Interferon-γ suppresses S100A4 transcription independently of apoptosis or cell cycle arrest

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The S100A4 protein has been associated with increased metastatic capacity of cancer cells, and recent studies have suggested a correlation between the expression level of S100A4 and the prognostic outcome for patients with various types of cancer. The knowledge about the mechanisms underlying the metastasis-promoting effects is still limited, and the aim of the present study was to elucidate signal transduction pathways involved in the regulation of S100A4. After treatment of human carcinoma cells with interferon-gamma (IFN-γ), we observed downregulation of S100A4 both at mRNA and protein levels. The effect was not dependent on IFN-γ-induced apoptosis or IFN-γ-mediated cell cycle arrest. Moreover, IFN-γ-mediated decrease in mRNA stability could not account for the observed decrease in S100A4 transcript level. Finally, microarray analysis suggests ISGF3G, ETV5, ZNF133 and CEBPG as possible candidate genes involved in IFN-γ-mediated repression of S100A4.

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MATERIALS AND METHODS

Materials

Interferon-gamma and thapsigargin were from Calbiochem (Nottingham, UK). PD98059 was from New England Biolabs (Beverly, MA, USA). Tumour necrosis factor-alpha (TNF-α), actinomycin D and transforming growth factor-beta (TGF-β) were all from Sigma Chemical Company (St Louis, MO, USA).

Cell culture

All the examined cell lines (OHS [Fodstad et al, 1986], II-11b [anti-S100A4-ribozyme transfected OHS cells, Mælandsmo et al, 1996],...
**Northern blotting**

Total RNA was isolated by a single-step RNA isolation method using Trizol® (GIBCO BRL, Life Technologies, Paisley, UK) according to the manufacturer’s protocol. A measure of 5 μg of total RNA was separated by electrophoresis through 1% agarose gels containing 6.6% formaldehyde in 0.02 M NaH2PO4 (pH 6.6) and transferred onto Hybond-N+ membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). After baking for 2 h and subsequent UV crosslinking, filters were hybridised with DNA probes labelled with 32P by the random priming technique (Feinberg and Vogelstein, 1983). The hybridisations were carried out in 0.5 M Na2HPO4 (pH 7.2) and 1% SDS according to Church and Gilbert (1984). For multiple hybridisations, the bound probe was removed by incubating the filters twice for 5 min in 0.1 × SSC and 0.1% SDS at 95–100°C. To correct for an uneven amount of RNA loaded in each lane, filters were rehybridised with a kinase labelled oligonucleotide probe specific for human 18S rRNA. The mRNA levels were measured using a Storm PhosphoImage™ scanner (Amersham Biosciences, Sunnyvale, CA, USA) for further analysis, including spot quantification using an ImageQuant software package (Amersham Biosciences, Sunnyvale, CA, USA). The cDNA probe for S100A4 was kindly provided by Dr CW Heizmann, University Hospital of Zürich, Switzerland.

**Western blotting**

Protein lysates were prepared in 50 mM Tris-HCl (pH 7.5), containing 150 mM NaCl and 0.1% NP-40 with 2 μg ml−1 pepstatin, aprotinin (Sigma Chemical company, St Louis, MO, USA) and leupeptin (Roche Diagnostics, Mannheim, Germany). Total protein lysate (40 μg) from each sample was separated by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA) according to the manufacturer’s manual. As a loading and transfer control, the membranes were stained with Bradford reagent. The rabbit polyclonal anti-S100A4 antibody (diluted 1:300, DAKO, Glostrup, Denmark), rabbit polyclonal anti-STATp191 (diluted 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse monoclonal anti-alpha-tubulin (diluted 1:250, Amersham Life Science, Buckinghamshire, England) in TBST containing 5% dry milk. When immunostaining with rabbit polyclonal antiphospho-STAT1 (Tyr-701) (diluted 1:1000, Cell Signaling Technology, Beverly, MA, USA), TBST containing 0.05% Tween 20 was used. After washing, the immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (diluted 1:5000 DAKO, Glostrup, Denmark), and the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, England).

