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Unresponsiveness to interferon associated with STAT1 protein deficiency in a gastric adenocarcinoma cell line

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Abstract HC class I expression can be up-regulated by interferons (IFN) and other cytokines. Both IFNα and IFNγ have been shown to exert their effects via a recently discovered signalling pathway by inducing tyrosine phosphorylation of their receptors. Receptors for interferons and other cytokines signal through the action of associated protein tyrosine kinases of the JAK family (Janus kinase) and latent cytoplasmic transcriptional activators from the STAT family (signal transducers and activators of transcription). Here we report a gastric adenocarcinoma cell line, AGS, that is defective in its response to either IFNα or IFNγ. AGS cells display selective alterations only in MHC class I inducibility and not in constitutive MHC class I expression. In nuclear extracts of AGS cells, no binding activity to interferon-responsive elements (GAS/ISRE) was observed. We found that AGS cells showed an extremely low level of STAT1 expression, which may be responsible for the absence of biological response to IFN. Because STAT1-deficient cells are highly sensitive to infection by virus, the absence of these proteins may also contribute to the tumor phenotype, giving the tumor a selective advantage, by inhibiting cell growth suppression mediated by IFN and abetting escape from the T cell antitumor response.

Key words Gene expression · Promoter · Transcriptional factors · Interferon response elements

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Introduction

The major histocompatibility complex (MHC) is a group of polymorphic membrane proteins involved in antigen recognition by immune cells [29]. MHC class I molecules are needed for endogenous antigen processing and target cell recognition by cytotoxic lymphocytes. MHC class I genes are constitutively expressed in nucleated cells, and are inducible by cytokines [3].

Cytokine-mediated MHC class I molecules are expressed as a result of transcriptional factors that act through class I gene regulatory elements [17, 32, 35]. Recent findings regarding tyrosine kinases of the JAK (Janus kinase) family and STAT (signal tranducers and activators of transcription) mediators have provided a nexus between cytokine action and gene activation [6, 16]. Type I (α/β) or type II (γ) interferons (IFN) can increase MHC class I expression through a transcriptional mechanism [20, 21, 32] IFNα binds to its specific membrane receptor; this results in activation of the enzymes JAK1 and TYK2, and in STAT1 and STAT2 phosphorylation. STAT1 and STAT2 are, respectively, p91/84 and p113 components of the transcriptional factor ISGF-3 [9, 10, 21]. ISGF-3 binds the interferon-stimulated response element (ISRE) in the nucleus of IFNα-treated cells and stimulates their transcription. JAK1, JAK2 and phosphorylation of STAT1 mediate IFNγ induction. Dimeric STAT1 molecules bind GAS regulatory sequences [33, 34]. Therefore, JAK1 and STAT1 are common elements of the IFNα/β and -γ signal transduction pathways [6].

IFNα/β increases MHC class I expression because ISGF-3 binds the class I ISRE regulatory sequence. Otherwise, IFNγ induction of MHC class I expression is mediated by IRF-1, the transcription of which is regulated through the GAS element [28].

MHC class I induction can increase the efficiency of recognition by T lymphocytes [18, 37]. MHC class I down-regulation is frequently observed in tumor cells [11, 12]. We studied the human tumor cell line AGS, in

which the absence of detectable levels of STAT1 may explain its unresponsiveness to IFNα or IFNγ. STAT1 appears to be specific for IFN pathways [8] and its absence in tumor cells may provide an additional mechanism by which the cells avoid an antitumor response [14].

Materials and methods

Cell cultures

The cell lines used in this study were AGS (derived from human gastric carcinoma), and HeLa (cervical carcinoma). Both cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, Md.). Cells were grown in RPMI medium (Gibco, Paisley, Scotland), supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 20 mM L-glutamine, 3.5 mg/ml sodium bicarbonate, 4.5 g/l glucose, 250 U/ml ampicillin and 20 mg/ml streptomycin. Cells were treated with either 500 U/ml IFNγ (Amersham, Little Chalfont, UK), or 1000 U/ml IFNα (Roferon-A, Basel, Switerland) and incubated for 30 min or 120 min for immunoblotting assays and for 72 h for immunofluorescence studies.

