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Research Article

Inhibition of proliferation by 1-8U in interferon-α-responsive and non-responsive cell lines

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Abstract. Interferon (IFN)-inducible proteins of the 1-8 gene family mediate homotypic adhesion and transduction of antiproliferative signals. Their induction correlates with inhibition of cell growth while they are often repressed in the course of malignant transformation and tumor development. Ras-mediated transformation of mouse mast cells is associated with downregulation of 1-8U expression and interferon-α (IFN-α) treatment reverts the proliferation rate to normal levels together with induction of 1-8U. Conversely, the antiproliferative responses of IFN-α in sensitive human melanoma cells are accompanied by 1-8U induction. Here we provide direct evidence that recombinant expression of 1-8U in human cell lines is sufficient to block cell proliferation. Based on the abundant expression and subcellular localization to the plasma membrane and exosome-like structures, we propose a model capable of explaining the pleiotropic functions of 1-8 family proteins in tumor cells and during normal development.

Key words. Interferon; gene expression; growth inhibition; 1-8U; membrane protein.

Interferons (IFNs) are multifunctional cytokines that play key roles in mediating antiviral, antiproliferative and immune-modulating responses. Type 1 (α and β) and type 2 (γ) IFNs signal through distinct but related pathways [1]. They bind to their cognate receptor and initiate a signaling cascade that eventually leads to the transcriptional induction of at least 70 genes whose products mediate IFN actions such as inhibition of proliferation [2, 3].

IFN-α therapy is utilized for the treatment of more than 14 types of cancer, including some hematological malignancies and certain solid tumors like melanoma and Kaposi's sarcoma [4]. The treatment is of clear efficacy in subgroups of patients, whereas no beneficial effects are observed in others [5]. The non-responsive phenotype is maintained in cells cultured from such individuals, which provides experimental access to study and compare the fundamental processes of IFN-α sensitivity and resistance.

Using microarrays we have demonstrated defects in gene expression in resistant cell lines affecting a subset of IFN-inducible genes which are possibly associated with antiproliferative responses induced by IFNs in sensitive cells [3]. The IFN-induced growth inhibition affects different phases of the mitotic cycle, including a block in G_1, lengthening of the S phase or a general prolongation of the cell cycle [6–8]. It is not clear which genes are involved in cell cycle control induced by IFN although several studies link the 9-27 surface protein, a member of the 1-8 family of IFN-inducible proteins, to antiproliferative activity. In B cells for example, the protein is part of a putative signaling surface receptor complex together with CD21, CD19 and CD81 [9]. Antibodies to 9-27 (identical to the Leu-13 antigen) trigger the antiproliferative activ-

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ity presumably by inducing receptor dimerization, which transmits the signal to the nucleus. In B cell lymphomas, induction of the 9-27 gene correlates with the sensitivity to IFN-induced inhibition of cell growth [10]. Tumor growth inhibition by high doses of IFN-α in a human melanoma xenograft model is also associated with an upregulation of 9-27 and 1-8U, another 1-8 family member, together with only four additional known IFN-α-inducible genes [C. Krepler, unpublished observations].

In contrast, 1-8 family genes are often downregulated when proliferation rates increase. Transcript levels of 9-27 for example are low in leukemic B cells compared to normal B lymphocytes and downregulation of 9-27 and 1-8U correlates with brain tumor progression [10, 11]. In a murine mast cell tumor model, expression of mouse 1-8U is consistently downregulated in tumors compared to the untransformed precursor cell line [12]. A rat homolog of the 1-8 family (*rat8*) is downregulated after ras transformation of rat fibroblasts [13].

The functional studies summarized above suggest that 1-8 family proteins are involved in the control of cell proliferation in vertebrates. Here, we show that regulation of 1-8U expression correlates with cell proliferation in sensitive and resistant human melanoma lines and we show that 1-8U overexpression alone is sufficient to inhibit proliferation. Differential centrifugation and immunohistochemistry show membrane association of 1-8U, and immunoelectron microscopy shows localization of the protein in distinct organelles resembling eukaryotic exosomes.

