Fra-1: a novel target for retinoid action

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Abstract Using a cDNA gene expression array system, we identified fra-1 as a novel target molecule for retinoid action in the human pancreatic carcinoma cell line Dan-G. Retinoid treatment resulted in a significant and time-dependent induction of the fra-1 expression on the post-transcriptional level. Supershift assays revealed that fra-1 participates in the activator protein 1 complex together with c-fos, c-jun and junB. Transient transfection experiments using a reporter plasmid containing an activator protein site upstream of the CAT reporter gene revealed that the phorbolester-induced CAT activity was suppressed by retinoids. Since fra-1 lacks a transactivation function, we therefore suggest that the retinoid-mediated induction of fra-1 might function as a negative regulator of the activator protein 1 activity in human pancreatic carcinoma cells.

Key words: Fra-1; Retinoic acid; Pancreas; Activator protein 1

1. Introduction

Retinoic acid (RA) modulates the cellular proliferation and differentiation in a variety of tissues in the adult organism as well as during the embryogenesis and development [1]. These pleiotropic effects of RA are transduced by nuclear RA receptors (RAR α, β, γ) and retinoid X receptors (RXR α, β, γ) which modulate gene expression through binding as ligand-activated heterodimers to RA responsive elements (RARE), located in the regulatory regions of target genes [2]. The anti-proliferative effects and the differentiating action of retinoids are thought to be mainly mediated through induction or repression of RA responsive genes. A number of RA-regulated genes have been characterized but attempts to identify genes involved in the anti-proliferative effects have so far been unsuccessful in the majority of cell types and tissues [3,4].

We have previously demonstrated that retinoids inhibit growth and induce cellular differentiation in a broad panel of human pancreatic carcinoma cells in vitro and in vivo [5–7]. We were therefore interested to identify novel retinoid-regulated genes in order to delineate the molecular targets which are responsible for the anti-proliferative effects of retinoids in pancreatic cancer cells. We used the Atlas cDNA Expression Arrays to identify differentially regulated genes in the human pancreatic carcinoma cell line Dan-G. Here, we present fra-1, a member of the fos protein family, as a novel target molecule for retinoid action. Furthermore, we provide evidence that retinoids might act as a negative regulator of activator protein 1 (AP-1) action through induction of fra-1 expression.

2. Materials and methods

2.1. Analysis of differential gene expression

To identify retinoid-mediated differences in gene expression, we used the AtlasTM cDNA Expression Arrays (Clontech, Palo Alto, CA, USA) according to the instructions of the manufacturer. Two nucleic acid arrays include 588 human cDNAs spotted as duplicates on a nylon membrane including several housekeeping genes as well as plasmid DNAs to control for the hybridization intensity and specificity. PolyA$^+$ RNA was isolated from DAN-G cells treated for 4 h either with all-*trans* RA or vehicle (0.1% DMSO). After RNase-free DNase I digestion, RNA was reverse-transcribed using the reagents provided and [α-^{32}P]dATP. The radioactively-labelled cDNA probes were separately hybridized overnight to the Atlas Arrays. After a high stringency wash, the hybridization pattern was analyzed by autoradiography.

2.2. RNA isolation and Northern blot

PolyA$^+$-enriched RNA was extracted from DAN-G cells using the PolyAT tract kit following the instructions of the supplier. For a quantitative analysis, RNA was analyzed in a 1% agarose-2% formaldehyde gel, blotted onto Hybond N$^+$ membranes (Amersham, Braunschweig, Germany) and immobilized by UV crosslinking. Hybridization was carried out using the full length human fra-1 cDNA, amplified by RT-PCR using specific 5′ and 3′ primers corresponding to bases 29–48 and 870–899 (accession number X16707). Hybridization was carried out using the QuickHyb reagent (Stratagene, Heidelberg, Germany).

