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Interferon regulatory factor 1 (IRF-1) mediates cell growth inhibition by transactivation of downstream target genes

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ABSTRACT

Interferon regularory factor 1 (IRF-1) is a DNA-binding factor which recognizes regulatory elements in the promoters of interferon (IFN)-β and some IFN-inducible genes. We observed that expression of transfected murine IRF-1 in different mammalian cell lines leads to down-regulation or stop of proliferation depending on the extent of expression. Expression of fusion proteins composed of IRF-1 and the hormone binding domain of the human estrogen receptor does not exhibit IRF-1 activity in the absence of estrogen. However, after estrogen treatment of the cells IFN-β promoters are activated and the cells stop growing. As shown by expression of IRF-1 mutants both functions of the IRF-1-protein require DNA-binding and transcriptional activation. Since secreted factors including IFNs are not responsible for the anti-proliferative effect of IRF-1 we suggest that IRF-1 may be regarded as a negative regulator of cell growth which acts by activation of down-stream effector genes.

INTRODUCTION

IRF-1 is a transcription factor belonging to a family of four DNA-binding factors. Three members of this group, IRF-1, IRF-2 and ICSBP accumulate in cells in response to interferons (IFNs), while ISGF3γ (p48) is activated by IFNs. All have structurally related DNA-binding domains at their N-terminus. IRF-1, IRF-2 and ICSBP recognize a central nine nucleotide core region, the IFN stimulated response elements (ISREs) of various IFN stimulated genes (ISGs) that are closely related to the PRDI of the IFN-β promoters (1, 2, 3). ISGF3γ binding requires an ISRE consensus sequence of more than 9 nucleotides for recognition. The DNA-binding region which IRF-1 shares with the other factors of the IRF-family contains an imperfect array of tryptophan repeats which is similar to the DNA-binding domain of the c-myb encoded oncoprotein, suggesting a common structural motif for DNA recognition (4, 5, 6). IRF-1 is a transcriptional activator: It has an activating domain at its C-terminus which does not show any structural homology with other activators.

The function of IRF-1 is obscure. Originally recognized as IFN regulatory factor it is able to bind to, and upon transient overexpression to induce IFN-β promoters (7, 8, 9, 10). Furthermore, its function as a transactivator seems not restricted to certain IFN type I genes but also to IFN-inducible genes, e.g. the presence of IRF-1 contributes to the constitutive expression of MHC/HLA genes (11). Its overexpression leads to the activation of MHC class I proteins, 2′5′ oligo A synthetase and ISG-15 (9, 11, 12, 13). It also is able to induce the complete set of anti-viral activities which is believed to require transcriptional activation of ISGs (13). The data have provided evidence to regard IRF-1 as a mediator for the diverse biological activities of IFNs. IRF-2 is able to antagonize the transcriptional activation of IRF-1 (8) and ICSBP acts as a negative regulatory factor on ISRE-containing promoters (14, 15, 16).

IRF-1 is transiently induced by IFNs, in particular by IFN-γ (17). In addition, a number of other inducers have been recognized including IL-1, IL-2, IL-6, TNFα, LIF, prolactin, GM-CSF, Concanavalin A, TPA as well as double-stranded (ds) RNA and a number of viruses have been also identified as inducers of IRF-1 and its mRNA (13, 18, 19, 20, 21).

In order to study the multifunctional effects of IRF-1 alone, we tried to stably overexpress IRF-1 in different cell lines. However, all the stable transfectants that were obtained did not demonstrate high levels of expression due to a negative effect of IRF-1 on cell growth. To overcome this growth arrest we have established a system in which the IRF-1 activity is inducible by an external agent, estradiol. This was used to study IRF-1 function within the cell. IRF-1 activation leads to a stop of cell growth and to the transcriptional activation of the IFN-β promoter. This requires DNA-binding and transcriptional activation. Besides its activity as a negative regulator of cell growth it seems that IRF-1 is part of the signal transduction machinery of IFN-mediated cell growth arrest.

MATERIALS AND METHODS

Expression vectors and plasmid constructions

The following expression vectors containing the indicated promoters were used: pMPSVHE (MPSV-LTR) (22), pBHE, pBEH (SV 40 early) (22), pSVM(2)6 (metallothioneine) (23), pGEM2 (phage T7) (Promega), pMT7HE (phage T7 promoter integrated into the MPSV-LTR between the TATA-box and the transcriptional start site).

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The coding region of the murine IRF-1 cDNA gene (7, 24) was amplified by PCR after reverse transcription of Ltk$^-$ RNA and inserted into pMPSVHE using oligonucleotide-born linker sequences. The sequence of the coding region was verified by dideoxy-sequencing. The amplified fragment was inserted into pMPSVHE, pBHE, pSVM(2)6, pGEM2 and pMT7HE in sense orientation and in pBEH and pGEM1 in antisense orientation with respect to transcription from the respective promoters. pMT7-hER was generated by integrating the insert of plasmid HE14 into the MCS site of pMT7HE. This insert encodes the hormone-binding domain of the human estrogen receptor (25). pMT7-hER-IRF-1 was constructed by inserting two PCR fragments into the MCS sites of pMT7HE. The 5′ fragment encodes the above described hER part and additional proline residues at its C-terminal end. Its 5′ end contains a sequence surrounding of the AUG which favours efficient translation initiation. The 3′ fragment contains the complete coding region of IRF-1. pMT7-IRF-1-hER was constructed by inserting the above mentioned fragment encoding IRF-1 with additional proline residues and the hER part into pMT7HE. pMPSV-CAT, pMPSV-LUC are described earlier (22). pT7-IRF-1: A DNA-fragment which contains the coding region of IRF-1 was inserted into pGEM2. The fragment appears in the sense orientation relative to the T7 promoter. pGEM1AS-1: A 400 bp fragment containing the 3′ part of the MPSV promoter from pMPSV-IRF-1 as well as the first 120 bp of the coding region of the IRF-1 gene was inserted into pGEM1. pSVM(2)6-IRF-1: A fragment containing the coding region of IRF-1 was inserted into pSVM(2)6. pMT7-IRF-1: The coding region of IRF-1 was integrated into the MCS of pMT7HE. pT7-Luc: A fragment containing the coding region of firefly luciferase and a polyadenylation site was ligated into pGEM2. pSVM(2)6-Luc: A luciferase fragment of pBHE-Luc (22) was inserted into pSVM(2)6. pMT7-M3: A fragment containing the 5′ region of IRF-1 (aa 1−198) was inserted into the MCS of pMT7HE. pMT7-M6: A deletion of the first 34 amino acids of the coding region of IRF-1 was generated with PCR. The fragment was inserted into pMT7HE. pSV2pac and pAG60 have been described by Vara et al. (26) and Colbère-Garapin et al. (27), respectively. pA15-TKCAT contains a synthetic promoter based on the Herpes Simplex Virus TK promoter which is complemented with 5 repeats of an IRF-1 binding sequence as indicated in Fig. 2.