Materials and methods

Cell lines and culture conditions

Mouse cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µM mercaptoethanol. Saturating amounts of conditioned medium from the interleukin (IL)-3-producing X63-mIL-3 line [14] were added for growth of PB-3c. PB-3c is a cloned, IL-3-dependent, nontumorigenic mast cell line from murine bone marrow [15]. V2D1, 15V4T21 and R56VT are IL-3-producing, autocrine tumor lines resulting from passage of ZIP-*ras-neo*-transfected subclones of PB-3c through mice [16, 17].

ME15, D10 and ME59 have been described elsewhere [18, 19]. ME59/4 and ME59/7 are subclones of ME59. ME59/cl7 was obtained after culturing ME59 in the presence of 1000 U/ml IFN-α for 4 weeks and selecting for IFN resistance. They were maintained in RPMI medium supplemented with 20% FCS, 2 mM glutamine, 1 mM sodium pyruvate, non-essential amino acids, antibiotics and 20 mM HEPES buffer.

EBNA cells were kept in Iscove's medium supplemented with 5% FCS and 100 units/ml penicillin, 100 µg/ml streptomycin.

Proliferation assays

Proliferation rates of mouse cells were measured as described previously [17]. Cells were grown in the presence or absence of IFN-α or IFN-γ. [^{3}H]-thymidine was added 24 h after the beginning of the experiment. After an additional 24 h, cells were harvested and incorporation of [^{3}H]-thymidine was measured. Mitogenicity of human cell lines was assayed using the CellTiter Aqueous One Solution Cell Proliferation Assay (Promega, Madison, Wis.). Assays were performed in triplicate.

Genomic DNA and RT-PCR

Genomic DNA was isolated using the DNA Isolation Kit for Cells and Tissues (Roche Diagnostics, Rotkreuz, Switzerland). PCR was performed using Taq Polymerase (Roche Diagnostics) in 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP.

For semiquantitative RT-PCR, total RNA was isolated with RNAzol B (TEL-TEST, Friendswood, Tex.). cDNA was synthesized from 2.5 µg of total RNA using the Superscript Choice System from GibcoBRL (Gaithersburg, Md.). Amplification was done as described above. The following primers were used:

5′-TTCAGGCACTTAGCAGTGGAGGCGTAGGC-3′
and
5′- GCCTGACCATGTGGTCTGGTCCCTGTTC-3′ for mouse 1-8U;
5′-GTCACACTGTCCAAACCTTCTTCTCTCC-3′ and
5′-CTATCCATAGGCCTGGAAGATCAGCAGT-3′ for human 1-8U;
5′-ATGAACCACATTGTGCAAACCTTCTCTC-3′ and
5′-CTACGCTGGGCCTGGACGACCAACA-3′ for human 1-8D;
5′-CAAAGGTTGCAGGCTATGGGCGGCTACTAG-3′
and
5′-AACACTTCCTTCCCCAAAGCCAGAAGATGC-3′
for human 9-27.

Sequencing

Fragments were subcloned into the pGEM-T-easy vector (Promega) and sequenced using the vector-specific primers 5′-ACGCCAGGGTTTTCCCAGTCAC-3′ and 5′-GGAAACAGCTATGACCAT-3′.

Western blot analysis

Polyclonal antibodies to the peptide NSGQPPNYEML-KEEQ (amino acids 11–25 of 1-8U), conjugated to keyhole limpit hemocyanin, were produced in rabbit by Medprobe (Olso, Norway). Western blotting was performed using the Super Signal chemoluminescence kit (Pierce, Rockford, Ill.) as recommended by the supplier. Primary

antibody was used at a dilution of 1 : 500, secondary goat anti-rabbit IgG (Bio-Rad, Hercules, Calif.) at 1 : 1000.

Oligonucleotide array analysis

Preparation of labeled targets, hybridization to the Affymetrix HuGene FL array (Santa Clara, Calif.) and data analysis were done as described elsewhere [12]. Three independent replicates were prepared for each condition. Change factors of at least 3 and a positive t test result at the 95% confidence level ($p < 0.05$) were used to identify and restrict the number of differentially expressed genes.