**Flow cytometry**

Cell cycle distribution of control and IFN-γ-treated cell cultures were analysed by flow cytometry. The cultures were trypsinized, fixed in ice-cold ethanol and stored at −20°C. Prior to analysis on a FACStar + flow cytometer (Becton Dickinson, San Jose, CA, USA), cells were washed in phosphate-buffered saline (PBS) and resuspended in 2 μg ml−1 Hoechst 33258 in PBS. Data analysis was carried out using the Modfit software (Verity Software House, Inc., Topsham, ME, USA). All experiments were conducted at least in duplicate and repeated three times.

**Apoptosis**

The amount of cells undergoing programmed cell death was estimated using the Cell Death Elisa® Plus Kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer’s manual. All assays were performed at least twice and in duplicate.

**Microarray analysis**

Microarray analysis using in-house printed slides containing 11.5 k polymerase chain reaction (PCR)-amplified cDNAs was performed hybridising with cDNA probes prepared from control and IFN-γ-treated (10 U ml−1 for 48 h) OHS and II-11b cell cultures. Total RNA was isolated using the GenElute Mammalian Total RNA Kit (Sigma Chemical Co., St Louis, MO, USA). Each probe was made using Cy3-dUTP and Cy5-dUTP in cDNA labelling reactions starting with 30 μg total RNA. Both untreated and IFN-γ-treated cultures were labelled with the two fluorophores and details for probe preparation and hybridisation conditions are included in the protocol located at http://www.med.uio.no/dnr/microarray. Totally, five hybridisations were performed (three OHS cDNA and two cDNA prepared from II-11b RNA), and the results are presented as average ratios from at least four hybridisations. Hybridised slides were scanned at a constant laser power of 100% for Cy3 and Cy5 and variable photomultiplier settings in a Scan Array 4000 instrument (GSI Lumonics, Inc., Billerica, MA, USA). Images obtained after being scanned were imported to the GenePix Pro 3.0 software (Axon Instruments, Inc., Union City, CA, USA) for further analysis, including spot
intensity measurements. Spots carrying technical-based artefacts were excluded manually.

RESULTS

Transcriptional regulation of S100A4

In attempts to elucidate cellular pathways involved in the regulation of S100A4 gene expression, four cell lines with S100A4 protein levels varying from low (II-11b and PM1) to medium (WiDr) and high (OHS) were treated with different signal inducers. In initial screening experiments, effects were monitored at the transcriptional level in PM1, WiDr and OHS cells. Under the experimental conditions used, gene expression was not influenced by the MEK1-inhibitor PD98059, the calcium ionophore thapsigargin, TNF-α or TGF-β (Figure 1). In contrast, IFN-γ induced a significant suppression of S100A4 transcription in all the cell lines (Figure 2A), and 12–24 h later, a corresponding downregulation of S100A4 was observed at the protein level. As measured by densitometric scanning, the S100A4 mRNA level was reduced four-fold in the high expressing OHS cells, and three-fold in the II-11b, WiDr and PM1 cells (Figures 1 and 2). The S100A4 response was observed at an earlier time point and at a lower dose in the osteosarcoma compared to the carcinoma cell lines (Figure 2A and B). Thus, maximal response was seen at 48 h with 10 U ml⁻¹ in the OHS and II-11b cultures, whereas for PM1 and WiDr cells, 200 U ml⁻¹ for 72 h was required to reach the same level of response. Notably, both WiDr and PM1 cells responded with induction of STAT1 after incubation with 50 U ml⁻¹ IFN-γ for 48 h (results not shown), suggesting that the difference in S100A4 induction of STAT1 after incubation with 50 U ml⁻¹ IFN-γ could not be explained by increased sensitivity to IFN-γ.

Activation of the Jak/STAT1 signalling pathway

It is well documented that STAT1 gene expression is induced upon activation of the IFN-γ/Jak/STAT1 signalling pathway (Boehm et al., 1997) and, in agreement with this, an induction of STAT1 was observed in all the examined cell lines (Figure 2B). To further confirm activation of STAT1, lysates from IFN-γ-treated OHS cells were stained with antiphospho-STAT1 (Tyr-701) antibody (Figure 3). The results showed that the treatment induced phosphorylation of STAT1 within a few minutes, thus confirming an active signalling pathway.