Monoclonal antibodies (mAb)

To test MHC class I expression on the cell surface before and after IFN treatment we used the mAb W6/32, which recognizes assembled HLA-A, B and C locus products. In immunoblotting assays the mAb used were anti-JAK1, which recognizes the amino acids 551–766 of the JAK1 protein (Transduction Laboratories, Lexington, Kentucky); anti-STAT1 mAb against the C-terminal fragment corresponding to amino acids 592–731 of STAT1 (Transduction Laboratories), anti-(IFNα/β receptor) against a fragment corresponding to amino acids 27–210 of human IFNα/β (Trafnsduction Laboratories) and anti-(IFNγ receptor) mAb (Genzyme, Cambridge, Mass.).

Cytofluorimetric analysis

MHC class I expression on the cell surface was determined using a standard indirect immunofluorescence method. Cells (10^5) were washed three times in phosphate-buffered saline (PBS), then incubated with the appropriate mAb for 30 min at 4 °C. Excess antibody was removed by washing three times in ice-cold PBS. Cells were stained with 1 μg fluorescein-isothiocyanate-labelled rabbit antimouse-[F(ab′)$_2$] immunoglobulin (Cappel, West Chester, Pa.). They were then washed again three times in PBS, and analyzed on a FACSort flow cytometer (Becton-Dickinson, San Jose, Calif.). Ten thousand events were collected and analyzed.

Plasmid construction

The plasmid A2-pXP1, containing HLA-A2 gene regulatory sequences upstream of the luciferase gene in the vector pXP1 [26], was made as follows. A fragment containing the HLA-A2 promoter region was obtained by polymerase chain reaction (PCR) amplification from the DNA of AGS cells, using the primers A201 Fw (5′-GAGCGCTTGGCACAGAAGCAG-3′) and A201 Bw (5′-AGTGGGCCTTCACTTTCCGTGTCTCCCC-3′). After PCR we obtained a 724-bp fragment from position − 312 to 412 of the HLA-A2 gene. This amplified fragment was used to generate a 224-bp fragment containing the enhancer elements in a second PCR reaction using A201 Fw and a nested primer HLA-A202Bw (5′-GCAAGTCGACACCCAATGGGAGTGAGAAC-3′), that contains a *Sal*I restriction site. The amplified fragment was digested with *Sal*I and ligated upstream of the luciferase gene in the pXP1 reporter plasmid cut with *Sma*I/*Sal*I (Fig. 2). The nucleotide sequence of the fragment was verified using the dideoxy-DNA sequencing procedure [1].

Transient transfection assays

Cells were transfected by electroporation (Electro Cell Manipulator 600, BTX Inc., San Diego, Calif.) as described [5]. Cells were adjusted to 3×10^6 cells/ml in HeBS medium (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose) and electroporated in a low-voltage mode (field strengths < 2.5 kV/cm), 130 V, 500 μF and 13 Ω in 500 μl HeBS medium in 2-mm cuvettes. Samples comprising 50 μg A2-pXP1 and 50 μg of pSV-β-galactosidase (Promega, Madison, Wis.) as the internal control were also included in the electroporation mixture to normalize transfection efficiencies. Luciferase activity was determined using the Luciferase assay system (Promega) and measured by luminometer (Lumat LB9501, Berthold). β-Galacosidase activity was evaluated using the β-galactosidase enzyme assay system (Promega) and measured by absorbance at 420 nm. Cells were stimulated 24 h after transfection with either IFNα or IFNγ prior to luciferase activity determination.

Cytoplasmic extracts and immunoblotting

Cytoplasmic protein extracts were obtained from 2×10^6 cells boiled for 5 min in 1 ml sample buffer (125 mM TRIS/HCl pH 6.8, 2% sodium dodecyl sulfate, 5% glycerol, 1% 2-mercaptoethanol), and the concentration was measured using a BioRad protein assay kit. A 10-μg sample of protein extract was electrophoresed through an 8% acrylamide gel with TRIS/glycine/SDS buffer (BioRad) (25 mM TRIS, 192 mM glycine, 0.01% SDS pH 8.3) at 10 V/cm, and then transferred to a polyvinylidene difluoride membrane in a semidry transfer system (transfer buffer: 25 mM TRIS, 190 mM glycine, 15% methanol) at 70 V/cm^2. After electrophoresis, the membrane was rinsed in blocking buffer (10 mM TRIS pH 7.5, 100 mM NaCl, 0.1% Tween 20, 5% bovine serum albumin) overnight at 4 °C. Immunodetection was performed by adding diluted anti-STAT1 (1:2000), anti-JAK1 (1:1000), anti-(IFNα/β receptor (1:2000), or anti-(IFNγ reeptor (1:400) mAb. The membranes were washed by agitation for 30 min with fresh changes of wash buffer every 5 min (wash buffer: 10 mM TRIS pH 7.5, 100 mM NaCl, 0.1% Tween 20). They were then incubated with biotin-conjugated sheep anti-(mouse Ig) (Boehringer Mannheim, Barcelona, Spain) diluted at 1:2000, and washed again. The blot was developed with 5-bromo-4-chloro 3-indolyl phosphate and 4-nitrobluetetrazolium chloride. As positive controls we used extracts from human endothelial cells for IFN receptor detection, and from A432 (human epidermoid carcinoma) and HeLa cells for JAK1 and STAT1 detection.