Fractionation of cells

Cells were washed in PBS, and homogenization buffer (10 mM HEPES pH 7.3, 320 mM sucrose, 0.1 mM EDTA) was added directly to the culture flask. Cells were harvested with a cell scraper and homogenized in a glass douncer with a tight pestle. The homogenate was centrifuged for 5 min at 1000 g, yielding a pellet containing intact cells and nuclei. The supernatant was centrifuged for 10 min at 10,000 g, producing a second pellet. The supernatant obtained after this step was again centrifuged for 30 min at 100,000 g, resulting in a pellet containing the membrane fraction and a supernatant consisting of the soluble cytosolic fraction. Proteins of all fractions were size-separated on SDS gels followed by immunodetection of 1-8U.

Immunofluorescence

Cells were fixed in 2% paraformaldehyde in PBS for 15 min, quenched in 0.05 M glycine in PBS for 10 min and blocked in PBS, 2% BSA, 0.05% saponin and 2% normal serum for 10 min. 1-8U was detected with a polyclonal rabbit 1-8U antibody (1 : 50 in PBS, 0.1% BSA, 0.05% saponin) and a secondary goat anti-rabbit FITC conjugate (Nordic Immunology, Tilburg, The Netherlands) or a Cy3-conjugated sheep anti-rabbit IgG (Sigma, Buchs, Switzerland) 1 : 100 in PBS, 0.1% BSA, 0.05% saponin, 2% normal serum. For double stainings, primary and secondary antibodies were incubated sequentially. MHC (HLA-A, -B, -C) proteins were detected with W6/32 mouse monoclonal antibody and secondary Cy2-labeled donkey anti-mouse (Jackson ImmunoResearch, Philadelphia, Pa.). Immunofluorescence images of the cells were recorded with a Leica TCS NT Confocal Microscope System using × 40 Plan Fluotar or × 63 Plan Apochromat Objective (Leica, Solms, Germany). Pinhole settings were 1 Airy unit for all images. Images were generated to satisfy the Nyquist criteria for sampling with 160-nm serial sections of x-y images along the z-axis [20]. For image processing, an SGI octane using the Imaris software (Bitplane, Zurich, Switzerland) was applied. Colocalization events were analyzed with the Bitplane Colocalization Software.

Electron microscopy

Ultrathin cryosections of IFN-α-treated ME59/cl4 cells were prepared and processed as described elsewhere [21, 22].

Cloning strategies and transfections

To produce tet-regulatable pUC-COMBI$^{cmv/zeo}$/1-8U, the 1-8U coding region was PCR amplified using the primers 5′-ATCCCGGGTAACCCGACCGCCGCTGGTC- 3′ and 5′-CCTGGCCTCAGTGATGCCTCCCGGGATCTATC-CATA-3′. The amplicon was inserted as a *Sma*I blunt fragment into the *Pme*I site of pUC-COMBI$^{cmv/zeo}$ [23]. The resulting vector coexpresses 1-8U under the control of the tet operator and the tet-regulatable transactivator under the CMV promoter. Transfection was performed with 1 µg plasmid DNA and 10 µl lipofectamine (GibcoBRL) according to the manufacturer's protocols. Tetracycline (0.2 µg/ml) and 200 µg/ml Zeocin (Invitrogen, Carlsbad, Calif.) for selection were added 24 h after the start of the transfection. Expression of 1-8U was assayed by Western blot analysis of induced and repressed cultures.

The SFV(PD) vector system has been described before [24]. It is based on two plasmid vectors: (1) an expression vector containing the non-structural viral genes required for RNA replication and the foreign gene of interest inserted behind the strong subgenomic 26S promoter and (2) a helper vector containing the viral structural genes (capsid and membrane protein genes) required for assembly of recombinant alphavirus particles. For expression vector SFV/1-8U, the 1-8U coding region was amplified using 5′-GCCGCTGGATCCTTCGCTGGAC-ACCATGAGTCA-3′ and 5′-CCTGGATCCTCAGTGATGCCTCCTGATCTATC CATA -3′. For the control vector SFV/co, a 200-bp stretch of the coding region of 1-8U was amplified with the primers 5′-ACGGATCCGCAGCGAGACCTCCGTGCC-3′ and 5′- CAGGGATCCCAGATGTTCAGGCACTTG-3′. The amplicons were ligated as *Bam*HI fragments into pSFV(PD) and the sense orientation verified by restriction digests. In vivo packaging of recombinant SFV particles was done as described elsewhere [25]. Briefly, in vitro-transcribed RNA from SFV/1-8U or SFV/co and pSFV-Helper2 was coelectroporated into BHK-21 cells and mature SFV particles harvested 24 h later. Prior to infection, the virus stock was activated with α-chymotrypsin. Expression of 1-8U was verified from SFV/1-8U-infected BHK-21 cells by metabolic labeling with [^{35}S]-methionine at 16 h post-infection. Cells of choice were transfected by adding the virus stock to the medium. For the luciferase reporter constructs, promoters of 1-8D, 1-8U and 6-16 were amplified using the following primers