Cell culture and gene transfer

Ltk$^-$ (ATCC CCL 1.3) (28) and C243 (29) cells were maintained in DME supplemented with 10% fetal calf serum, antibiotics and glutamine if not otherwise stated. DNA was transfected using the calcium phosphate precipitate technique (30). The medium was changed 4 h prior to transfection. Cells for transient expression were seeded in 9.1 cm^2 wells of culture dishes with 2.5 ml of medium. The precipitates composed of 15 μg DNA in a final volume of 0.25 ml were supplied to this medium. It was renewed by fresh medium 20 h post transfection. Cells were harvested 48 h after transfection.

Stable cell clones were obtained by cotransfection of plasmids bearing either the puromycin resistance gene (pSV2pac) or the neomycin resistance gene (pAG60) with the indicated effector and reporter genes in a 1:10 ratio. The seletive medium was added 48 hours after transfection.

Induction protocols

Transiently transfected cells were induced 20 hours after transfection. Induction was done with either 100 μM $ZnCl_2$ and 2 μM $CdCl_2$, 2.5 μM 4-hydroxy-tamoxifen, 2.5 μM 17β-estradiol or antibodies against 2500 U α- and IFN-β or as indicated. In the case of the proliferation determination induction takes place 24 h after seeding of the cells and stopped after the indicated time.

Cells were induced with inactivated *Newcastle Disease virus* (NDV) or mock induced for 1 h in serum free DME (31). Induction was carried out by addition of 10 plaque-forming units of NDV per cell. After the induction time the cells were washed and incubated with DME with serum. Cells were harvested 20 h after induction for CAT assays or after 8 h for RNA preparation. Supernatants were taken for determination of endogenous IFN titers as described earlier (31). In the case of 17β-estradiol induction cells were harvested as indicated.

Reporter gene assay

CAT assays were performed as described earlier (31). Extracts were prepared from transfected cells by freezing and thrawing and taken for determination of CAT enzyme activity. Acetyl CoA was purchased from Pharmacia and [^{14}C] chloramphenicol (spec. act. 50−60 mCi/mmole) was obtained from Amersham. Luciferase activity was measured in a luminometer (Berthold) as desribed by de Wet et al. (32). In transient expression experiments the CAT enzyme activity was normalized to the expression of luciferase or *vice versa*. Additionally, CAT and luciferase activities were normalized according to the cell number as determined by measurement of the protein concentration.

S1 mapping analysis

Total cellular RNA was prepared by the guanidine thiocyanate method as described in Sambrook et al. (33). RNA probes were synthesized *in vitro* using pGEM1AS-1 for IRF-1 antisense transcription. After XbaI cleavage run-off transcription generates a probe that is complementary to the endogenous IRF-1 mRNA for 120 bases and to the recombinant IRF-1 mRNA for 131 bases. The internal standard was obtained by inclusion of *in vitro* transcribed mRNA of pBSPK which contains the murine pyruvate kinase gene to the S1 assay as described earlier (34). S1 analysis was performed according to Sambrook et al. (33). The protected fragments of 2.5 μg total RNA were subjected to a 6% polyacrylamide 8 M urea analysis.

Expression of IRF-1 and derivates *in vitro*

Plasmids pMT7-IRF-1, pMT7-M3 and pMT7-M6 and were linearized with EcoRI. Run-off RNA was produced using the T7 RNA polymerase (Boehringer/M) in the presence of m7GpppG. RNA synthesis was quantified by incorporation of [^{32}P]-GTP (Amersham). For the production of recombinant protein in a rabbit reticulocyte lysate (Amersham) 50 ng RNA template was used. Each translation was done twice, one was done in presence of [^{35}S]-labeled methionine to control the translation efficiency, the other with unlabeled methionine. The labeled proteins were separated on polyacrylamide gels overnight at 50 V, fixed in isopropanol/acetic acid solution, dried and autoradiographed. The rainbow marker (Amersham) served to identify the size of the synthesized proteins. The translation procedure was performed as described by the manufacturer's instructions. Normally 1 μl of a translation reaction was used for a gel retardation assay.

Electromobility shift assay (EMSA)

EMSA analysis was done according to the protocol of Fried and Crothers (35). Proteins were incubated with 20,000 cpm of

labeled $(AAGTGA)_3$ in the presence of 1 μg poly[d(IC)] in 10 mM HEPES pH 8.0, 5 mM $MgCl_2$, 50 mM KCl, 0.025% bromophenolblue, 0.025% Xylen Cyanole, 10% ficoll and 3% glycerol. The samples were loaded on a pre-electrophoresed 6% polyacrylamide gel. After drying, the gels were autoradiographed.

FACS analysis

FACS analysis was performed by using FACSCAN (Becton-Dickinson). A mouse antibody directed against H-$2K^k$ was used to determine the expression of H-$2K^k$ Antigen. An second fluoresceine conjugated goat-anti-mouse antibody (Tago) was used to detect the first antibody.

Proliferation assay

Cells were seeded as indicated. The determination of living cells was done in 96 well plates using the tetrazolium salt conversion to blue formazan (Promega). The tests were done as described in the manufacturer's instructions.