Cellular extracts and gel-mobility-shift assays

Cell nuclear extracts were prepared as described [7] with minor modifications [31], from nuclei isolated from lysed cells. The protein concentration in the nuclear extracts was determined using the BioRad protein assay kit (Richmond, Calif.). A typical 20-μl reaction involved 5–10 μg nuclear protein, 1 μg poly(dI′dC) (Pharmacia, Uppsala, Sweden), 12 mM HEPES (pH 7.6), 10 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 3.5% glycerol, and 20000 cpm probe (0.05 ng). In competition experiments 50 ng or 100 ng double-stranded unlabeled competitor oligonucleotide was added to the reaction 5 min before addition of the probe. All reactions were done at room temperature. The products were electrophoresed through 4% low-ionic-strength acrylamide gels (29/1), 0.4 × TBE (1 × TBE = 0.089 M TRIS base, 0.089 M boric acid, 0.93 g/l Na_2 EDTA), fixed in 10% acetic acid, dried and exposed on XAR film (Kodak) at −80 °C.

The duplex oligonucleotides used (Table 1) were GAS/ISRE consensus oligonucleotide, containing overlapped GAS and ISRE

Table 1 Oligonucleotide probes used in electrophoretic-mobility-shift assays. *GAS*, *ISRE* interferon-stimulated response element

Oct-1	5′-AATTGCATGCCTGCAGGTCGACTCTA- GAGGATCCATGCAAATGGA- CGTACGGACGTCCAGCTGA- GATCTCCTAGGTACGTTTACCT- -TCCCCGGGTACCGAGCTC-3′ -AGGGGCCCATGGCTCGAGTTAA
GAS/ISRE consensus	5′-AAGTACTTTCAGTTTCATATTACTCTA-3′ TTCATGAAAGTCAAAGTATAATGAGAT
GAS/ISRE mutant	5′-AAGTACTTTCAGTGGTCTATTACTCTA-3′ TTCATGAAAGTCACCAGATAATGAGAT

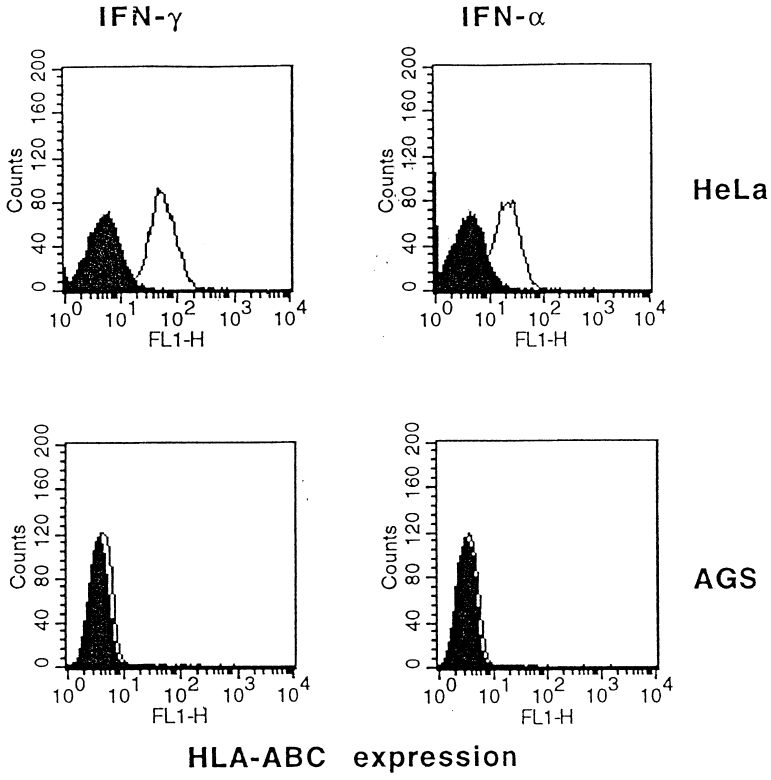

Fig. 1 Flow-cytometric assays of AGS and HeLa cells after immunofluorescence staining with mAb W6/32. Cells were incubated with medium alone (■) or with interferons (IFN; □), and stained with mAb. Data were obtained using a FACSort flow cytometer. HLA class I expression was not induced by IFN in AGS cells