Half-life of S100A4 protein

In order to explain the time gap between the observed downregulation of S100A4 mRNA and the corresponding decline at the protein level, we used a radioactive incorporation assay to determine the half-life of the protein. The estimated half-life of S100A4 in OHS was 24 h (results not shown), which might explain the observed lag period between mRNA and protein response (Figure 2A and B).

Effects on cell proliferation and cell cycle distribution

As measured by the MTS assay, IFN-γ inhibited proliferation in OHS and WiDr cells by approximately 50%, while II-11b and PM1 cells were less influenced by the treatment, showing only 13 and 17% reduction in proliferation rate, respectively (Table 1). The cell cycle distribution of IFN-γ-treated cell cultures was analysed by flow cytometry. Both treated and untreated WiDr cells demonstrated an accumulation of cells in the G0/G1 phase. This may be explained by the long incubation period (72 h), while neither the treatment nor the long incubation time had any clear effect on cell cycle distribution in the OHS, II-11b or PM1 cell lines.

IFN-γ-mediated induction of apoptosis

To study the possible effects of IFN-γ treatment on apoptosis, an immunoreagent-based apoptosis detection kit was utilised. A 3.5- and 5.7-fold increase in apoptosis was seen for the OHS and WiDr cell lines, respectively, as compared to the corresponding control cultures. For the low S100A4-expressing cell line II-11b, a 1.6-fold increase in apoptosis was found, whereas the fraction of apoptotic PM1 cells was unchanged by the treatment. These results are in agreement with the cell survival data. Thus, whereas the II-11b and PM1 were only slightly influenced by IFN-γ, OHS and WiDr cell survival was reduced to approximately 50% (Table 1). Notably, independent of the induction of apoptosis, S100A4 expression was downregulated by IFN-γ in all the cell lines.
Analysis of S100A4 mRNA stability

To study whether IFN-γ-mediated downregulation of S100A4 expression could be explained by decreased mRNA stability, OHS cells were incubated with IFN-γ for 24 h before adding actinomycin D, a general inhibitor of transcription. Cells were harvested at different time points (0–24 h) after the addition of actinomycin D, total RNA was extracted and the amount of S100A4 transcript was analysed by means of Northern blotting and densitometric scanning. As shown in Figure 4, no significant differences in S100A4 mRNA decay between IFN-γ-treated and untreated cultures were demonstrated.

IFN-γ-responsive genes as measured by cDNA microarray

To search for candidate genes involved in IFN-γ-mediated repression of S100A4 transcription, cDNA microarray analysis was performed, focusing on DNA-binding genes/transcription factors/repressors that were significantly up- or downregulated by the treatment. By defining three-fold increase/decrease in both cell lines as a cutoff level, four candidate genes (ISGF3G, ETV5, ZNF133, CEBPG) were identified (Table 2). All four cDNAs have been confirmed by sequencing, and the IFN-γ-mediated upregulation of all four genes as well as IFN-γ-mediated downregulation of S100A4 has been verified by Northern blotting. Whether any of these four factors are involved in IFN-γ-mediated regulation of S100A4 remains to be determined.

Table 1

| Cell line | IFN-γ (U ml⁻¹) | Hours of treatment | % cells in G0/G1 (s.d.) | % cells in S (s.d.) | % cells in G2/M (s.d.) | MTS assay (proliferation) % of control (s.d.) | Apoptosis fold induction (s.d.) |
|-----------|-----------------|--------------------|------------------------|-------------------|----------------------|------------------------------------------|--------------------------|
| OHS       | —               | 0                  | 46 (7)                 | 16 (6)            | 38 (7)               | 100                                      | 1                        |
|           | 10              | 0                  | 55 (10)                | 11 (6)            | 43 (7)               | 52 (15)                                  | 3.5 (2.4)                |
| II-11b    | —               | 0                  | 38 (6)                 | 4 (11)            | 51 (10)              | 100                                      | 1                        |
|           | 10              | 0                  | 48 (7)                 | 15 (7)            | 41 (5)               | 87 (13)                                  | 1.6 (0.6)                |
| PM1       | —               | 0                  | 68 (4)                 | 14 (5)            | 18 (1)               | 100                                      | 1                        |
|           | 200             | 72                 | 70 (8)                 | 9 (5)             | 20 (7)               | 83 (5)                                  | 1.0 (0.1)                |
| WiDr      | —               | 72                 | 40 (12)                | 18 (6)            | 42 (11)              | 100                                      | 1                        |
|           | 200             | 72                 | 66 (9)                 | 5 (5)             | 29 (12)              | 53 (8)                                  | 5.7 (0.1)                |