RESULTS

Constitutive overexpression of IRF-1 leads to cell growth inhibition

In order to study the function of the murine interferon regulatory factor 1 (IRF-1), we have tried to overexpress it constitutively in different mammalian cell lines. The number of stable transfectants following transfection experiments in which an IRF-1 expression plasmid is cotransfected together with a selectable marker plasmid is significantly reduced compared to control plasmids (Tab. 1). In the initial experiments IRF-1 was driven by strong constitutive promoters, such as the SV40 early promoter or the LTR from MPSV. Low efficiency transfection rates were not only seen in mouse L cells but also in other mouse and hamster cell lines (C243, BHK-21). Obvious negative effects on the cells upon transient expression of IRF-1 were not detected. Since the low numbers of stable cell clones was not due to contaminations in the DNA preparation of the expression plasmids we concluded that a long-term overexpression of IRF-1 leads to cell damage or a block of proliferation. To exclude the possibility that IRF-1 exerts adverse effects to the target cells *per se* we have tried to express the IRF-1 cDNA gene under the control of weaker promoters or inducible promoters. Tab. 1 shows that even when the IRF-1 gene is expressed under the control of weak promoters like the uninduced metallothioneine promoter or the prokaryotic T7 promoter very few transfectants are obtained in Ltk^- cells. On the other hand, the same cDNA fragment in antisense orientation in the expression plasmid does not lead to any obvious negative effect on transfection efficiency or creation of stable transfectants.

The few cell clones following transfections with IRF-1 cDNA in sense orientation could be explained by the absence or inactivity of the IRF-1 gene. Alternatively, these cell clones could exhibit a very low expression of IRF-1 which is tolerated by the cells. Analysis of 21 clones revealed that some were normally growing, while the rest were more or less retarded in growth. In order to correlate IRF-1 expression with cell growth, we have determined MHC class I protein expression on the cell surface of these transfectants (Fig. 1). Most transfectants (18 from 21) showed slightly elevated levels of MHC class I molecules on their cell surface. In two cell lines (clones E6 and H3), which showed a significant growth inhibition, the presentation of MHC class I molecules on their cell surface was significantly higher compared to the other cell clones (Fig. 1A). This stimulation of MHC class I expression is not due to the production of IFN as a result of IRF-1 overexpression since we could not detect any antiviral activity in the supernatant of these cell clones (data not shown).

S1-analysis revealed that endogenous and exogenous IRF-1 mRNA can be distinguished (Fig. 1B). The figure shows that an extremely low level of endogenous IRF-1 mRNA (not visible) is inducible by virus or IFN-β, which are both weak inducers of IRF-1. Exogenous IRF-1 mRNA is between 8- and 10-fold higher compared to endogenous IRF-1 in non-induced cells and 3-fold higher than in virus-induced cells. Assuming that the efficiency of translation from exogenous and endogenous IRF-1 mRNA is comparable we conclude that a continuous more than ten-fold elevation of endogenous IRF-1 leads to a significant retardation of cell growth.

We have analysed the presence of IRF-1 protein which is synthesized as a consequence of the transfected cDNA. Nuclear extracts of the two cell clones with retarded cell growth and enhanced MHC class I expression were prepared. IRF-1 could be detected by an electromobility shift assay (EMSA) in both cell clones, while endogenous IRF-1 was not visible (Fig. 1C).

Intrinsic transmission of the negative effect of IRF-1 on cell growth

IRF-1 overexpression leads to the activation of several IFN type I genes (10, 36). Therefore, the observed growth inhibitory effect could be explained by IRF-1 induced IFN secretion. IFNs in turn would act on the producer cells and inhibit cell growth. In order to check this possibility we have tried to get stable transfectants of IRF-1 in C243 cell by growing the transfectants in the presence of neutralizing antibodies directed against type I IFNs. The result which is described in Tab. 2 shows that the neutralizing antibodies do not lead to a higher number of IRF-1 transfectants. Furthermore, IFN activity, as determined by the antiviral assay, was not detectable in the supernatants after transfection with

Table 1. IRF-1 exerts a negative effect on cell growth even at low expression levels

Promoter[1]	Luciferase expression[2]	Number of stable transformants[3]
MPSV-LTR	300	<10
SV40 early	100	<10
Metallothioneine	13	<10
Metallothioneine[4] +	64	<10
T7	6	<10
SV40 early antisense IRF-1	–	>200
–	–	>200

1. The cDNA genes of murine IRF-1 and firefly luciferase (32) were integrated into expression plasmids which harbour different promoters: MPSV-LTR (pMPSV-IRF-1, pMPSV-Luc); SV40 early (pBHE-IRF-1, pBHE-LUC); semisynthetic murine metallothioneine (pSVM(2)6-IRF-1, pSVM(2)6-LUC); *E.coli* phage T7 (pT7-IRF-1, pT7-LUC). As a control IRF-1 was inserted in antisense orientation (pBEH-IRF-1). As another control MPSV-vectors without inserts (–) were used.
2. The relative strength of the promoter was tested by measuring luciferase activity in extracts of stable C243 transfectants (clone mixtures) from the luciferase expression plasmids. Expression from the SV40 early promoter was arbitrarily set to 100. Results represent mean values of three individual measurements of luciferase.
3. The amount of stable cell clones from independent transfections with IRF-1 expression plasmid was counted. The results are from three independent transfections with different DNA preparations for determination of stable cell clones.
4. The cells were treated with heavy metal ions as specified in Materials and Methods.

