Cell. Mol. Life Sci. 63 (2006) 2387–2396
1420-682X/06/202387-10
DOI 10.1007/s00018-006-6256-7

Cellular and Molecular Life Sciences

Research Article

Interferon-alpha and transforming growth factor-β co-induce growth inhibition of human tumor cells

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Received 1 June 2006; received after revision 13 July 2006; accepted 9 August 2006
Online First 20 September 2006

Abstract. A hallmark of resistance to type I interferons (IFNs) is the lack of antiproliferative responses. We show here that costimulation with IFN-α and transforming growth factor beta-1 (TGF-β) potentiates antiproliferative activity in a sensitive (ME15) and resistant (D10) human melanoma cell line. A DNA microarray-based search for proliferation control genes involved that are cooperatively activated by IFN-α and TGF-β, yielded 28 genes. Among these are the insulin-like growth factor-binding protein 3 (IGFBP3) and the calcium-binding protein S100A2; we demonstrate, that recombinant IGFBP3 protein is a potent growth inhibitor requiring TGF-β activity. The antiproliferative activity of S100A2 is significantly enhanced by IFN-α in stably transfected ME15 or D10 cell lines. We show for the first time that IFN-α is a potent inducer of intracellular calcium release required for activation of S100A2. Our study provides a functional link between IFN-α and TGF-β signaling and extends the function of IFN signaling to calcium-sensitive processes.

Keywords. Calcium signaling, cell proliferation, interferon-alpha, transforming growth factor-beta, S100 proteins.

Introduction

Interferon (IFN) was first discovered as an interference activity produced by heat-inactivated influenza virus [1]. Today, about twenty different IFN molecules are known, subdivided into two main categories with distinct activities: class I IFNs (-α, -β) have a wide variety of biological effects that include antiviral, antiproliferative and immunomodulatory activities, while type II (γ) IFNs are involved in adaptive and innate immune responses [2]. For two decades, recombinant human IFN-alpha-2A (IFN-α) has been used as the main therapy to treat viral infections and oncological disorders. Naturally occurring IFN-α resistance, severe toxicity including gastrointestinal disorders, hypo- or hypertension, tachycardia or headache are side effects that severely limit the clinical efficacy of IFN-α therapy [3].

At the molecular level, all type I IFNs bind the dimeric IFN-I receptor, which is associated with tyrosine kinase 2 and Janus kinase (Jak) 1 [2]. This ultimately leads to the formation of IFN-stimulated gene factor 3 complexes (ISGF3), which translocate to the nucleus and initiate the transcription of target genes containing IFN response elements (IREs) within the regulatory region.

Several studies suggest an association between biological and transcriptional activity, which implies that occurrence of resistance and side effects are also linked to gene expression. A hallmark of IFN-α non-responsiveness is the lack of antiproliferative activity, which can be demonstrated in cell lines established from tumors. Re-

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cently, we have shown that IFN primary response genes (IPRGs) with authentic response elements are induced with equal efficiency in a sensitive (ME15) and resistant (D10) melanoma cell line, while IFN secondary response genes (ISRGs) lacking such IREs show markedly reduced activation in the non-responsive line. It is thus plausible to propose that full activation of the ISRGs is required for the antiviral and antiproliferative activities of IFN-α [4].

Efficient IFN signaling requires activity of other signaling pathways [2]. The Rac1/p38 mitogen-activated protein kinase pathway (MAPK) is required for IFN-α-dependent transcriptional activation [5, 6], and IFN-γ prevents the activation of TGF-β inducible genes by induction of SMAD7, which inhibits the interaction of SMAD3 with the TGF-β receptor [7]. In addition, TGF-β induces G1 arrest in most cell types, and in epithelial cells this process is controlled by a direct interaction of the c-Myc oncogene with the transcription factor SMAD3 [8–10]. The phosphatidylinositol 3-kinase pathway is activated in response to both type I and II IFNs [11]. Common to these pathways is the transmission of growth inhibitory signals, and consequently a downstream signaling defect could account for lack of antiproliferative activity in IFN-α-resistant cell lines. Attachment of polyethylenglyol moieties to IFN-α for instance, cannot negate non-responsiveness *in vitro* and *in vivo,* although pegylation extends serum half-life from 1 day to an entire week [12, 13], which highlights the need for alternative strategies.

We show here that costimulation of resistant human melanoma cells with pegylated IFN-α (IFN-α^{K134}) and TGF-β restores antiproliferative activity and induces the cooperative activation of 28 genes. We demonstrate that the activity of the small calcium-binding protein S100A2 or the insulin-like growth factor binding protein 3 (IGFBP3) alone is sufficient to arrest cell growth and requires either IFN-α or TGF-β, respectively.

Materials and methods

Reagents. For IFN-α stimulation, we used the monopegylated isomer K134 (IFN-α^{K134}), the variant with the highest transcriptional and antiproliferative activity relative to the other positional isomers (K_{31}, K_{49}, K_{70}, K_{80}, K_{112}, K_{121}, K_{131}, K_{134}, K_{164}) contained in the commercial formulation (Pegasys®; [14]). Recombinant TGF-β was purchased from Calbiochem, Germany. All other reagents and chemicals were of the highest purity available.