Figure 2  Optimisation of the IFN-γ response. (A) Northern blots showing transcriptional regulation of S100A4 in time–response experiments. OHS and II-11b cells were incubated with 10 U ml⁻¹ IFN-γ, and WiDr and PM1 cells were incubated with 200 U ml⁻¹ IFN-γ. Expression of 18S rRNA serves as loading control. (B) Western blots showing regulation of S100A4 and STAT1 by IFN-γ in time–response experiments. Incubation conditions as in (A). Immunostaining of α-tubulin serves as loading control.

Figure 3  Confirmation of active Jak/STAT1 signalling pathway. Western blot stained with antiphospho-STAT1 (Tyr-701). OHS cells were treated with 1000 U ml⁻¹ IFN-γ as indicated. Cells were harvested at indicated time points and analysed by Western blotting as described in Materials and Methods.

Table 1  Cell cycle distribution, proliferation and apoptosis in IFN-γ treated cell cultures

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CONSIDERING THE FUNDAMENTAL BIOLOGICAL EFFECTS INDUCED BY THE MAP KINASE CASCADE, AND ALSO BY TGF-β AND TNF-α, IT WAS SOMETHOW SURPRISING THAT NONE OF THESE SIGNALLING PATHWAYS WERE FOUND TO MODULATE THE TRANSCRIPTION OF S100A4. SEVERAL STUDIES HAVE INDICATED THAT S100 PROTEINS, DUE TO THEIR Ca²⁺-BINDING PROPERTIES, ARE INVOLVED IN REGULATING INTRACELLULAR Ca²⁺ HOMEOSTASIS OR Ca²⁺-DEPENDENT CELL SIGNALLING (BONI ET AL, 1997; DUARTE ET AL, 1998). IN ADDITION, AN INTRACELLULAR RELLOCATION OF S100A4 UPON TREATMENT WITH THAPSIGARGIN HAS BEEN REPORTED (MUELLER ET AL, 1999). WE DID NOT, HOWEVER, OBSERVE ANY CHANGES IN S100A4 EXPRESSION CAUSED BY THAPSIGARGIN, INDICATING THAT NO DIRECT ASSOCIATION BETWEEN TRANSCRIPTIONAL REGULATION OF S100A4 AND Ca²⁺-DEPENDENT CELL SIGNALLING EXISTED IN OUR CELL SYSTEMS. IN AGREEMENT WITH OUR RESULTS, TAKENAGA (1999) SHOWED THAT THE EXPRESSION OF S100A4 IN HUMAN COLON ADENOCARCINOMA CELL LINES WAS REGULATED BY IFN-γ, BUT NOT INFLUENCED BY IL-1, IL-6, VARIOUS GROWTH FACTORS OR IFN-α AND -β. TAKEN TOGETHER, THE EXPRESSION OF S100A4 SEEMS TO BE RATHER STABLE IN CANCER CELLS, AND ONE MIGHT SUGGEST THAT SIGNALLING PATHWAYS REGULATING S100A4 GENE TRANSCRIPTION IN CULTURED CANCER CELLS ARE WELL DEFINED, WITHOUT EXTENSIVE CROSSTALK BETWEEN THE DIFFERENT PATHWAYS. WHETHER THIS IS THE CASE IN CANCER CELLS IN VIVO REMAINS TO BE INVESTIGATED.