sequences; GAS/ISRE mutant oligonucleotide with a TTCA→GGTC substitution in the overlapping region of the GAS- and ISRE-DNA-binding elements (Santa Cruz Biotechnology Inc.) and Oct-1. Double-stranded oligonucleotides corresponding to GAS/ISRE elements were labelled with (γ-^{32}P)dATP using T4 polynucleotide kinase; Oct-1 was labelled with (α-^{32}P)dATP by the filling-in reaction using Klenow enzyme. The specificity of binding was also examined by competition with unlabelled oligonucleotides.

Results

AGS MHC class I expression cannot be induced by IFN

We have previously demonstrated that, in the AGS cell line, MHC class I antigen expression is not induced in response to IFN in fluorocytometry and Northern blot analyses [1]. As shown in Fig. 1, after IFN treatment, AGS showed no MHC class I antigen induction in comparison with HeLa cells. In order to rule out the possibility that the defect in IFN induction was due to a defect in the *cis*-regulatory element of MHC class I genes, transient transfection was performed. The results of a representative experiment using the AGS HLA-A2 enhancer A/ISRE region fused to a reported luciferase gene, are shown in Fig. 2. Transcriptional activation was calculated with reference to the activity obtained under non-induced conditions. Luciferase activity was about threefold greater in response to IFNα, and about two-fold greater in response to IFNγ in Hela cells; no significant differences were obtained in AGS cells. As expected, the HLA-A2 promoter was active, under non-inducing conditions in both AGS and HeLa cells.

AGS IFN unresponsiveness is not due to the absence of receptors

Although the inability of a cell to respond to IFN could be due to the absence of both types of IFN receptors, this possibility did not seem likely. In fact, in immunoblotting assays with specific IFNα/β and -γ receptor mAb (Fig. 3), protein extracts from AGS yielded bands similar to those obtained with the extracts used as