Antiproliferative activity assay. The human melanoma primary cell lines ME15 and D10 are a gift from Prof. Giulio Spagnoli (University Basel, Switzerland) and described elsewhere [15, 16]. Cells were grown in RPMI medium supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM nonessential amino acids and HEPES buffer as described [13]. The cell proliferation assay was started with approximately 1000–1500 cells/well in a flat-bottom 96-well plate over a period of 7 days with or without cytokines as described [14]. The number of living cells was then determined using a cell staining kit (Promega, Madison, WI) based on the colorimetric detection of the cleavage of the tetrazolium salt MTS into formazan. The MTS reaction solution was added according to the manufacturer's recommendation and absorption at 490 nm was measured in a spectrophotometer. IFN-α^{K134} was used at a concentration of 100 U/mL and TGF-β at 20 μg/ mL as recommended by the supplier.

Western blotting. Immunoblots were performed with normalized total protein extracts derived from 5×10^6 cells. Sample integrity and the protein concentration were determined by Coomassie Blue staining of aliquots by SDS-PAGE. Proteins were detected using rabbit polyclonal antibodies to 1-8U [17] or rabbit polyclonal antibodies to S100A2. Protein antibody complexes were visualized with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Inc., Hercules, CA) and a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent; Pierce Chemical Co.).

Oligonucleotide array analysis. The melanoma cell lines were incubated in triplicate for 2 and 6 days with cytokines supplemented as indicated in the figure legends. After this period, the cells were harvested by scraping and the total cellular RNA was extracted from each culture with RNA-Bee™ (AMS Biotechnology, Bioggio, Switzerland). Total cellular RNA (10 μg) from individual cultures were processed for hybridization to Affymetrix U95Av2 human microarrays following the manufacturer's instructions and using commercial kits as recommended. After washing, cell intensities were collected by laser scanning, the raw data were stored in database followed by normalization, and differential expression analysis using RACE-A software was performed as described [4]. We considered only genes with a standard deviation smaller than the absolute change in signal intensity and the calculated confidence level of a gene was set greater than 97% (p value < 0.03).

Intracellular Ca^{2+} assay. The melanoma cell lines were plated in 96-well plates (10 000 cells/ well) for 24 h. Mobilization of intracellular calcium was measured using a FLEXstation system (Molecular Devices, Sunnyvale, CA) and the calcium assay kit recommended for the instrument by the manufacturer. The fluorescence was monitored at λ_{ex} = 485 nm, λ_{em} = 525 nm and relative expression units are reported.

Cell transfection of S100A2 in ME15 and D190 cells. A 970-bp fragment, containing the entire coding region of the S100A2 cDNA, was inserted into the mammalian cell expression vector pcDNA3 and designated pcDNA3-S100A2. The ME15 and D10 cell lines were transiently transfected with 2.0 µg of the appropriate plasmid DNA employing LipofectAMINE Plus (Invitrogen), according to the manufacturer's instructions. Transfectants were selected in medium with Geneticin (800 µg/mL) and individual clones were isolated and screened by Western blotting.

Comparative genomics analyses. Genomic sequences for human, dog, mouse, and rat were downloaded from the http://genome.ucsc.edu web site (entries: NCBI35/UCSC hg17, UCSC canfa1, NCBI34/UCSC mm6, UCSC rn3). For the cow, genomic sequences from the EMBL database (http://www.ebi.ac.uk/embl), release 83, section 'wgs_cow', were used. Homologous genomic loci were identified based on BLAST searches [18] and the corresponding sequence stretches were aligned using MLAGAN [19]. Transcription factor binding sites were predicted using the binding site matrices from TransFac 8.4 [20]. Potential matches to the obtained binding site matrices were obtained individually for the genomic sequences and mapped to each other by close proximity in the genomic sequence alignment. Transcription factor binding sites conserved in all species were further analyzed.

Results

TGF-β enhances the antiproliferative activity of IFN-α in both a sensitive and a resistant human melanoma cell line. Efficient Jak-STAT signaling requires cooperation with other pathways such as the MAPK or the phosphatidylinositol 3-kinase signaling cascades, which led to the hypothesis that a defect in an associated pathway causes lack of IFN inducible antiproliferative activity. We thus tested, whether effective doses of interleukin (IL)-1α, IL-1β, IL-6, IL-10 or TGF-β inhibit the growth of IFN-α-responsive ME15 cells. As it turned out, only TGF-β showed significant growth inhibition, while the other cytokines had no or only marginal effects (data not shown). We next explored a possible linkage of TGF-β and IFN-α signaling and incubated ME15 and resistant D10 cells with a combination of IFN-α^{K134} and TGF-β and the individual cytokines as control. Growth inhibition of ME15 cells commences at day 4 under all conditions and costimulation induces the highest levels of antiproliferative activity (Fig. 1a). As expected, IFN-α^{K134} alone did not inhibit growth of D10 cells and TGF-β induced significant antiproliferative activity at day 6. However, costimulation with both cytokines enhanced this activity to levels typical for responding cell lines (Fig. 1a). The antiproliferative activity in D10 at day 6 is comparable to day 4 in M15, and this delay is probably related to the slower cell cycle of D10 [17]. The cooperative induction of antiproliferative activity by IFN-α and TGF-β suggests functional linkage of these pathways and supports previous conclusions from signaling experiments [5].