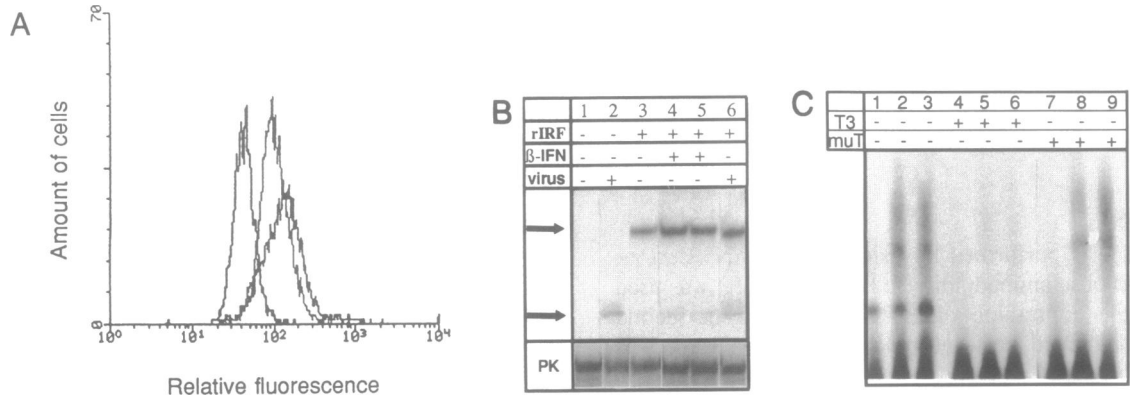


Figure 1. Expression of IRF-1 in stable transfectants. Ltk$^-$ cells were cotransfected with expression plasmids encoding the puromycin resistance gene and the IRF-1 gene (pMPSV-IRF-1). Individual cell clones were picked, expanded and tested for growth rates and MHC class I (H-2K^k) presentation. Two of the slowest growing cell clones (E6 and H3) showed enhanced H-2K^k presentation. Both cell clones were expanded for preparation of nuclear extracts and RNAse protection analysis. **A:**Cell surface expression of MHC class I molecules by indirect immunofluorescence and FACS analysis. The distribution of mock transfected cells (control) and cells of clones H3 and E6 are shown from left to right. **B:**RNAse protection analysis of endogenous and exogenous IRF-1 mRNA. 2.5 μg of total RNA from cells which were treated as indicated were hybridized to the labeled probe. The probe is 131 bp homologous to the spliced mRNA derived from recombinant IRF-1 (upper arrow) and 120 bp to the endogenous mRNA (lower arrow). Mock transfected cells: lanes 1 and 2; clone E6: lanes 3, 5; clone H3: lanes 4, 6. The slots in the bottom row show the protected bands of a simultaneously hybridized pyruvate kinase probe. **C:**Electro mobility shift assay (EMSA) of nuclear extracts from mock transfected cells (lanes 1, 4, 7) and cells from clones E6 (lanes 2, 5, 8) and H3 (lanes 3, 6, 9). The labeled oligonucleotide $[(AAGTGA)]_3$ represents a consensus sequence for IRF-1 recognition. In lanes 4, 5 and 6 competition with 500 fold molar exess of unlabeled oligonucleotide $[(AAGTGA)]_3$ (T3) is performed. In lanes 7, 8 and 9 competition with a mutated sequence $[(GAGTGA)]_3$ (muT) was carried out.

IRF-1. This is substantiated by the fact that stable cell clones which slightly overexpress IRF-1 do not produce IFN but are significantly retarded in cell growth (Fig. 1).

Other factors which are secreted upon IRF-1 overexpression might be responsible for the observed proliferation inhibition. If this would be the case the secreted compounds from the IRF-1 transfectants would be able to inhibit cell growth of cocultivated mock-transfected cells. To control this possibility, transfectants with a selectable marker (puromycin resistance gene) were mixed with cotransfectants containing the IRF-1 gene plus the selectable marker and grown under selective pressure (Tab. 2). The number of stable cell clones obtained in the mixed transfections indicates that the growth inhibitory effect is only seen in the IRF-1 transfectants and is not transmitted to the cocultivated cells. We therefore conclude that the negative effect of IRF-1 to cell proliferation is not a result of an autocrine mechanism but is mediated by an intracellular pathway.

Table 2. The effect of IRF-1 on cell growth is not mediated by secreted factors

Transfection 1	Transfection 2	Number of stable cell clones
Puro	–	176
Puro + CAT	–	126
Puro + IRF-1	–	< 10
Puro	Puro + CAT	304
Puro	Puro + IRF-1	210
Puro + IRF-1*	–	< 10

The cDNA genes encoding puromycin resistance (Puro), chloramphenicol acetyl transferase (CAT) and IRF-1 were integrated into expression vectors (pSV2pac, pMPSV-LUC and pMPSV-IRF-1, respectively). Transfections with indicated plasmids were carried out as described in Materials and Methods. Two days after transfection the cells were detached. 1/3 of transfection 1 and, if stated, 1/3 of transfection 2 were mixed and replated in the presence of selective drug (puromycin). Two weeks later the number of stable cell clones was determined. The asterisks indicates that this culture after replating was kept in the presence of antibodies neutralizing 2500 u/ml of both, murine IFN-β and murine IFN-α.

Hormone-dependent activation of IRF-1 functions by fusion proteins composed of IRF-1 and an estrogen receptor fragment

In order to confirm the interrelationship between IRF-1 expression and cell growth inhibition, conditional IRF-1 expression is required. Transient expression experiments are not acceptable since the transfection by itself affects the IFN system (37) and the amount of the gene product is only available for a restricted time in a variable amount per cell. Since we could not find an inducible promoter which is absolutely silent in the non-induced state in C243 cells we have established a system in which IRF-1 is constitutively expressed but is only activated by external modulators. The binding of steroid hormones to their receptor leads to nuclear translocation and transcriptional activation of target genes. This results in nuclear translocation and an activation of the trans-activating domain (38, 39). This concept was also shown to be valid in fusion proteins (myc, fos, rel, myb, E1A) composed of DNA-binding proteins and the hormone-binding domain of the steroid receptor (40, 41, 42, 43, 44). We have fused the estrogen-binding domain of the human estrogen receptor (hER) (25) either to the N-terminus or to the C-terminus of the complete murine IRF-1 protein (Fig. 2).

To determine whether the fusion proteins act as hormone-dependent transactivators, we transiently transfected IRF-1 and the hER-constructs into murine C243 cells which stably express an IRF-1 inducible reporter gene (A15-TKCAT). The transfected cells showed hormone-dependent activation of the reporter gene in a similar extent to the activation of the unfused IRF-1 protein (Fig. 2). The unfused hER expression construct did not exert any effect on the reporter gene. Thus, it appears that the fusion proteins are estrogen-dependent for transcriptional activation of a reporter gene.