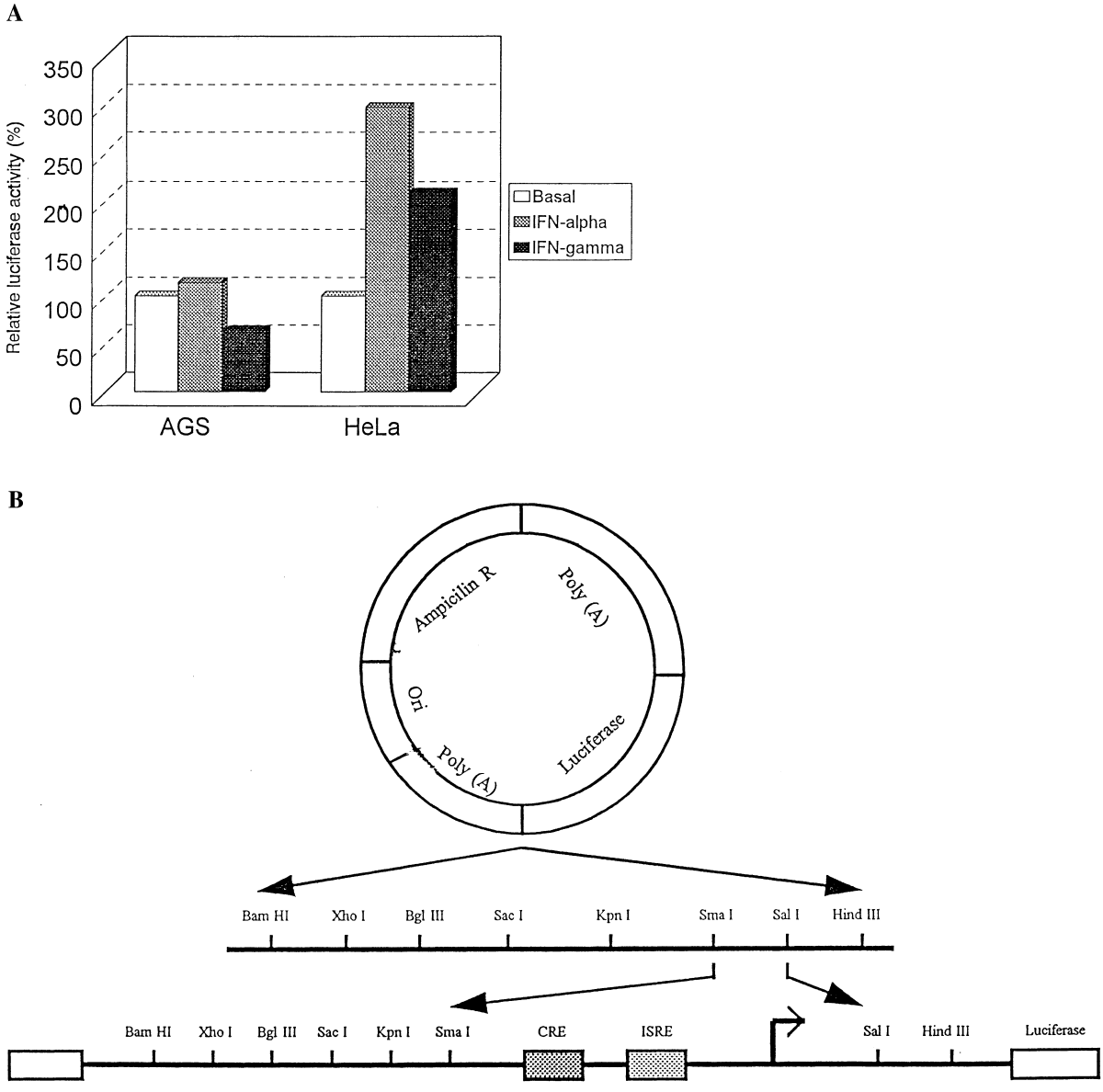

Fig. 2 **A** Functional analysis of HLA-A2 promoter activity in AGS and HeLa transfected cells. A fragment containing the promoter sequences was obtained from AGS DNA and cloned 5′ to the luciferase gene at *Sma*I/*Sal*I sites in the pXP1 reporter plasmid. Cells were stimulated with either IFNα or IFNγ prior to the assay for luciferase activity. The results of a representative experiment are shown, in which transfection mixtures were tested in triplicate. Comparative basal and IFN-treated luciferase activities are expressed relative to basal conditions (100%) after normalization to β-galactosidase activity measured in the same sample. **B** Diagram of the HLA A2 luciferase reporter construct

controls: a 102-kDa band for the IFNα receptor and a 80- to 90-kDa for the IFNγ receptor.

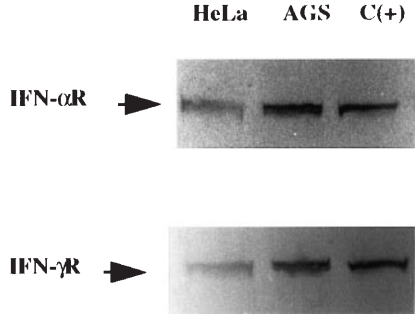

Fig. 3 Immunoblot analysis of cellular IFNα/β and IFNγ receptors. Protein extracts were quantified as described in materials and methods. Specific antibodies against IFNα/β and IFNγ receptors were used. Protein extracts from AGS, HeLa and human endothelial cells were compared. Specific bands are indicated by arrows

IFNα and -γ do not induce ISRE/GAS binding activity in AGS

Transcriptional induction of IFN is related to a *cis*-acting factor that binds to the ISRE and GAS elements

of inducible genes. Both ISRE and class I regulatory element (CRE) are necessary for IFN induction [17], and are conserved in AGS MHC class I genes [1]. We found that both functioned normally in transient transfection assays (Fig. 2). This finding may be explained by the absence of nuclear transcriptional factors binding to the interferon response element. We therefore investigated IFN-dependent DNA binding factors in electrophoretic-mobility-shift assays. Cells were exposed to IFNα and IFNγ for 30 min and 2 h, and nuclear extracts were obtained. When AGS cells were treated with IFN, nuclear extracts were incapable of binding to ISRE/GAS signal-response DNA elements, in contrast with the results in Hela cells (Fig. 4). An ISRE/GAS mutant probe was unable to detect DNA-protein complex in Hela extracts (Fig. 5). Competition experiments confirmed the specificity of the band. In addition, to rule out the possibility that the absence of binding activity in AGS was due to the quality of the protein extracts, we measured Oct-1 binding activity in both cells. Similar binding activity was obtained in extracts from IFN-treated and untreated cells (Fig. 6).