Overexpression of the small IFN-α inducible membrane protein 1-8U results in an almost complete growth arrest of transfected EBNA cells [17]. Therefore, we had to consider the possibility that cooperative up-regulation of this proliferation control protein by IFN-α^{K134} and TGF-β induces growth inhibition particularly in D10 cells. However, neither TGF-β nor the combination of both cytokines causes further up-regulation of 1-8U mRNA above IFN-α^{K134}-induced levels (Fig. 1b). We thus exclude the possibility that additive up-regulation of 1-8U is involved in growth control of ME15 or D10, and we proceeded with the identification of proliferation control candidate genes by a global DNA microarray approach.

Identification of genes with positive transmodulation by IFN-α and TGF-β. The individual antiproliferative activity of IFN-α and TGF-β is well documented and both cytokines induce individually the differential expression of several hundred genes [21, 22]. We thus searched for genes with enhanced and cooperative induction by both cytokines at the day of maximal antiproliferative activity in each cell line. RNA from triplicate ME15 and D10 cells cultures, grown and stimulated as above, was extracted and processed for microarray analysis using commercial chips (U95A; Affymetrix Inc.) with a content of about 13 000 probe sets covering 9100 human genes. In ME15, 325 genes were up- or down-regulated at least twofold ($p = < 0.03$) at day 2, while only 217 genes were modulated in D10 at this time point (data not shown). After 6 days in culture, the number of modulated genes increased to 513 in ME15 and to 371 in D10. As expected, IFN-α^{K134} and TGF-β modulate distinct groups of target genes, and costimulation generates in general a merged activation pattern of the individual profiles with some interesting exceptions: we identified clusters of IFN-α^{K134}-stimulated genes that are repressed by TGF-β, and also the opposite scenario, where IFN-α^{K134} suppresses TGF-β target genes. This novel trans-suppression of certain genes by IFN-α or TGF-β genes supports the linkage of these pathways at the transcriptional level and deserves further investigation.

We next tried to identify growth control candidate genes that are co-induced and show the highest expression level at the time point of maximal antiproliferative activity in either cell line. In particular, a candidate gene had to fulfill the following criteria: (i) additive, at least twofold co-activation by IFN-α^{K134} and TGF-β at day 6 in D10, (ii) IFN-α^{K134}-dependent induction at day 2 in ME15, and