To investigate the effect of the fusion proteins on cell growth we co-transfected the expression constructs encoding the fusion

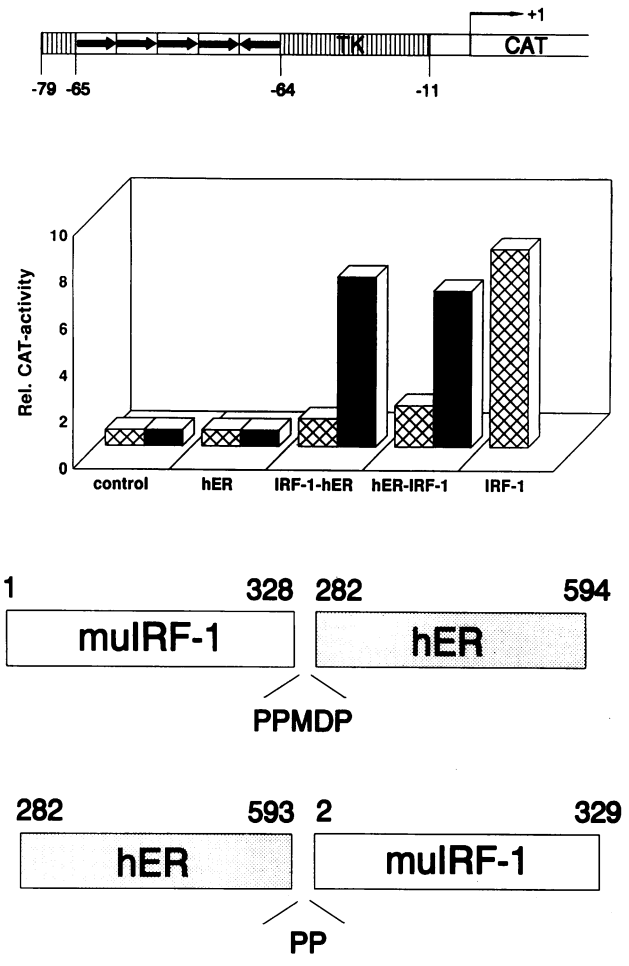


Figure 2. Activation of an IRF-1 responsive synthetic promoter by IRF-1 and IRF-1 fusion proteins. The IRF-1 responsive reporter gene A15-TKCAT (upper) was stably transfected in C243 cells. Each arrow represents the sequence [(AA-GTGA)]$_3$. Expression vectors with the indicated cDNA inserts were transiently super-transfected. After 24 hours the cells were treated for further 24 hours with 2,5 μM 17β-estradiol (black bars) or left untreated (crossed bars). The cells were harvested and CAT-activity was determined in the extracts. The middle part of the figure shows the activity of the reporter gene. The lower part of the figure depicts the structure of the fusion proteins with the complete IRF-1 and the hormone binding domain of the human estrogen receptor (hER). Both represent inserts of a mammalian expression vector used for transient expression. The linker peptides between the protein domains are given in single letter code.

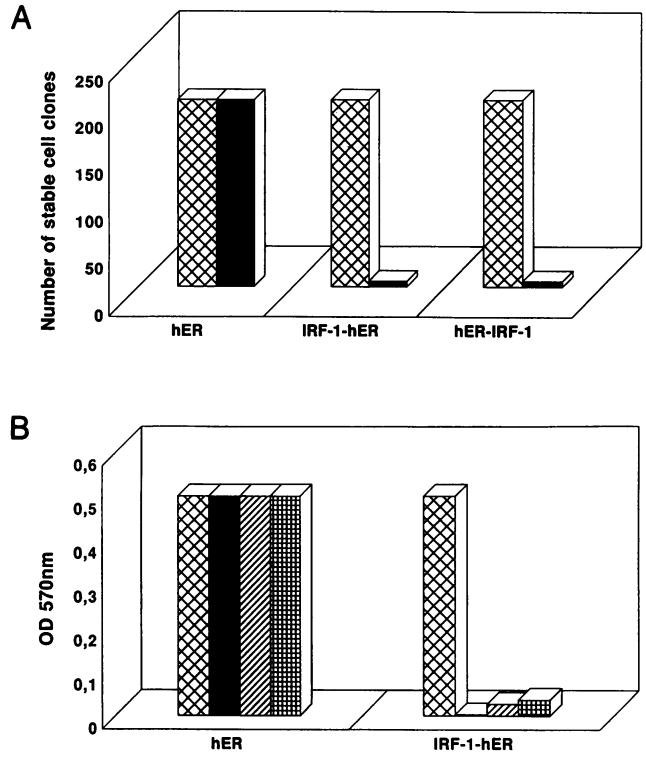


Figure 3. Estrogen-activated cell growth inhibiton of IRF-1 in fusion proteins with the human estrogen receptor. **A:**C243 cells were transfected with expression vectors containing the indicated DNAs as inserts together with the puromycin resistance gene as a selective marker. 48 hours after transfection the drug was added in presence (black bars) or absence (crossed bars) of 1 μM β-estradiol in the medium. After two weeks of selection the number of cell clones was counted. **B:**125 stably transfected cells with the indicated DNAs were seeded in each well of a a microtiter plate. One day later the following agents were added: crossed boxes: no agent, black boxes: 2.5 μM 17β-estradiol, hatched boxes: 2.5 μM OHT, squared boxes: antibodies directed against 2500 u/ml IFN-α and IFN-β, each. After 6 days of treatment the number of living cells was determined as described in Material and Methods. Stable transfectants of hER or IRF-1-hER expression plasmids were selected in the absence of 17β-estradiol.

proteins together with a selectable marker gene into C243 cells to generate stable clones. The number of stable clones in the absence of estrogen during selection is comparable to control transfections (Fig. 3A). When the selection for stable transfectants was carried out in the presence of estrogen their number was markedly reduced, in a similar fashion to that found in transfections with the unfused IRF-1. The data indicate that the IRF-1 activity in fusion proteins is dependent on the presence of estrogen.