AGS cells are STAT1-deficient

Although IFNα and IFNγ bind different receptors, JAK1 tyrosine kinase and STAT1 protein are two common elements in IFNα and -γ pathways, and are activated after treatment by each ligand. The lack of these common factors originates from mutant lines unresponsive to both cytokines [22, 27]. The absence of specific ISRE/GAS binding activity in AGS cells may be related to the unresponsiveness to IFN, and may reflect the lack of an IFNα- and IFNγ-dependent transcriptional factor. To investigate common IFN signal-transduction pathway mediators, Western blot analyses were done. Immunoblotting assays using mAb specific to JAK1 and STAT1 showed that total JAK1 levels were similar in AGS cells and controls. In contrast, the level of STAT1 proteins in AGS cells was almost undetectable (Fig. 7).

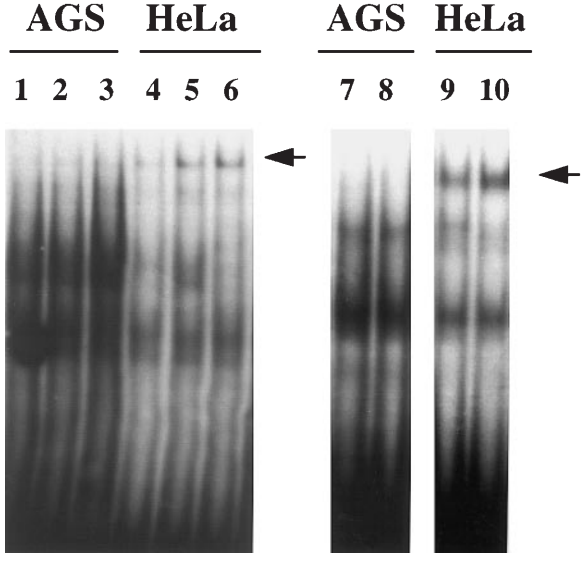

Fig. 4 Interferon-stimulated response element (GAS/ISRE) binding acitivity in AGS and HeLa cell nuclear extracts under basal conditions (*lanes 1*, *4*) and after 2 h of IFNα (*lanes 2*, *5*) or IFNγ treatment (*lanes 3*, *6*). Cells were also treated for 30 min with IFNα (*lanes 7*, *9*) or IFNγ (*lanes 8*, *10*). The probe used was GAS/ISRE consensus. Only the upper migrating complex was specific according to competition analysis. GAS/ISRE binding activity (marked by arrow) was detected in IFNα-treated (*lanes 5*, *9*) and IFNγ-treated (*lanes 6*, *10*) HeLa cells, but not in IFN-treated AGS cells (*lanes 2*, *3*, *7*, *9*). DNA binding activities detected at 30 min were stronger than in cell extracts treated for 2 h