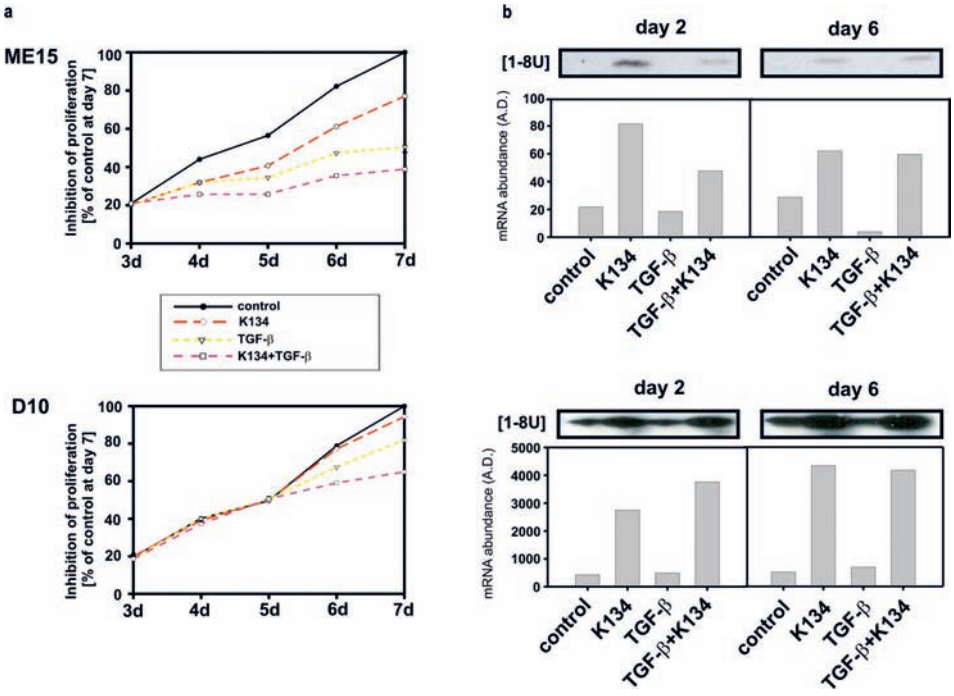

Figure 1. TGF-β restores the antiproliferative activity of IFN-α in D10 cells. IFN-α-sensitive ME15 or resistant D10 cells were incubated over 7 days with either K134 (a pegylated form of IFN-α-2A), TGF-β or a combination of both cytokines (*a*). Proliferation was measured with a colorimetric assay and is given as% of control at day 5. Note the delayed onset of antiproliferative activity under all conditions in D10 relative to ME15. Expression of the proliferation control protein 1-8U is specifically induced by IFN-α^{K134} and incubation with TGF-β or co-incubation has no detectable impact on mRNA or protein levels (*b*). The levels of 1-8U mRNA were measured on microarrays and the 1-8U protein was detected by immunoblotting with rabbit serum against 1-8U.

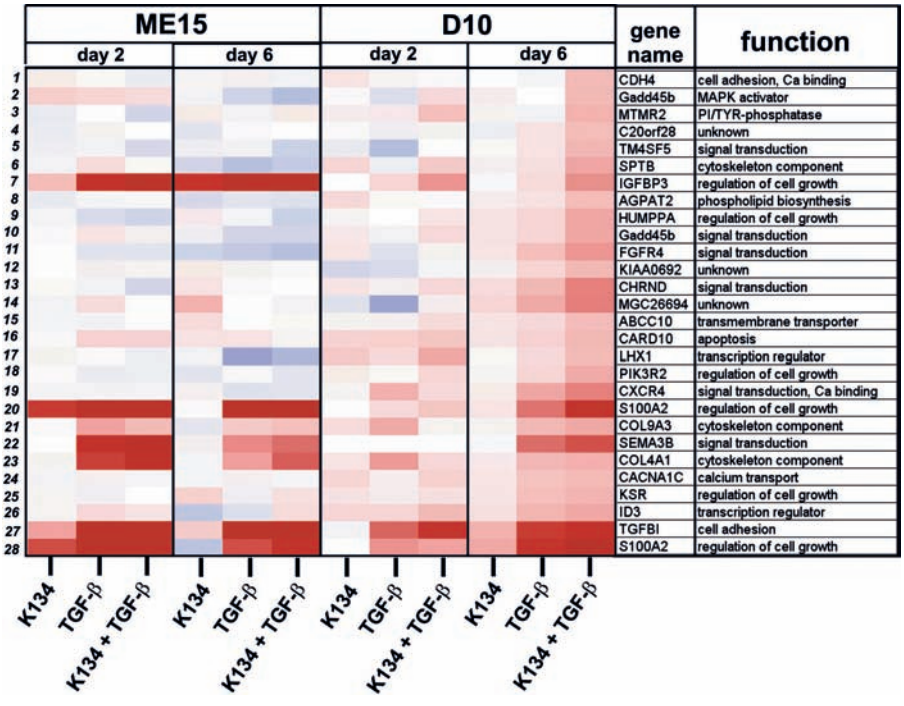

Figure 2. Proliferation control candidate genes with additive induction by IFN-α^{K134} and TGF-β. ME15 and D10 cell cultures were treated with IFN-α^{K134}, TGF-β or both followed by total RNA extraction after 2 or 6 days in culture. After processing, the cRNA was hybridized to commercial DNA microarrays containing probe sets for about 11 400 transcripts. Down-regulated transcripts are displayed in blue and up-regulation is represented in red and maximum intensity indicates a change factor of ± 10-fold. The microarray data represent mRNA expression average data obtained from three individual cultures per treatment and time point. The heat map displays genes that are up-regulated at day 6 by IFN-α^{K134} and TGF-β and inducible by IFN-α^{K134} alone in ME15 based on the maximum antiproliferative activity.

(iii) low expression at day 2 in D10. Only 27 genes fulfilled all conditions and consistent with our working hypothesis none of these growth control candidate genes is known to be IFN-α- or TGF-β-inducible except the TGF-β inducible collagen-associated adhesion protein TGFB1 (Fig. 2; [23]). In ME15, co-stimulation with IFN-α^{K134} and TGF-β induces maximal expression of five genes, namely IGFBP3, S100A2, SEMA3B, COL4A1 and TGFBI at day 2, which correlates with the onset of antiproliferative activity (Fig. 2). The small calcium-binding protein S100A2 and the IGFBP3 show also co-operative up-regulation at day 6 in D10, which selects them together with their documented function in cell growth and proliferation as the most promising candidates for growth inhibition (Fig. 2; [24–26]).