2.3. Western blot analysis

Cell lysates were prepared using a buffer containing 1% SDS (w/v), 0.1 M Tris, pH 7.5, 0.05 M EDTA, 0.02 M EGTA, 0.1 M sucrose, 0.1 M β-mercaptoethanol and 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF). 20 μg protein per lane was run on a 12% SDS-PAGE, transferred to nitrocellulose and probed with specific polyclonal antibodies (Santa Cruz, CA, USA). The detection was performed using a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Dianova, Hamburg, Germany) and the ECL detection system (Amersham, Braunschweig, Germany) or with an alkaline phosphatase conjugated anti-rabbit goat antibody (Promega, Heidelberg, Germany).

2.4. Electrophoretic mobility shift assay

Nuclear extracts from DAN-G cells were prepared by the micropreparation technique described by Andrews and Faller [8] and the electrophoretic mobility shift assay was performed exactly as described previously [9]. For supershift assays, 0.1 ng of polyclonal antibodies specific for c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB and JunD were added to the reaction mixture and incubated for 2 h at 4°C before adding the ^{32}P-labelled double-stranded oligonucleotide containing the AP-1 consensus sequence: 5′-CTAGACTGAACGGTGACTCAAACTGCCGCTGCA-3′ [10].

2.5. Nuclear run on transcription assay

Nuclei were prepared by sucrose gradient centrifugation. In vitro

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transcription, prehybridization and hybridization were carried out exactly as previously described [9].

2.6. Transient transfections and CAT assay

Using the Lipofectamine Reagent (Gibco BRL, Bethesda, MD, USA), DAN-G cells were transfected with 6 μg of the MMP-1 (matrix metalloprotease 1) promoter/CAT plasmid −518/+63 and the mutant construct −518/+63-mTRE containing three base pair changes in the AP-1 consensus sequence [11,12]. 12 h after transfection, cells were incubated for 24 h with 1 μM 13-*cis*-RA, 0,1 μM 12-*O*-tetradecanoylphorbol-13 acetate (TPA), or with a combination of 13-*cis*-RA and TPA. The CAT activity was assayed using the CAT Enzyme Systems (Promega, Heidelberg, Germany) following the instructions supplied by the manufacturer.

3. Results

3.1. The retinoid-mediated induction of fra-1 expression

Using the Atlas cDNA Expression Arrays (Clontech Laboratories, Palo Alto, CA, USA), we identified the fos-related protein fra-1 which was specifically up-regulated by ATRA in the human pancreatic tumor cell line DAN-G within 4 h (data not shown) as compared to vehicle-treated controls.

To confirm the retinoid-mediated induction of fra-1, we performed Northern and Western blot analyses in DAN-G cells. ATRA treatment resulted in a significant and time-dependent increase of fra-1 mRNA and protein concentrations, reaching a maximal induction at 24 h (Fig. 1). A prolonged incubation for up to 48 h did not result in a further increase of the fra-1 expression, neither did incubation with vehicle alone (data not shown). In dose-response experiments, we observed that 13-*cis*-RA, the stereoisomer of ATRA, was equipotent in terms of the fra-1 induction as assessed by Northern and Western blotting (data not shown).

To elucidate the mechanism of retinoid-induced fra-1 expression, we investigated the effects of ATRA on the fra-1 gene transcription using a nuclear run on analysis. Neither the fra-1 nor GAPDH gene transcription, which served as an internal control, were altered by ATRA treatment at 4 h or 24 h (Fig. 2), suggesting a primarily post-transcriptional mechanism of induction.