The effect of IRF-1 expression on cell growth

Mixtures of stable cell clones transfected with IRF-1-hER were treated with estrogen in order to activate the IRF-1 functions. Six days later, the amount of living cells was determined by a biochemical assay (see Materials and Methods). Fig. 3B shows that while untreated cultures had grown normally, the estrogen-treated culture contained in comparison less than 3% the number of living cells, indicating that the cells either died or were retarded in growth. The endogenous IFN-β promoter is induced by IRF-1-activation and anti-viral activity is detectable in the cell supernatants (2000 units IFN per ml). However, the effects of IRF-1 activation on cell growth are not mediated by secreted IFN, since similar results were obtained when the cells were treated with estrogen plus neutralizing antibodies directed against IFN type I (Fig. 3B).

In order to determine the time course of growth retardation by the estrogen-activated IRF-1, different amounts of cells in a time-dependent manner, different amounts of cells containing the fusion protein IRF-1-hER were treated with estrogen and tested for the proportion of living cells (Fig. 4). The results show that independent of the initial concentration the amount of living cells remains roughly constant for 7 days. The level of fusion protein in the IRF-1-hER-transfectants is expected to be high due to the strong promoter utilized in the expression construct. At high concentrations of estrogen all molecules should be activated. This holds true for experiments in which ≥ 1 μM estrogen are used. At this concentration the amount of cells remains constant for 7 days (Figs. 3B and 4). The concentration of estrogen should

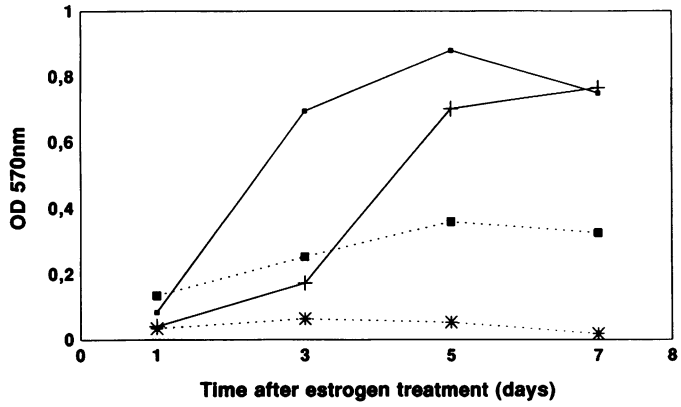


Figure 4. Inhibition of cell proliferation after IRF-1 activation. Different amounts of C243 cells stably transfected with IRF-1-hER were seeded in wells of microtiter plates and were grown for the indicated time with (dotted line) or without 2.5 μM 17β-estradiol (solid lines). The number of living cells was determined using the tetrazolium staining method. Rectangles indicate the time kinetics of cells which were seeded at a concentration of 1000 cells/well; crosses depict cells which were grown at an initial concentration of 250 cells/well.

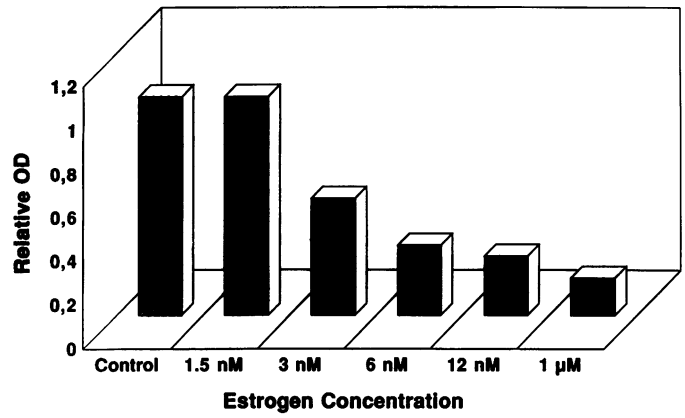


Figure 5. The strength of proliferation inhibition depends on the estrogen concentration. Stable transfectants of C243 cells expressing IRF-1-hER were seeded in microtiter wells (500 cells/well). 6 days after treatment of the cells with the indicated concentrations of 17β-estradiol the amount of living cells was measured. The initial concentration of cells was OD 0.2.