Comparative genomics analysis of IGFBP3 and S100A2 genomic loci. The relatively late, co-operative induction of IGFBP3 and S100A2, classifies these genes as ISRGs and consequently we did not expect typical *bona fide* IRE- or SMAD-binding sites in the promoter regions. This was indeed the case based on a computer-aided, global search for conserved transcription factor binding sites in the equivalent loci from human, dog, cow, mouse and rat loci using a comparative genomics approach. For human IGFBP3, the analyzed chromosomal region spanned about 15 kb and included all exons and in addition, six strongly conserved non-coding sequence stretches. We identified 50 conserved nucleotides 1.1 kb upstream of the translation initiation codon, which lacks any homology with known transcription factor binding motifs and a TATA box at equivalent position in all species (data not shown). Interestingly enough, we discovered a conserved IRE in the first intron of the IGFBP3 gene, and it remains to be confirmed whether this unusual IRE is functional (position +2326; Fig. 3a). For the S100A2 gene, we screened about 11 kb of genomic DNA and recovered several sequence stretches with significant inter-species conservation. We identified a known tandem AP-1 binding site 1.9 kb downstream of the transcription start site (data not shown) and a well-conserved E box (E47) enhancer element 5 kb upstream of the initiation codon (Fig. 3b). Furthermore, we could not identify transcription factor binding motifs that are present in both genes and could explain co-activation. In summary, this *in silico* analysis eliminates direct activation of IGFBP3 and S100A2 expression by IFN-α or TGF-β and suggests an alternative mode of co-operative transcriptional activation.

Direct inhibition of proliferation by IGFBP3 and S100A2 proteins. IGF-binding proteins prolong the half-life of IGF and regulate the bioavailability of free ligand. Secreted IGFBP-3, but not IGFBP-1, inhibited Hs578T human breast cancer cell growth by a mechanism independent of IGFs [27]. In addition, the antiproliferative activity requires binding of IGFBP3 to the cell surface. Therefore, we supplemented cell cultures with 1, 10 or 80 nM recombinant IGFBP3 protein and measured antiproliferative activity. For these experiments, we have purposely chosen serum-free media to exclude interference from generic growth factors present in serum-containing cell culture media. Under these conditions, addition of 80 nM IGFBP3 had only a moderate effect on cell growth (20%) starting at day (Fig. 4). Strikingly, co-incubation of IGFBP3 with 10 µg/mL TGF-β induced significant antiproliferative activity, even at the lowest concentration of IGFBP3. In contrast, incubation with IFN-α^{K134} did not activate IGFBP3 (data not shown), and we conclude that antiproliferative activity of IGFBP3 requires TGF-β-inducible components.

The low molecular weight calcium-binding protein S100A2 has recently been identified as a potential tumor suppressor molecule that is transactivated by p53 in a calcium-dependent fashion [25, 28]. Expression studies showed that S100A2 is down-regulated in several tumors like melanoma and breast carcinoma, which led to the view that S100A2, has antiproliferative activity [29]. In Figure 5a, we show that the highest levels of S100A2 mRNA and protein occur indeed at time points with peak antiproliferative activity. So far, direct growth inhibition by S100A2 has not been demonstrated, and we generated stable ME15- and D10-derived cell lines, that overexpress S100A2 about 20-fold (data not shown). From each cell line, we selected one representative clone (ME15-3 and D10-5) and measured cell proliferation over a period of 7 days together with the parental lines (Fig. 5b). In both lines S100A2 overexpression reduced cell proliferation by about 20% (Fig. 5b, left panel). Co-incubation with TGF-β did not enhance this activity (data not shown), but stimulation with IFN-α boosted the basal activity to 60% in ME15 and to about 40% in D10 (Fig. 5b). In contrast to IGFBP3, activation of S100A2 requires IFN-α-inducible co-factor(s) and the most obvious candidates are in fact calcium ions, which induce a conformational change and target protein binding [30]. We treated ME15 and D10 cells with IFN-α or TGF-β, and measured intracellular calcium release seconds after stimulation with a standard fluorescence-based assay. IFN-α induces a fast and significant raise of intracellular calcium levels, while TGF-β has no detectable effect (Fig. 5c). This result provides a plausible explanation for enhanced antiproliferative activity of S100A2 transfected cells in the presence of IFN-α, and extends the potential function of IFN-α signaling to calcium-sensitive pathways that regulate cell-to-cell communication, membrane excitability, secretion or apoptosis [31].

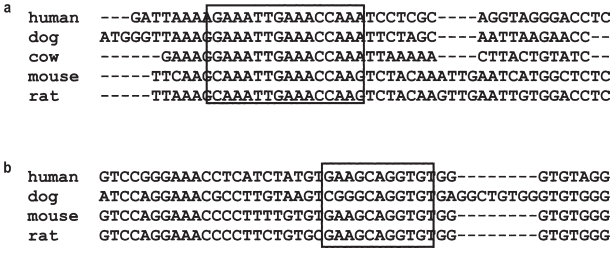

Figure 3. Conserved non-coding sequences with matches to known transcription factor binding sites. (*a*) Putative IRE located in intron 1 of IGFBP3. (*b*) Putative E box (E47) located about 5 kb upstream of the S100A2 coding region. Alignments were generated as described in the Materials and methods.