**IFN-γ INDUCED APOPTOSIS BUT NO CELL CYCLE ARREST**

IFN-γ may inhibit cell proliferation and induce cell cycle arrest and/or apoptosis through diverse mechanisms. Since no differences in cell cycle distribution between treated and control cell cultures were revealed, we propose that downregulation of S100A4 expression could not be explained by IFN-γ-mediated cell cycle arrest.

Using an immunoreagent-based apoptosis detection kit, we found that the two cell lines, OHS (high expression of S100A4) and WiDr, were more susceptible to IFN-γ-induced apoptosis (up to five-fold increase) than the low S100A4 expressing II-11b and PM1 cell lines. Notably, the latter was completely resistant to IFN-γ-induced apoptosis. Recently, GRIGORIAN ET AL (2001) REPORTED A SIGNIFICANT INVERSE CORRELATION BETWEEN EXPRESSION OF WILD-TYPE p53 AND S100A4 IN A PANEL OF TUMOUR CELL LINES, AND SUGGESTED THAT S100A4 COULD COOPERATE WITH WILD-TYPE p53 IN TRIGGERING APOPTOSIS (GRIGORIAN ET AL, 2001). PM1 IS THE ONLY CELL LINE IN OUR STUDY HARBOURING WILD-TYPE p53 (UNPUBLISHED). ACCORDING TO OUR RESULTS, WILD-TYPE p53 COMBINED WITH A LOW EXPRESSION OF S100A4 PROTECTED THE CELLS AGAINST IFN-γ-MEDIATED APOPTOSIS. INTERESTINGLY, II-11b CELLS WITH A LOW EXPRESSION OF S100A4 WERE SIGNIFICANTLY LESS SUSCEPTIBLE TO IFN-γ-MEDIATED APOPTOSIS THAN THE HIGH EXPRESSING COUNTERT PART, OHS. WHETHER THESE DIFFERENCES IN INDUCTION OF

**DISCUSSION**

**TRANSCRIPTIONAL REGULATION OF S100A4**

Transcriptional regulation of S100A4 in response to treatment with different signal inducers was examined in cell lines expressing different levels of the protein. IFN-γ-MEDIATED DOWNREGULATION OF S100A4 WAS OBSERVED IN ALL THE EXAMINED CELL LINES, BUT THE SENSITIVITY VARIED WITH RESPECT TO DOSE AND TIME OF INCUBATION. WHEN EXPOSED TO A LOW-DOSE IFN-γ (AND FOR A SHORTER INCUBATION PERIOD), STAT1 WAS INDUCED IN ALL FOUR CELL LINES, INDICATING THAT THE RECEPTOR IS PRESENT, AND THAT THE EXTENT OF S100A4 TRANSCRIPTIONAL DOWNREGULATION COULD NOT BE ATTRIBUTED TO A GENERAL DIFFERENCE IN IFN-γ SENSITIVITY.

**Figure 4** Effect of IFN-γ on S100A4 mRNA stability in OHS cells. Northern blot analysis demonstrating S100A4 mRNA stability in OHS control cultures and cultures treated with 10 U ml⁻¹ IFN-γ. The cells were then harvested at different time points as indicated. Isolation of total RNA and Northern blotting were performed as described in Materials and Methods. The chart is a graphical presentation of the band intensities as measured by means of densitometric scanning. The figure shows one of three independent experiments with similar results.

γ-mediated repression of S100A4 expression is currently under investigation.

| ID       | ID name  | Common name                          | Functiona | Ratiob |
|----------|----------|--------------------------------------|-----------|--------|
| 724588   | ISGF3G   | Interferon-stimulated transcription factor 3 gamma, p48, IRF-9 | Responsible for the initial stimulation of IFN-α- and β-responsive genes, it recognises and binds to the IFN-stimulated response element, or ISRE within the regulatory sequences of target genes. ISGF3 plays a primary role in the transmission of a signal from the cell surface to the nucleus. | 9       |
| 796542   | ETV5     | ERM, ETS-related protein              | Binds to DNA sequences containing the consensus nucleotide core sequence ggaa | 6       |
| 50794    | ZNF133   | Zinc-finger protein 133               | May be involved in transcriptional regulation as a repressor | 4       |
| 455121   | CEBPγ    | CCAAT/enhancer binding protein gamma  | Transcription factor that binds to the enhancer element PRE-i (positive regulatory element-i) of the IL-4 gene. Might change the DNA-binding specificity of other transcription factors and recruit them to unusual DNA sites | 4       |