a) for the 1-8D promoter: 5′-CAGCAGACTCGAGGC-CTGTGACAAG-3′ and 5′-GTGACCAAGCTTGGCG-GTCGGGTTACC3′

b) for the 1-8U promoter: 5′-TGCTCCCTTGGGCTCT-CGAGAGGAG-3′ and
5′-AAGCTTGTCCAGCGAAGACCAGCGGCGGTC-3′
c) for the 6-16 promoter: 5′-GAAGCAGCCAGCCTC-GAGAGAACAATG-3′ and 5′AGTAAACGGTTCTC-CGGCTGAAGCTTGGC-3′.
All amplicons were cloned as *Xho*I-*Hin*dIII fragments into the pUHC13-2 expression vector, which had previously been digested with *Xho*I and *Hin*dIII. pUHC13-2 was a kind gift of Dr. U. Deuschle [26]. Digestion with *Xho*I and *Hin*dIII removes all regulatory promoter elements and leaves the luciferase gene as the sole transcriptional unit. D10 and ME15 were transiently transfected in six-well plates with 1 µg of plasmid DNA and 5 µl lipofectamine using the manufacturer's protocol. Serum was added 3 h after the start of transfection. Twenty-one hours later, the cells were split and further cultivated either in the presence or absence of 1000 units/ml IFN-α for another 24 h before luciferase activity was measured [23].

Results

High proliferation rate of *ras*-transformed mouse mast cells is reversible by IFN treatment

A microarray-based analysis of differential gene expression in a murine mast cell tumor model revealed downregulated transcript levels of four IFN-inducible genes in tumors compared to precursor cells. One of them, 1-8U, is transcribed at intermediate levels in slowly dividing, normal precursor cells and fully downregulated after *ras* transformation in three independent tumors [27]. This observation is consistent with findings in humans where downregulation of 1-8 family genes correlates with tumor growth and metastasis [10–12]. Conversely, induction of the 1-8 family correlates with growth arrest of tumors [3, 10; C. Krepler, unpublished observation]. This circumstantial evidence suggests that members of the 1-8U protein family are involved in cell cycle control.
We addressed this hypothesis and first asked whether the high proliferation rate of *ras*-transformed tumor cells returns to normal levels upon IFN stimulation, which is expected to induce expression of the murine 1-8U gene. The three tumor lines (V2D1, 15V4T21 and R56VT) and the common precursor PB-3c of the original *ras* transformation experiment [27] were stimulated with IFN-α and also IFN-γ, because the human 1-8U promoter responds, in contrast to other IFN-inducible genes, to both cytokines [28]. Cells were thus grown in the presence of IFN-α or IFN-γ and proliferation was assayed by conventional [H^3]-thymidine incorporation (fig. 1). Consistent with the proposed function of 1-8U, both cytokines reduced the tumor cell proliferation rate in a dose-dependent fashion to levels comparable to the untransformed precursor cell line. RT-PCR analysis confirmed IFN induction of 1-8U in the tumors. Cytokine treatment did not modulate proliferation of the precursor cells, where 1-8U is constitutively expressed. Thus, the reversal of the proliferation rate by IFNs in tumor cells and the concomitant 1-8U ex-