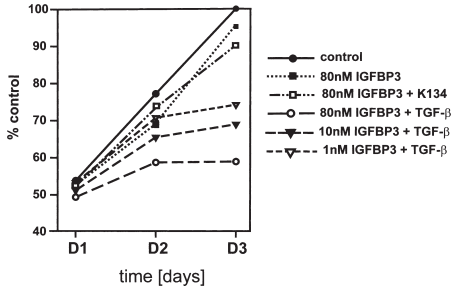

Figure 4. Efficient inhibition of proliferation by IGFBP-3 requires TGF-β signaling activity. Cell culture medium was supplemented with commercial IGFBP-3 at 1, 10 and 80 nM with or without TGF-β (10 µg/mL). ME15 cell proliferation was monitored using a colorimetric assay (see Materials and methods).

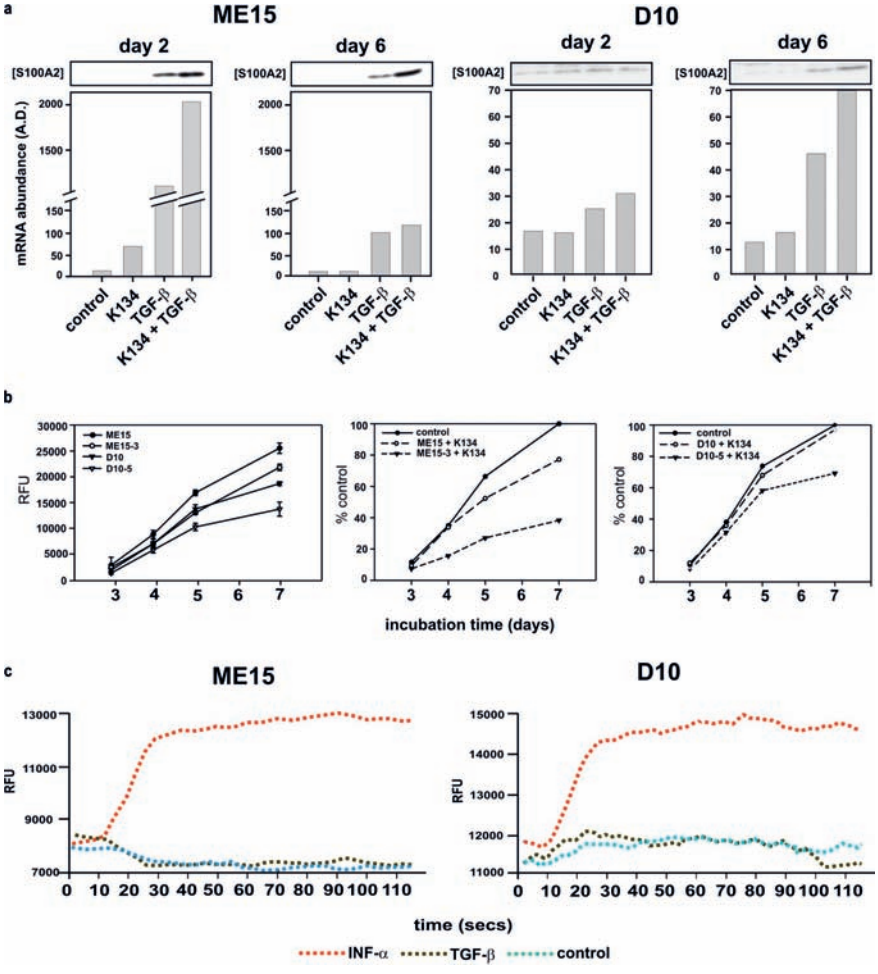

Figure 5. Enhancement of S100A2 antiproliferative activity by IFN-α. mRNA and protein abundance in lysates of ME15 and D10 cells. Expression of S100A2 protein correlates with mRNA abundance (*a*). Total protein lysates were probed with rabbit serum against S100A2 protein and mRNA abundance was measured using microarrays. ME15 and D10 cells were cultured and stimulated as in Figure 1. Note the reduced apparent levels of S100A2 protein and mRNA at each time point relative to ME15. Growth rates of ME15 and D10 cells stably transfected with S100A2 cDNA (*b*). On the left is shown the growth rate of non-transformed and stably transformed ME15 and D10 cells without cytokine stimulation. Note the early onset of antiproliferative activity in D10 compared with the growth rate shown in Figure 1a. The adjacent graphs show proliferation assays of the same cells in the grown in the presence of 100 U/ mL K134. Incubation with TGF-β has no impact on the growth rate (data not shown). IFN-α is a rapid and efficient inducer of intracellular calcium release (*c*). ME15 or D10 cells were stimulated with non-pegylated IFN-α-2a or TGF-β and intracellular calcium release was measured using a fluorescent calcium-release reporter assay. Note that an efficient and rapid release occurs with equal efficiency in ME15 and D10. Calcium release levels are given in relative fluorescence units (RFU).