*Descriptio of genes are modified from Rebhan et al. (1997). URL: http://bioinformatics.weizmann.ac.il/cards. OHS and II-11b cells treated with IFN-γ (10U ml⁻¹; 48h) vs untreated control, average ratio from four hybridisations.
apoptosis can be ascribed to the disparate expression level of S100A4 is currently under investigation. Importantly, expression of S100A4 was downregulated in all four cell lines, independent of the level of apoptosis. Based on this observation, we concluded that the observed repression of S100A4 was not caused by IFN-γ-mediated apoptosis.

IFN-γ mediated no change in S100A4 mRNA stability

The observed IFN-γ-mediated downregulation of S100A4 might be caused by decreased mRNA stability, or alternatively by repression of transcriptional activity. In contrast to previous results (Takenaga, 1999), treatment of the OHS cells with actinomycin D revealed no significant difference in RNA stability upon the addition of IFN-γ. One possible explanation for this discrepancy is that different cell lines were used. We concluded that in OHS cells, IFN-γ-mediated downregulation of S100A4 was most likely caused by repression of S100A4 gene transcription and not a result of decreased mRNA stability.

Candidate genes involved in repression of S100A4 transcription as revealed by microarray analysis

Our results indicated that the S100A4 promoter carries elements/regions that are susceptible to IFN-γ-mediated signaling. Notably, the time gap between phosphorylation of STAT1 protein (within a few minutes) and detectable reduction of S100A4 transcription (24 h) suggested that the latter response was not a direct effect of STAT1 binding to elements in the S100A4 promoter. In line with this, no IFN-GAS was found in the promoter comprising 2500 base pairs (bp) upstream of ATG. Several other proteins may interact with STAT1 and modulate its transcriptional activity, for example, the histone acetyltransferases CBP/p300 and the tumour suppressor BRCA1 (Zhang et al, 1996; Ouchi et al, 2000). It has also recently been published that IFN-γ may activate other signalling pathways, like the MAP kinases, independent of STAT1 activation (Gil et al, 2001; Ramana et al, 2001). This complex picture of proteins involved in the regulation of IFN-γ-induced gene expression led us to perform microarray analysis in an attempt to identify candidates participating in the observed repression of S100A4 gene expression. We identified four genes, of which ISGF3G (IRF-9/p48) was the most pronounced upregulated transcript (nine-fold), while a member of the CCAAT-enhancer-binding protein family, CEBP-gamma (CEBPG), was upregulated four-fold. Notably, it was recently shown that another member of the family (CEBPG) was transcriptionally induced by IFN-γ, and subsequently regulated the transcription of ISGF3G through the IFN-γ-activated transcriptional element (GATE) (Roy et al, 2002). Members of the CEBP family have been shown to interact with CBP/p300 and members of the Ets family of transcription factors (Mink et al, 1997; McNagny et al, 1998). ETF5, which is an Ets family member, was found to be upregulated more than six-fold in our IFN-γ-treated cells. ZNF133 has previously been associated with transcriptional repression in other cell systems (Visging et al, 1995). We were, however, not able to find binding sites for ZNF133 or ISGF3G when examining 1000 bp upstream of the human S100A4 translation start site. In contrast, several potential binding sites for factors of the CEBP family, Ets family and p300 were detected (Schug and Overton, 1997).

In conclusion, we have found that IFN-γ downregulates S100A4 expression in osteosarcoma, breast and colon carcinoma cell lines. Our results indicate that this downregulation was mediated by inhibition of the S100A4 gene transcription, and not, as previously reported, caused by the IFN-γ-mediated decrease of S100A4 mRNA stability. S100A4 transcriptional activity was repressed independently of IFN-γ-mediated apoptosis and cell cycle effects. Therefore, we are currently investigating the S100A4 promoter in order to identify responsible transcriptional elements and mechanisms involved in IFN-γ-mediated inhibition of S100A4 gene transcription.

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