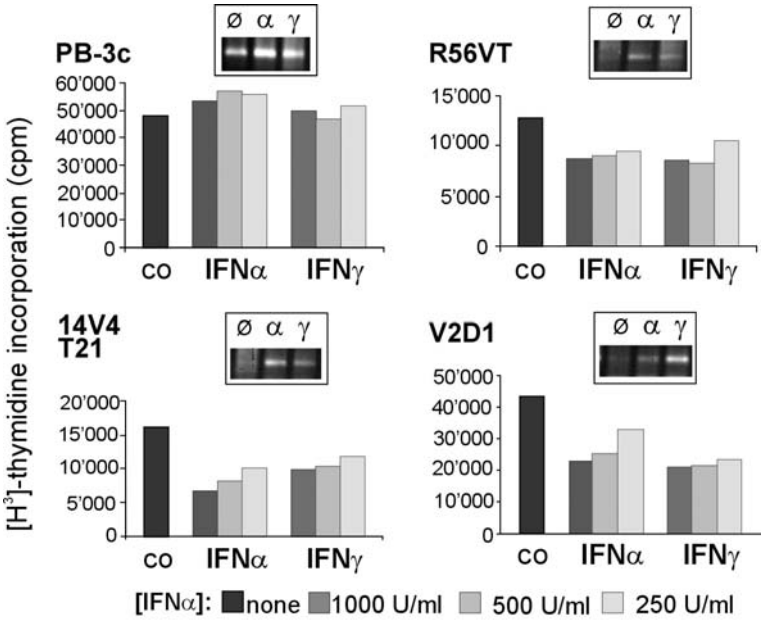

Figure 1. IFN treatment results in growth inhibition of all three tumor lines but not of the precursor line PB-3c. Inhibition is accompanied by an induction of 1-8U gene expression shown by RT-PCR (boxes). To assay mitogenicity, cells were grown in the presence or absence of IFN-α or IFN-γ and [^{3}H]-thymidine incorporation was measured 24 h after the beginning of the treatment. Semiquantitative RT-PCR was carried out with RNA isolated after treating the cells with 250 U/ml IFN for 48 h (α, IFN-α; γ, IFN-γ; ∅, untreated).

pression favor the view that 1-8U activity is involved in control of proliferation.

Expression pattern of 1-8U in IFN-α-resistant and -sensitive primary melanoma cells

Melanoma cells from patients maintain their sensitivity or resistance to the growth inhibitory effect of IFN-α in cell culture. According to our working hypothesis, we expect differential expression of 1-8U in responders and non-responders. ME15 (sensitive) and D10 (resistant) are a well-studied pair of human melanoma cell lines, generated by culturing cell suspensions derived from surgically excised tumors [18, 19]. By RT-PCR we determined the transcript levels of 1-8U, 1-8D and 9-27 after IFN-α stimulation in both lines in order to correlate differential transcription with proliferation (fig. 2). In D10, control of 1-8U expression is apparently lost since the constitutive mRNA levels were similar to the fully IFN-α-induced state of ME15. Cytokine treatment of D10 further upregulated 1-8U expression about twofold. Western blot analysis using a polyclonal antibody against 1-8U confirmed the upregulation at the protein level. 9-27 is fully IFN-α responsive in both lines, while 1-8D, the third member of the family, is not inducible in either line, probably due to a base pair transition in the IFN-stimulatable response element (ISRE) in its promoter. This conversion has been shown to abolish 1-8D promoter activity in HeLa cells [29]. Interestingly, the mutation does not prevent deregulation of 1-8D expression in unstimulated D10 cells.

Based on this transcript analysis of the 1-8 family in ME15 and D10, 1-8U is the only gene of this small family whose expression mode correlates with inhibition of proliferation. Note that the full responsiveness of the 9-27 gene in both lines illustrates that the basic IFN-α signaling pathway operates properly in D10.

The differential expression of the 1-8U family genes described above could be a result of the different genetic background of the donors from which the cell lines were derived. We addressed this possibility by selecting IFN-resistant cells from a culture of IFN-α-sensitive primary cells (ME59) which represent a heterogenous cell population containing a small subset of resistant variants [19]. ME59 was cultured for 4 weeks in the presence of 1000 U/ml IFN-α, which yielded the resistant clone ME59/cl7. By limiting dilution we also isolated a sensitive clone (ME59/cl4) without IFN selection from the same initial population.