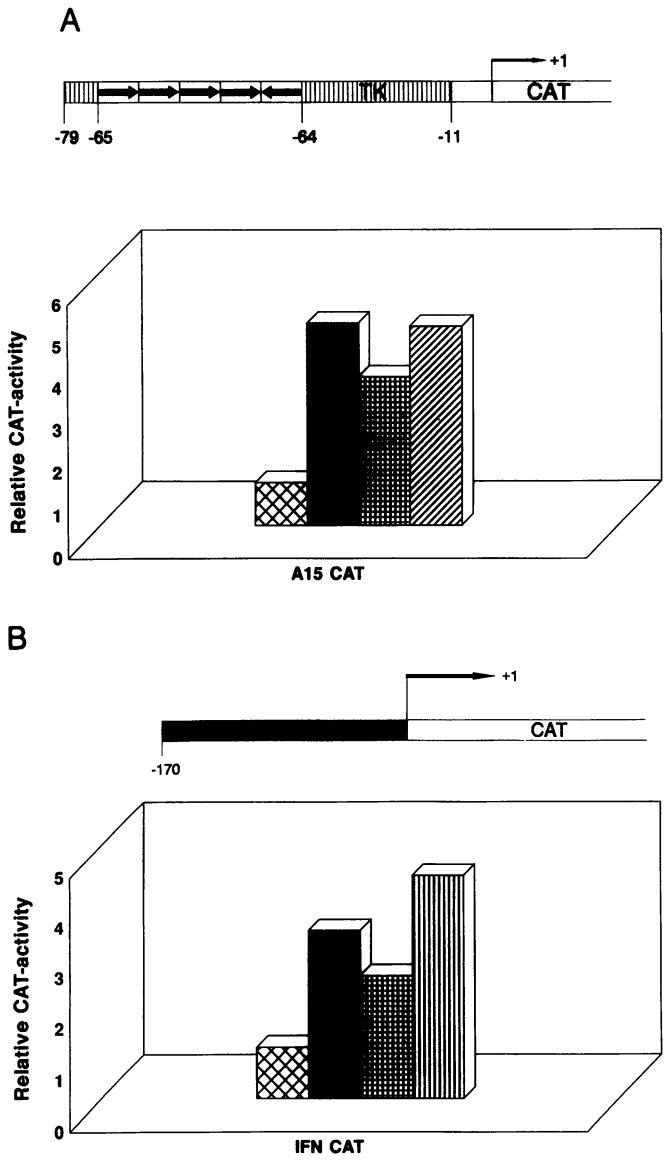


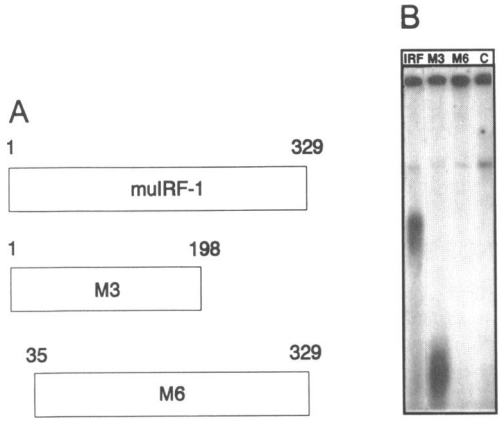
Figure 6. Induction of promoters by IRF-1 activation. Transfectants containing the indicated CAT reporter constructs were stably supertransfected with IRF-1-hER. The CAT genes in the reporter constructs are driven by the following promoters. **A:** A synthetic promoter composed of 15 repeats of AAGTGA (5' to the truncated HSV TK promoter, compare Fig. 2). **B:** The murine IFN-β promoter. Stable supertransfectants were treated for 24 h with 17β-estradiol (black bars), for 24 h with 2.5 μM OHT (squared bars), for 24 h with 17β-estradiol plus anti IFN-antibody (diagonal striped bars), or for 1 h with virus (vertical striped bars). Cells were harvested after 24 h.

determine the amount of activated IRF-1. We therefore titrated the concentration of estrogen in order to find conditions at which the growth inhibition is half-maximal. This was achieved at a concentration of 3 nM (Fig. 5). These results indicate that growth inhibition is dependent on the level of activated IRF-1.

DNA-binding and -transactivation is a prerequisite for IRF-1 function

Hydroxy-Tamoxifen (OHT) which binds to the native hER induces nuclear translocation and DNA-binding, but does not allow the activation of the hER-activator domain (45). The administration of OHT, which also binds to the hER-fragment results in the same effect on cell growth as estrogen. This implies that the fusion protein is bound to the target DNA sequences and that the transactivation domain of IRF-1 is inducing transcription. Estrogen- as well as Hydroxy-Tamoxifen-activation of IRF-1 lead to the induction of the promoter of human IFN-β and a synthetic IRF-1-responsive promoter (Fig. 6).

The data suggest that the estrogen-induced IRF-1 functions by its property to bind DNA-motifs of endogenous promoters and transactivate their genes. To confirm this, two IRF-1 mutants, one (M3) lacking the C-terminus, the other (M6) lacking 34 N-terminal amino-acids were expressed in C243 cells. The C-terminal deletion leads to inactivation of the transacting domain (10). The N-terminal deletion eliminates two tryptophan residues which have been suggested to belong to a DNA-binding motif related to c-myb (6). In an EMSA we demonstrate that *in vitro*



C

Transfected DNA	Transactivation of A15-TKCAT	Number of stable cell clones
IRF-1	+	<10
M3	-	>200
M6	-	>200

Figure 7. DNA binding and transcription activation of IRF-1 is essential for inhibition of cell growth. Wild type and deletion mutants of IRF-1 were tested for DNA-binding, transactivation and cell growth inhibition. **A:** Fragments of the IRF-1 coding region as inserts in the expression vector pMT7HE are shown. Numbering refers to the amino acid sequence of wild type murine IRF-1. **B:**EMSA of *in vitro* translated wild type and mutant IRF-1. Expression plasmids described in A were linearized 3′ to the coding insert sequences and transcribed *in vitro*. The resulting RNA was translated in a reticulosate lysate. The ^{35}S-labeled proteins were tested for successful translation of the expected lengths by SDS-PAGE (not shown) and simultaneously tested for DNA-binding to ^{32}P-labeled [$(AAGTGA)_3$] in an EMSA. **C:** Expression of wild type and mutant IRF-1 in C243 cells. The expression plasmids described in A were tested for transient transactivation of the synthetic IRF-responsive promoter as described in Fig. 2 and for efficiency in the formation of stable transfectants as described in Tab. 1.

translated M6 is not able to bind DNA specifically, as the wild type IRF-1 and the M3 mutant do so (Fig. 7). M3 as well as M6 give rise to stable transfectants in C243 cells indicating that they have lost their ability to mediate cell growth inhibiton. Furthermore, none of these mutants are able to induce any other effects which are typical to IRF-1 (Fig. 7). This finding confirms that the cell growth inhibiting effect of IRF-1 is due to the activation of (a) cellular target gene(s).

DISCUSSION

It is believed that IFN, upon contact with its cell surface receptors stimulates the activation of a series of genes, the IFN-stimulated-genes (ISGs). Although the functions of many of these genes are not known some have been studied in detail and recognized to be involved in cell growth control. These genes include 2′5′ oligo A synthetase (OASE), dsRNA-dependend protein kinase (dsI) and indolyl-oxygenase (IDO) (47, 48, 49, 50). Furthermore, IFNs have been shown to lead to indirect effects involved in cell growth inhibition, namely suppression of c-myc expression, phosphorylation of the retinoblastoma gene product (pRb), induction of its synthesis (reviewed in 51), induction of p34 phosphorylation and cyclin A synthesis (52).