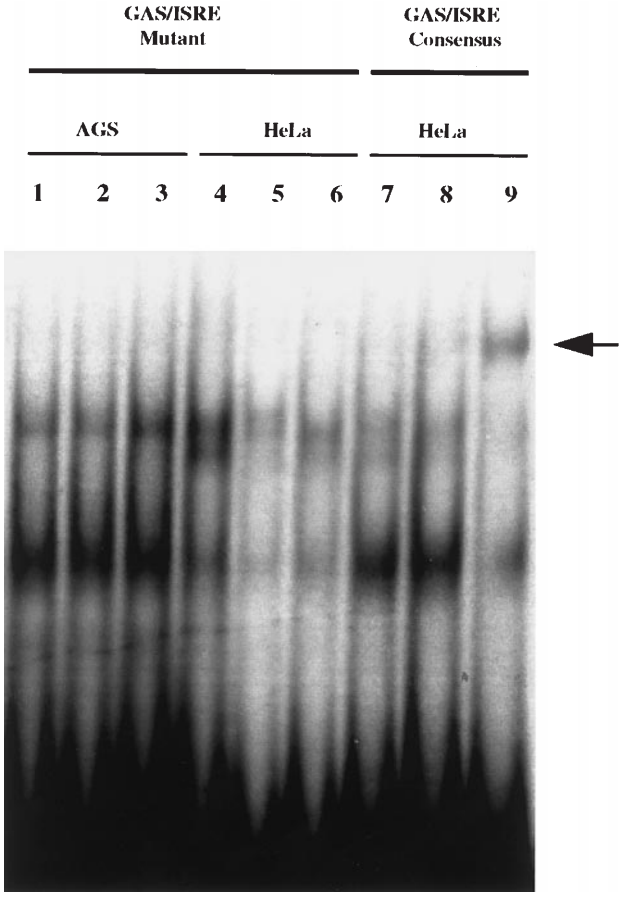

Fig. 5 Electrophoresis mobility shift assay (EMSA) using a mutated GAS/ISRE probe. Point mutations within GAS/ISRE prevented the binding of transcriptional factors. EMSA were carried out using nuclear extracts from AGS and HeLa cells under basal conditions (*lanes 1*, *4*), and after 30 min of IFNα (*lanes 2*, *5*, *7*, *9*) or IFNγ treatment (*lanes 3*, *6*, *8*). The probes used were GAS/ISRE mutant (*lanes 1–6*) and GAS/ISRE consensus (*lanes 7–9*). The GAS/ISRE mutant probe did not detect any binding activity (*1–6*). In competition experiments, a 100-fold unlabelled GAS/ISRE consensus probe removed the binding activity in HeLa extracts (*lanes 7*, *8*). In contrast, competitive pre-absortion with GAS/ISRE mutant did not remove GAS/ISRE consensus binding activity from IFNα-treated HeLa cell extract [9]. The gel shows a representative assay from three independent experiments

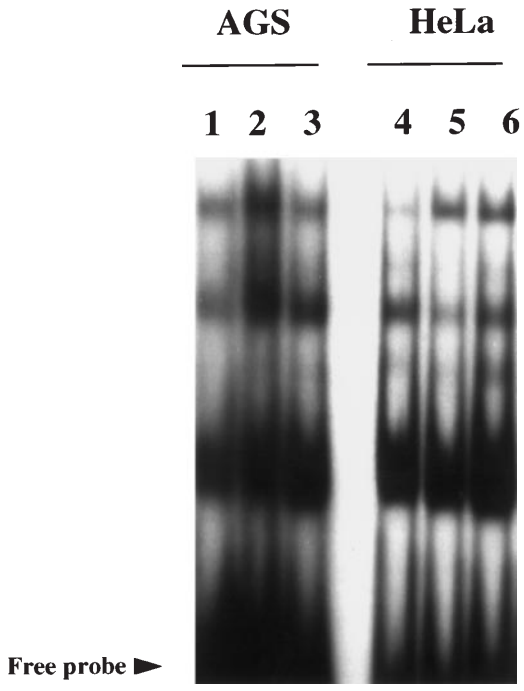

Fig. 6 Binding of nuclear extracts from AGS and HeLa cells to the octamer motif (probe Oct-1). Electrophoretic-mobility-shift assays were done with nuclear extracts from IFN-treated or untreated cells. Extracts were obtained from cells under basal conditions (*lanes 1*, *4*), and after IFNα (*lanes 2*, *5*), or IFNγ treatment (*lanes 3*, *6*)

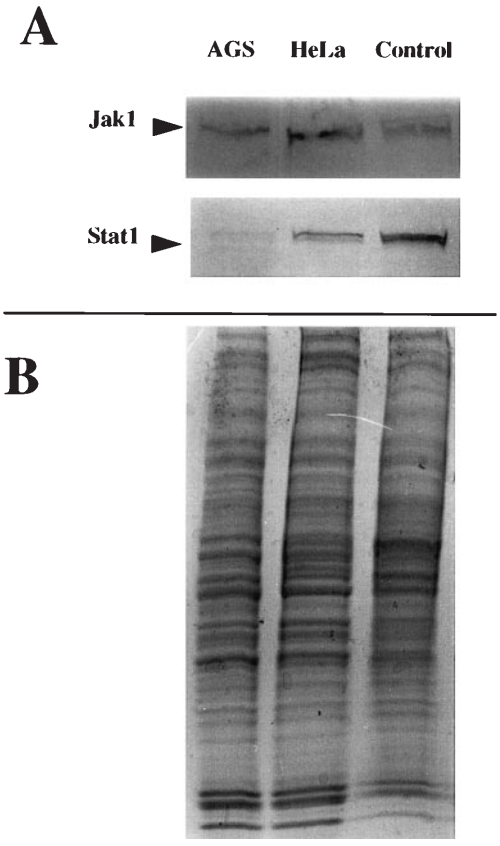

Fig. 7A, B Immunoblotting assays of JAK1 and STAT1 mediators, showing that AGS cells produced a limited amount of STAT1. **A** The results of probing the membrane with anti-JAK1 and anti-STAT1 mAb. **B** A simultaneous electrophoresis done with the same amounts of protein extracts as used for immunoblotting, and stained with Coomassie blue. *C+* extracts from A432 (human epidermoid carcinoma) and HeLa cells for JAK1 and STAT1 detection

