipitated AP-2 α (Fig. 4C, left). We also provide initial evidence for AP-2 α /APC/ β -catenin interactions in a primary human colorectal cancer; β -catenin antibody immunoprecipitated AP-2 α , and AP-2 α antibody pulled down full-length APC (Fig. 4C, right).

4. Discussion

The main goal of this investigation was to extend the prior work which implicated AP-2 α as a possible tumor suppressor. Inasmuch as *in vivo* gene delivery of AP-2 α inhibited intestinal polyps in the *Apc*^{min} mouse, and protected against the development of anemia and splenomegaly, we can conclude that our findings are consistent with a tumor suppressor role for this transcription factor. With appropriate modification of the *in vivo* gene delivery protocol, such as shortening the duration between injections, it might be feasible to inhibit polyp formation to a greater extent than observed here, although this possibility awaits experimental verification.

Consistent with its proposed tumor suppressor function, AP-2 α expression tended to be lower in polyps than adjacent normal-looking tissue, although this was not universally true. By the time polyps arise in *Apc*^{min} mice the adjacent normal-looking tissue is scarcely “normal”, and microadenomas or other precursor lesions might conceivably affect AP-2 α levels. Moreover, immunoblotting and immunohistochemical studies might not provide the complete picture. In human colorectal cancer cells transfected with exogenous AP-2 α , immunoblotting of nuclear extracts revealed high levels of AP-2 α without changes in β -catenin or TCF-4; however, co-immunoprecipitation experiments revealed that β -catenin/TCF-4 nuclear interactions were attenuated, whereas AP-2 α - β -catenin/APC interactions had increased [11]. We extended these

observations and showed, for the first time *in vivo*, that AP-2 α /APC/ β -catenin interactions were detected in the intestinal mucosa of *Apc*^{min} mice and in a primary human colorectal cancer.

We postulate that early stages of colorectal cancer might benefit most from therapy targeted at AP-2 α , because the AP-2 α /APC/ β -catenin complex requires at least one full-length copy of APC (Fig. 1B), and in late-stage colon cancers loss of heterozygosity can delete both wild type APC alleles [23]. APC is known to shuttle in and out of the nucleus [24]; thus, it is possible that nuclear APC, along with AP-2 α , sequesters β -catenin away from TCF transcription factors as a means of controlling β -catenin/TCF signaling. Consistent with this paradigm, polyps with low levels of AP-2 α expression had high levels of Cyclin D1, a down-stream β -catenin/TCF target. However, it is premature to conclude from the present investigation that AP-2 α /APC/ β -catenin complex formation was the only (or even primary) mechanism for tumor suppression. For example, Cyclin D1 and other downstream β -catenin/TCF targets might be influenced by AP-2 α as a transcriptional regulator. Additional studies are warranted to clarify the precise mechanisms involved. The current preclinical model and *in vivo* gene delivery approach has advantages in terms of simplicity and speed, but it is not ideal in that AP-2 α was targeted primarily to liver. A conditional model targeting AP-2 α to the intestinal and/or colonic mucosa might provide more definitive insights into the role of AP-2 α as a tumor suppressor in the gastrointestinal tract.

In summary, we have shown for the first time that AP-2 α suppresses the development of intestinal tumors arising spontaneously in a mouse model of dysregulated Wnt signaling. The findings are consistent with studies in primary human colon cancer and cultured colorectal cancer cells indicating that AP-2 α associates with APC/ β -catenin, with the potential to disrupt β -catenin/TCF interactions. Further work is needed to corroborate whether this mechanism might provide a therapeutic avenue in clinical cases of colorectal cancer.

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Fig. 1. Full-length APC is required for AP-2 α /APC/ β -catenin complex formation: (A) scheme showing wild type APC and truncated APC proteins with different functional domains. *Positions of nuclear export signals and (B) ³⁵S-labeled AP-2 α as well as unlabeled APC fragments were synthesized *in vitro* and incubated with Sepharose-bound GST- β -catenin. Beads were washed, and bound ³⁵S-labeled protein was eluted, followed by SDS-PAGE and autoradiography. The presence (+) or absence (-) of GST- β -catenin, [³⁵S]AP-2 α , or APC fragments was as indicated above each lane.

Fig. 2.

AP-2α over-expression in mouse liver and intestine. *Apc*^{min} mice were injected with an AP-2α expression construct using an *in vivo* gene delivery system. (A) Myc-His-AP-2α was detected in total cell lysates of mouse liver by immunoblotting with anti-myc-tag antibody; β-actin, loading control. Representative liver (B, C) and ileum sections (D, E) immunostained for AP-2α. Arrows, cells magnified (inset) to show high AP-2α expression. IHC, immunohistochemistry.

Fig. 3.

AP-2α suppresses intestinal polyps in mice. *Apc*^{min} mice were injected with AP-2α, or with the corresponding vector control, using an *in vivo* gene delivery system: (A) inverse association between tumor multiplicity and AP-2α plasmid dose; (B) suppression of tumor multiplicity by AP-2α in *Apc*^{min} mice; (C) fewer polyps developed in the ileum of *Apc*^{min} mice injected with AP-2α; (D) in *Apc*^{min} mice, AP-2α protects against severe anemia, as indicated by the packed red cell volume (hematocrit) and (E, F) splenomegaly in *Apc*^{min} mice is attenuated by AP-2α. In A–D, data = mean ± SD; *P < 0.05; **P < 0.01. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

Fig. 4. AP-2α interacts with APC and β-catenin *in vivo*: (A) immunoblotting for AP-2α in *Apc*^{min} mouse small intestine lysates; β-actin, loading control; PC; positive control for AP-2α (cell lysate from human embryonic kidney 293 cells). The blot shows data for three mice injected with AP-2α and three vector controls, and are representative of findings from six mice in each group. Adjacent bar graph shows the densitometry data, mean ± SD, *n* = 6; **P < 0.01; (B) immunoblot analyses of AP-2α, β-catenin, and Cyclin D1 protein expression in tumors (T) and adjacent normal-looking (N) intestinal mucosa; β-actin, loading control and (C) immunoprecipitation (IP) was performed with the antibodies indicated, followed by immunoblotting (IB) with anti-Apc and anti-AP-2α antibodies, in lysates obtained from

Apc^{min} mouse small intestine (left). In a primary human colorectal cancer (right), IP was performed with β catenin antibody followed by IB for AP-2 α and β -catenin, or alternatively IP was performed with antibody to AP-2 α followed by IB for full-length APC. No antibody (no Ab) and HA-tag antibody served as IP controls.