In this study we have shown that overexpression of IRF-1 leads to cell growth inhibition or to a complete stop of proliferation of several cell lines. IRF-1-mediated IFN-secretion is not the reason for proliferation inhibition. Experiments using specific monoclonal antibodies to eliminate active IFN in the supernatants as well as mix-experiments (Tab. 2, Fig. 3B) have ruled out this possibility. However, we cannot fully exclude that IFN synthesis may help the intrinsic effect in a synergistic manner. But this is definitely not the main activity. Assuming that translation of recombinant IRF-1 mRNA and endogenous mRNA are identical a 10-fold amount of IRF-1 compared to non-stimulated control cells is sufficient to significantly block cell proliferation (Fig. 1). This amount of IRF-1 is transiently achieved by physiological modulators. In the present study we have used type I IFNs or viruses for the induction of endogenous IRF-1 mRNA. However, it is known that IFN-γ is a more potent inducer (53) which would, therefore, lead to the production of similar amounts of IRF-1 as present in the transfected cell clones (Fig. 1). The effect of elevated IRF-1 levels produced in response to natural modulators such as IFNs lead to typical growth inhibitory effects. This might imply that IRF-1 is a central mediator of the IFN-induced anti-proliferative response.

IRF-1-mediated proliferation inhibition was reported in two other studies. Antisense oligonucleotides directed to block IRF-1 mRNA translation have led to a partial abbrogation of cell growth inhibition in IL-6 and LIF treated M1 cells (20). These cells differentiate upon IL-6-treatment, produce large amounts of IRF-1 and stop growing. Yamada et al. (54) have constructed transgenic mice carrying IRF-1 under the control of an immunoglobulin heavy chain enhancer. These mice showed a specific depletion of the B-cell population which could be interpreted as a result of inhibition of cell proliferation in a cell population which is able to activate the IgG enhancer.

Antagonists of IRF-1 function should be able to abbrogate the anti-proliferative effect. IRF-2 has been reported to counteract IRF-1 function as transcriptional activator (8, 9). IRF-2, which possesses a long half-life-time is not regulated during the cell cycle, has been shown to act as an oncogene upon overexpression in a recent report (55). In this case IRF-1 might intrinsically counteract cell growth activation allowing the induction of the side effects but not cell proliferation at the same time.

IRF-1 is expressed in most differentiated cells at a very low level. The IRF-1 protein and its mRNA are very unstable, the half-life time is about 30 min. (56). This is in agreement with the fact that all its inducers lead only to a transient induction of IRF-1 transcription and protein accumulation.

The constitutive expression of IRF-1 might contribute to the continuous transcription of the genes which it is able to stimulate. Antagonists like ICSBP and its N-terminal region are able to reduce the activity of these genes (14, 15). In contrast to these experiments the natural stimulations of most ISGs might be due to ISGF-3 and requires the complete ISRE sequence. In this context it is of interest to recognize that IRF-1 is able to bypass the ISGF3 activation without needing IFN stimulation upon overexpression.

IFN production due to transiently expressed IRF-1 has been observed (8, 9, 10). Induction of IFN-β transcription by activation of the IRF-1-hER fusion protein with estrogen confirms that the mere overexpression of IRF-1 is sufficient to trigger this activity without the influence of the calcium phosphate precipitates stimulate transcription of various genes including IFN type I genes (37). Nevertheless, it is still obscure whether IFN induction by

IRF-1 overexpression mimics a physiological effect, since most IRF-1 inducers do not lead to the secretion of IFN-β. To explain this contradiction, different assumptions can be made: First, the IRF-1 inducers could simultaneously induce other metabolic effects which abbrogate IFN induced secretion. One of these abbrogators could be IRF-2 which is often simultaneously induced together with IRF-1 (17). A second possibility would be, that a post-translational modification of IRF-1, e.g. as shown by Pine et al. (3) influences its activity concerning the activation of target promoters. Overexpression of IRF-1 by genetic manipulation could result in differentially (incomplete) modified IRF-1 protein with altered properties. Third, the regulation of IFN-β expression could be on a post-transcriptional level. Pine (13) has also observed IFN-β gene transcription but found neither mRNA nor secreted functional IFN as a consequence of constitutive IRF-1 overexpression. We did not follow IFN-β secretion resulting from the IRF-1-hER fusion protein, after estrogen-activation. It is well possible that IFN-β secretion declines after an initial burst. This might explain the absence of IFN secretion in the stable transfectants which slightly overexpress wild type IRF-1 (Fig. 1).

Elucidation of the mechanism of IRF-1-hER activation by estradiol is beyond the scope of this study. The portion of the hER used in this study contains a transcription activation domain which becomes activated by estrogen-binding. The fusion protein, therefore, should contain two trans-activation domains which both could activate the target gene promoters. We have used hydroxy-tamoxifen (OHT) to show that the IRF-1 activation domain is sufficient to mediate trans-activation. OHT has been reported to promote nuclear translocation and demasking of estrogen receptors but prohibits the activation domain from being active. This is true also for fusion proteins derived thereof (57). Because all effects of IRF-1-hER upon estrogen activation could be reproduced with OHT we conclude that the activated fusion protein behaves closely related to that of wild type IRF-1.

IRF-1 is regarded as transcriptional activator. It is therefore conceivable to assume that it mediates proliferation inhibition by activating down-stream signal genes or target genes have to be activated by IRF-1 in order to achieve this specific activity. The data in Fig. 7 confirm this assumption showing that the proliferation inhibition activity of IRF-1 requires DNA-binding and activation. We do not regard c-myc or Rb as targets of IRF-1, since those have been described to abrogate GO/G1 progression. dsI, OASE and IDO are more likely candidates. The proliferation inhibition initiated by IRF-1 does not lead to a specific block within the cell cycle (data not shown). Recent functional studies of the dsI have demonstrated that it acts as a repressor of cell growth. Furthermore, transdominant mutants, devoid of phosphorylation capacity, function as oncogenes (49, 58). We have shown that by the estrogen mediated activation of IRF-1-hER the dsI is induced (data not shown). However, its involvement in the anti-growth effect initiated by IRF-1 overexpression remains to be demonstrated.

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