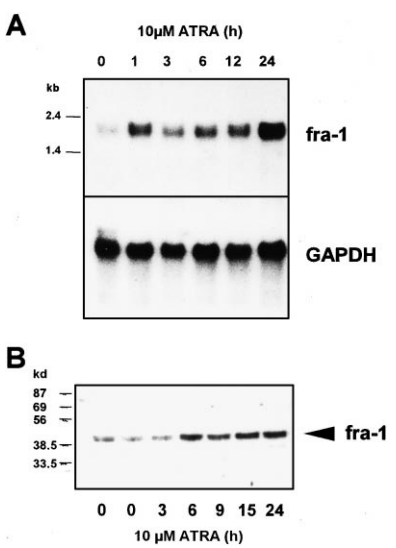

Fig. 1. Effects of ATRA on fra-1 expression in DAN-G cells. DAN-G cells were incubated for the indicated time points with 10 μM ATRA. 1A: Northern blot. Shown is a representative of three experiments. 1B: Western blot. Shown is a representative of two independent experiments yielding identical results. Controls were analysed in duplicate.

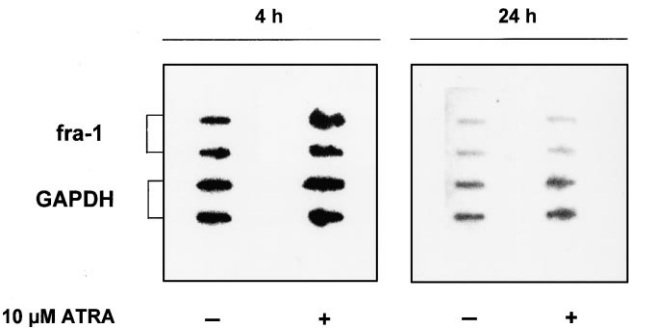

Fig. 2. Effects of ATRA on fra-1 gene transcription. DAN-G cells were treated with 10 μM ATRA or vehicle either for 4 h or 24 h and the fra-1 and GAPDH transcription were assessed by nuclear run on analysis. Duplicates for each cDNA were slotted. Shown is a representative of three independent experiments yielding identical results.

3.2. Molecular constituents of the AP-1 complex in DAN-G cells

To investigate which members of the jun and fos family participate in the AP-1 complex formation in DAN-G cells, we performed supershift assays using antibodies directed against specific components of the AP-1 complex and a specific oligonucleotide containing the AP-1 consensus sequence. To determine the specificity of binding to the AP-1 consensus sequence, a 50–500-fold excess of unlabelled oligonucleotide was added to the nuclear protein extracts prior to the addition of labelled oligonucleotide. As seen in Fig. 3, the dimers binding to the AP-1 oligonucleotide are composed of c-fos, fra-1, c-jun and junB proteins, fra-1 being the most prominent AP-1 component. In contrast, neither fosB, fra-2 or junD were detected in the formation of the AP-1 complex in DAN-G cells.

3.3. Effects of ATRA on the protein expression of AP-1 components

To examine whether the retinoid-mediated induction of fra-1 expression was selective for this AP-1 component, we inves-

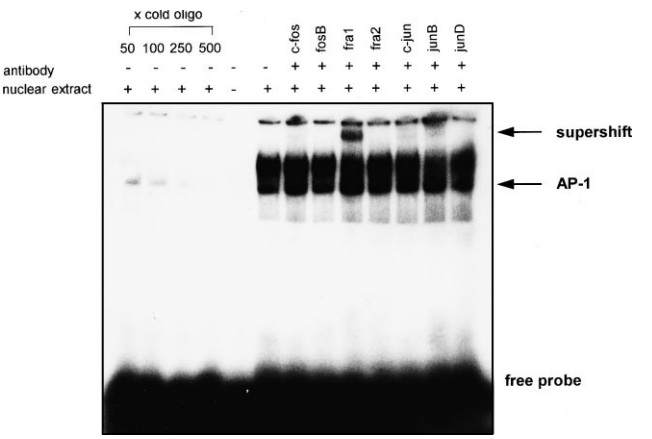

Fig. 3. A supershift assay of AP-1 components in DAN-G cells. Nuclear extracts were incubated with the radioactively labelled AP-1 consensus oligonucleotide together with specific antibodies directed against c-fos, fosB, fra-1, fra-2, c-jun, junB and junD. For competition experiments, a 50–500-fold excess of unlabelled oligonucleotide was added.