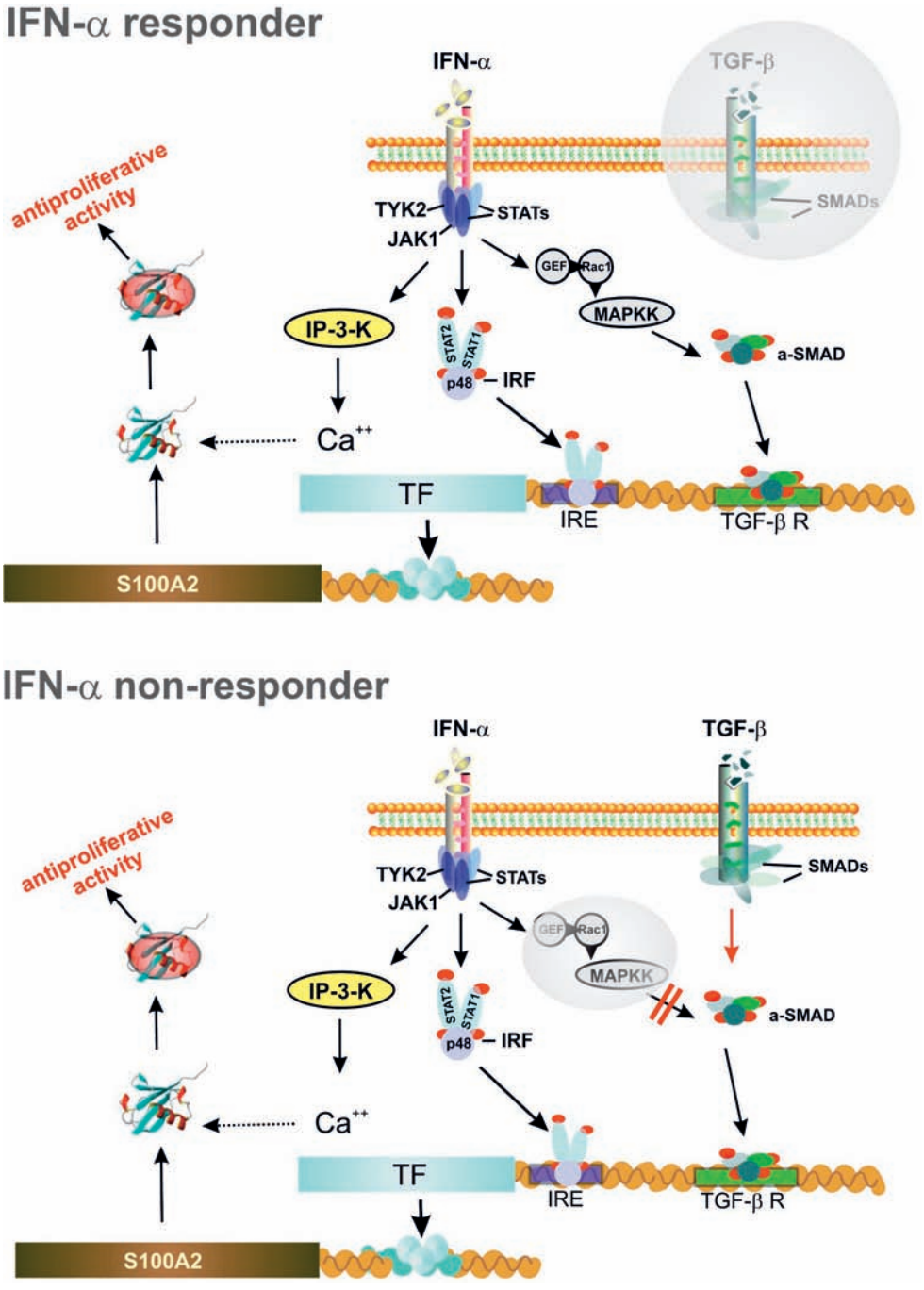

Figure 6. Proposed model for cooperative signaling activity of IFN-α and TGF-β in IFN-α-sensitive (left panel) and -resistant cell line (right panel). In IFN-α responders, co-activation of the parts of the TGF-β pathway occurs through MAPK stimulation, which leads to induction of an hypothetical transcription factor (TF) with growth control target genes. In non-responders this co-activation is defective and is complemented by co-stimulation with TGF-β.

Discussion

We have shown that co-stimulation with IFN-α and TGF-β induces antiproliferative activity in a human melanoma cell line, which is normally resistant to the growth inhibitory activity of IFN-α. By microarray profiling, we identify 28 genes with cooperative induction by IFN-α and TGF-β at the time point of maximal antiproliferative activity. We show that S100A2 and IGFBP3 proteins, identified by transcriptional profiling, have antiproliferative activity, which requires IFN-α or TGF-β costimulation, respectively.

The focus of our studies was the elucidation of a mechanism responsible for resistance to IFN-α and as an initial step, we investigated this process using a pair of well-characterized human melanoma cell lines [4, 13]. We confirm in this study, that transcriptional activation in D10 is less efficient than in ME15 but not impaired. This suggested to us that some genes relevant for the control of proliferation are not properly expressed in D10, which in turn causes unresponsiveness. We show that costimulation with TGF-β restores antiproliferative activity in D10 most likely through joint induction of the proliferation control proteins S100A2 and IGFBP3. Since these genes lack IREs, we propose a mechanism in which IFN-α and TGF-β co-induce sufficient levels of an IFN-α and TGF-β co-inducible transactivator protein, which then induces expression of the proliferation control target genes (Fig. 6).