Discussion

Interferons are pleiotropic cytokines that can be induced in a variety of cells. After IFN treatment, a multiple combination of STAT complexes translocates to the nucleus and activates transcription by binding to specific promoter elements [15, 19]. Targeted disruption of the STAT1 gene transforms cells into an IFN-unresponsive state, making them susceptible to viral disease [12, 23]. In addition, antitumor T cells could be grown only from cells with high class I expression cells, suggesting that the recognition of tumor cells by antitumor T cells is enhanced by specific up-regulation of molecules related to antigen processing and presentation [30]. This may occur because optimal IFNγ production is required to generate Th1 $CD4^+$ cells and cytotoxic T lymphocytes [37].

We report here an IFNα-, IFNγ-unresponsive state in a human tumor cell line, AGS, derived from a gastric adenocarcinoma. This cell line was previously found to lack MHC class I inducibility (Fig. 1). We show here that MHC class I inducibility is not the result of a deficiency in IFN receptors and a similar level of expression was obtained in protein extracts from HeLa and human endothelial cells used as controls (Fig. 3). Lack of responsiveness to both IFN is not restricted to MHC class I genes, as other IFN-inducible genes were also affected (not shown). In fact, several findings strongly suggest that the lack of MHC class I inducibility in AGS cells is the result of the absence of transcriptional activation and a failure in the JAK/STAT signal-transduction pathways. As shown in Fig. 2, the HLA-A2-gene-regulatory elements from AGS fused to a luciferase reporter failed to respond to either IFNα or IFNγ in transfected AGS cells, whereas in Hela cells they were activated two- or threefold (Fig. 2). In electrophoretic mobility shift assay experiments with an ISRE/GAS probe, no DNA-protein complexes were found in nuclear extracts from IFN-treated AGS cells in comparison with nuclear extracts from HeLa cells (Fig. 4). DNA binding activity was represented by a specific band, which appeared in IFNα and IFNγ-treated Hela cells. The binding to the ISRE/GAS mutant site was defective for this complex (Fig. 5) and a 100-fold molar excess of the unlaballed probe for the ISRE/GAS mutant site did not compete efficiently for binding of ISRE/GAS in Hela extracts (Fig. 5, lane 9). Because JAK1/STAT1 connects the ligated IFNα and IFNγ receptor and the downstream branch points, we believe that the extremely low level of STAT1 expression may be responsible for the absence of biological responses to the two IFN. The elucidation of the JAK-STAT pathway has been made

possible by the demonstration that mutagenized cells lacking these proteins were unresponsive to IFN. In fact, complete lack of responsiveness to either IFNα or IFNγ has been described in the mutant cell line U4, which contained a truncated JAK1 transcript [24] or in U3 A cells, which were also unresponsive to both IFN and lacked mRNA for the STAT1 protein [25]. In the present study we describe a similar defect in a human tumor cell line. Although the level of expression of JAK1 was similar to that found in control cells (Fig. 7A), a very weak signal was obtained in AGS cells, indicating either that the level of expression of STAT1 was extremely reduced, or that a small proportion of AGS cells within the bulk population maintained normal expression. Recently, similar defects have been shown in a human melanoma cell line [36, 38]. To establish the biological relevance of this finding, further studies will need to search for a similar defect in human tumor tissues. STAT1 protein is essential for cell growth suppression in response to IFN [4], and a lack of responsiveness may be a common phenomenon responsible for reduced cellular responsiveness of melanomas and other tumors to IFN and also contribute to the tumor phenotype tumorigenesis [38]. In addition, MHC class I down-regulation is a widespread phenomenon in tumor biology [13, 2, 12] and may have profound effects on T cell and NK cell antitumoral responses [14]. Unresponsiveness to IFN may also contribute to tumoral escape from T cell responses, because IFNγ production up-regulates HLA class I molecules and certain accessory molecules including ICAM-1, and is required to generate cytotoxic T lymphocytes [37].

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