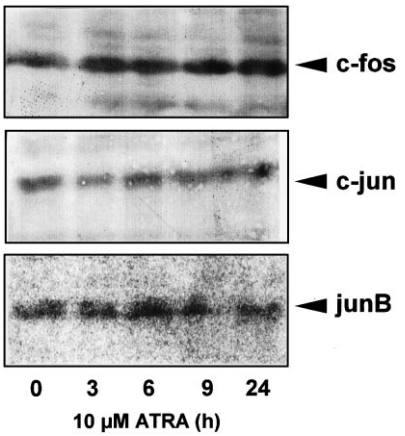

Fig. 4. Effects of ATRA on c-fos, c-jun and junB protein expression. DAN-G cells were incubated for the indicated time points with 10 μM ATRA. Shown is a representative Western blot.

tigated the effects of ATRA on the protein expression of the other AP-1 constituents. A Western blot analysis revealed that ATRA had no effect on the c-fos, c-jun and junB expression in DAN-G cells (Fig. 4), indicating that fra-1 is the only component of the AP-1 complex that is regulated by retinoids.

3.4. RA inhibits TPA-stimulated AP-1 activity

To corroborate the functional significance of retinoid-mediated up-regulation of fra-1 expression, we investigated the AP-1 activity in DAN-G cells using transient transfection experiments. As a reporter construct we used a −518/+63-CAT plasmid which contains an AP-1 site from the human MMP-1 gene upstream of the CAT gene [11,12]. Control cells were transfected with the −518/+63-mTRE-CAT plasmid which contains three base pair changes in the AP-1 consensus sequence resulting in a loss of function. TPA stimulation for 24 h resulted in a significant increase of the CAT activity compared to controls and cells incubated with 13-*cis*-RA (TPA: 2.93×10^4 cpm, 13-*cis*-RA: 0.25×10^4 cpm, control: 0.17×10^4 cpm) (Fig. 5). The stimulatory effect of TPA on CAT was inhibited to approximately 50% by simultaneous incubation of cells with 13-*cis*-RA (1.44×10^4 cpm). These results indicate that retinoids inhibit the TPA stimulated AP-1 transactivation activity in DAN-G cells.

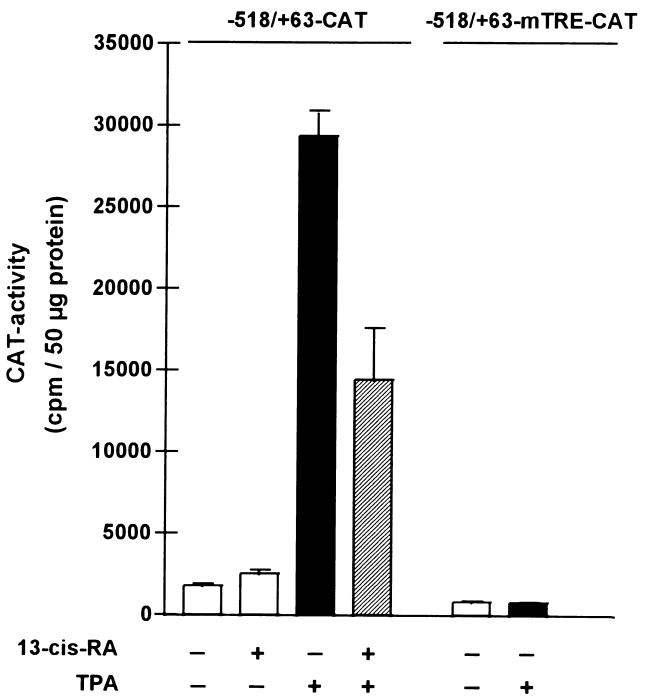

Fig. 5. Effects of RA on the TPA-stimulated CAT activity. DAN-G cells were transiently transfected with the −518/+63-CAT or −518/+63-mTRE-CAT reporter plasmid and incubated for 24 h with 1 μM 13-*cis*-RA, 0.1 μM TPA or 13-*cis*-RA plus TPA. The CAT activity was determined by liquid scintillation counting and is expressed as cpm per 50 μg protein. Shown are the mean and S.E.M. of three independent experiments.