Alternatively, chromatin structure-dependent activation by initiation complexes, which have multiple redundant targets, could regulate these genes. C-myc transcriptional activation complexes for instance contain transcription factors, histone acetylation related proteins or zinc finger-binding proteins that recognize a large number of target genes. The oncoprotein c-myc can activate transcription of suppressor proteins, which block as heterodimeric complexes transcription from the core promoter of target genes, and such a mechanism may explain the observed transcriptional suppression of IFN-α target genes by TGF-β and *vice versa* [32]. TGF-β for instance induces expression of *P15NK4B*, which encodes an inhibitor of Cdk4 and Cdk6 kinase. C-myc suppresses this activation by interaction with MIZ-1 at the core promoter [8, 10].

Interestingly, the full antiproliferative activity of either IGFBP3 or S100A2 protein also requires TGF-β or IFN-α signaling, respectively. IGFBP3 is the major serum transport protein for IGFs, but also inhibits cell proliferation in a number of cell types [27, 33]. Consistent with previous studies, we show that the activity of IGFBP3 is enhanced by TGF-β, which was also demonstrated in T47D cells, where costimulation potentiates SMAD phosphorylation [34]. The same group demonstrated that IGFBP3 binds to the TGF-β receptor II, which results in SMAD phosphorylation and transcription of downstream target genes [35]. Such a mechanism is consistent with our data, because maximal antiproliferative activity correlates with the time point of secondary response genes expression.

The small calcium-binding protein S100A2 is frequently down-regulated in a number of tumor tissues such as melanomas [36] or breast carcinoma [37] and it was identified by subtractive hybridization between normal and tumor tissue as a tumor suppressor [38]. More recently, specific interaction with p53 and modulation of transcriptional activity has been demonstrated [23]. These studies associate S100A2 with control of proliferation, and we show here directly by recombinant expression that this protein inhibits cell growth. Interestingly, IFN-α^{K134} is an efficient enhancer of the antiproliferative activity of S100A2, and we show that triggering of the IFN receptor results in a fast intracellular calcium release, which is the most plausible explanation for the observed potentiation of antiproliferative activity (Fig. 5). Interestingly, additional IFN-α^{K134} and TGF-β co-activated genes namely the chemokine receptor 4 (CXCR4), the voltage-dependent calcium channel (CACNAC1) and the calcium-binding protein cadherin 4 (CDH4) require calcium for signaling or activation. Activation of another calcium binding, S100C/ A11 protein requires either high Ca^{2+} levels or TGF-β activity to block the growth of human epidermal keratinocytes [39]. Calcium release connects type I IFN signaling to other cellular functions, such as the control of cell proliferation, apoptosis, signal transduction or cell-to-cell communication through gap junctions [31, 40]. For example, Ca^{2+} can interact with the MAPK signaling pathway by activating the proline-rich tyrosine kinase 2, which leads ultimately to the expression of MAPK target genes [41]. It is thus possible that at least some of the ISRGs, that typically lack IREs, are regulated by Ca^{2+}-sensitive transcriptional complexes. Viruses such as hepatitis C virus induce release of type I IFNs upon liver infection and Ca^{2+}-mediated signal transmission through gap junctions may transmit the primary signal through the entire organ, resulting in efficient and fast induction of immune and antiviral defense mechanisms [42].

In summary, the initial observation, that TGF-β restores antiproliferative activity in D10 correlates with the cooperative induction of the cell cycle control proteins IGFBP3 and S100A2, which in turn require either TGF-β or IFN-α for activation. Based on our data, we propose a model in which downstream components of TGF-β are necessary for efficient Jak/STAT signaling and cell proliferation. In IFN-α-sensitive cells essential components of the TGF-β signaling pathway are co-induced, which leads ultimately to the expression of a co-inducible transcription factor, which activates in turn secondary response genes such as the proliferation control proteins S100A2 and IGFBP3 (Fig. 6, left panel). In non-responding cells cross-talk between these signaling cascades is defective and can be complemented by costimulation with TGF-β, which leads to sufficient induction of transcription factor and ultimately to the expression of ISRGs (Fig. 6, right panel). This model provides a plausible explanation for the data presented above and implies that IFN-α and TGF-β signaling is functionally linked. To identify transcription factor candidate proteins, we are currently comparing the protein maps of untreated and treated ME15 cells. At this point, we an only speculate that co-stimulation with TGF-

β may overcome resistance in patients that fail to respond to IFN-α monotherapy.

Acknowledgements. We thank Adriana Ille for outstanding technical assistance and Patricia McLoughlin for suggestions and critical reading of the manuscript and Clemens Broger for bioinformatics support. We are grateful to Peter Nesböck for support in calcium assays, to Giulio Spagnoli for cell lines and to Ciro Caravaggio and Klaus Lindpaintner for continued interest in this work. Part of this work (C.W.H.) was supported by the Swiss National Fond.

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