4. Discussion

We have previously demonstrated that retinoid treatment results in inhibition of growth and induction of cellular differentiation in human pancreatic cancer cells in vitro and in vivo [5–7]. These biological effects of retinoids are based on a coordinated regulation of gene expression. Therefore, the identification of retinoid responsive target genes which are involved in the retinoid-mediated growth inhibition and induction of differentiation, is of particular interest in order to further understand how these pleiotropic effects of retinoids are brought about.

Using the Atlas cDNA Expression Arrays, we have identified fra-1 as a novel target for the retinoid-regulated gene expression. fra-1, along with c-fos, fosB and fra-2, belongs to the family of fos-related genes [13,14]. These proteins function cooperatively as transcriptional regulators in a heterodimeric complex with the jun-related proteins c-jun, junB and junD (reviewed in [15]). These dimeric AP complexes (AP-1) interact with specific DNA binding sites (TRE) to regulate the transcription of genes, usually associated with the induction of proliferation. The activity of AP-1 is therefore modulated by various molecules involved in the growth regulation such as growth factors, cytokines, oncogenes and tumor promoters like TPA.

We observed a time-dependent induction of fra-1 mRNA and protein levels by ATRA. A run on analysis revealed that ATRA did not alter the transcription rate of the fra-1 gene suggesting that retinoids post-transcriptionally increase the fra-1 expression via an indirect mechanism. Post-transcriptional modification of gene expression by retinoids has been described in other systems and might, for example, involve mRNA stabilization by various mechanisms [16]. To evaluate if fra-1 expression plays a role in the AP-1 complex formation in pancreatic carcinoma cells, we performed supershift assays. We observed that c-fos and fra-1, in cooperation with c-jun and junB, participate in the AP-1 complex in this cell line. In addition, retinoids specifically up-regulate the fra-1 expression and had no effect on the c-fos, c-jun and junB expression.

To investigate the biological consequence of fra-1 induction by retinoids, we performed transactivation assays using a transiently transfected reporter plasmid containing a functional TRE of the human MMP-1 promoter. We observed a pronounced suppression of the TPA-stimulated CAT activity by retinoids suggesting that the enhanced formation of fra-1/jun heterodimers resulted in a less active AP-1 complex. Wisdom and Verma [17] demonstrated that fra-1, unlike c-fos and fosB, lacks a C-terminal transactivation domain as well as

N-terminal sequences with a transactivation function. Moreover, no transforming potential of fra-1 has been detected in rat fibroblasts. Hence, fra-1 is considered to be a negative regulator of the AP-1 activity. Moreover, Suzuki et al. [18] demonstrated that fra-1, like fra-2, is able to inhibit the AP-1-dependent transactivation by c-fos and c-jun in transiently transfected embryonal carcinoma cells. In addition, serum growth factors stimulated the transcription of fra-1 with delayed kinetics relative to those of c-fos and fosB [19], suggesting that one function of fra-1 might be to limit the transcriptional activity of c-fos and fosB by sequestering jun partner proteins into less active AP-1 complexes. However, in a mouse mammary carcinoma cell line, fra-1 has been shown to induce morphological transformation [20], suggesting the existence of a species- and cell type-specific biological function.

In summary, we have identified fra-1 as a novel target for RA in human pancreatic carcinoma cells. Retinoid-mediated induction of fra-1 might inhibit the AP-1 transactivation activity. Therefore, in addition to the known antagonism between retinoids and AP-1 due to direct protein-protein interaction between retinoid receptors and AP-1 components [21,22], induction of the fra-1 gene expression represents a novel level of molecular interference between these two signalling cascades.

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