Both clones were grown in the presence or absence of IFN-α followed by RT-PCR and immunoblot analysis of 1-8U expression. Similar to ME15 and D10, 1-8U is fully inducible in ME59/cl4 and again deregulated in the resistant clone ME59/cl7 (fig. 3). Thus, IFN-α resistance is accompanied by deregulation of 1-8U expression in D10 and the selected clone ME59/cl7, suggesting an association between the two features.

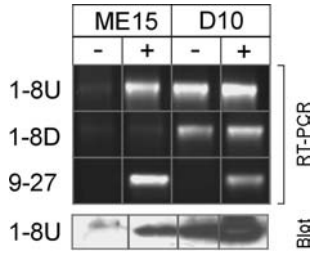

Figure 2. Expression of 1-8U is constitutive in the IFN-α-resistant cell line D10 but not in the sensitive line ME15. 1-8D is deregulated in D10 but uninducible by IFN-α, while 9-27 is induced in both lines. Cells were cultured for 48 h in the absence (–) or presence (+) of 1000 U/ml IFN-α and expression patterns of the 1-8 family genes were assayed. RT-PCR was performed to analyze mRNA expression levels. Deregulation of 1-8U was confirmed by Western blot analysis using a polyclonal antibody specific for 1-8U.

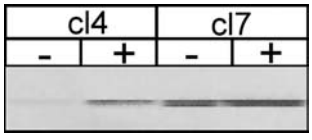

Figure 3. Expression of 1-8U is deregulated in the IFN-α-resistant cell line ME59/cl7. Protein levels of 1-8U in IFN-sensitive ME59/cl4 and resistant ME59/cl7 melanoma cell lines were analyzed by Western blotting. Cells were cultured in the presence (+) or absence (–) of 1000 U/ml IFN-α for 48 h prior to protein extraction.

Transcription profiling of ME59/cl4 and ME59/cl7

We next analyzed the expression modes of other genes in ME59/cl4 and ME59/cl7 by transcript imaging using commercial oligonucleotide arrays with probe sets for all known human transcripts including every known IFN-inducible gene. Cells were cultured with or without IFN-α followed by RNA extraction and sample processing for microarray analysis. In both lines, about 40% of the probe sets available on the arrays were detected which indicates sample integrity (data not shown). When focusing on IFN-α-inducible genes we found three major expression modes of transcription illustrated in table 1. A significant number of classical IFN-α response genes like p78 or STAT1 responded in both lines (cluster 1). The transcription factor STAT1, for example, is part of the complex required for the induction of most IFN response genes explaining why candidate downstream genes are inducible in both lines [1]. Cluster 2 shows the genes which were not upregulated by IFN-α in the resistant line ME59/cl7 but induced in the sensitive line ME59/cl4. Cluster 3 shows the genes that were controlled like 1-8U. The microarray analysis reveals rather complex and distinct expression modes of IFN-inducible genes and shows that only a subset of genes is affected by dysregulation, which argues against major defects affecting all IFN-α-inducible genes in the resistant cell lines studied here.

Sequence and functional analysis of 1-8U promoters

At this point, mutations or defects affecting only 1-8U and the remainder of the dysregulated genes shown in table 1 could explain the loss of expression control. The two classical ISREs in the 5′ region of the 1-8U promoter, for example, are necessary and sufficient for IFN-induced gene expression [29, 30]. We thus PCR amplified and cloned the 1-8U published promoter regions from ME15, D10, ME59/cl4 and ME59/cl7. Based on the studies above, unsurprisingly, all promoter sequences were identical and matched the published database sequence (em_hum5 : hs18U; data not shown). It is thus reasonable that a general, epigenetic defect in D10 and ME59/cl7 is responsible for the dysregulation of 1-8U.

To prove this possibility, we constructed luciferase reporter gene constructs using an IFN-α-responsive1-8U promoter isolated from EBNA, a common human embryonal kidney cell line (data not shown). As an assay control, we used the 6-16 gene promoter, which is not responsive in D10 [3, 31], and 1-8D. After transient transfection of ME15 and D10, all three reporter genes showed the same IFN response mode as their endogenous counterparts (fig. 4). These results further eliminate the possibility that promoter mutations cause deregulation of 1-8U in D10 (table 1).

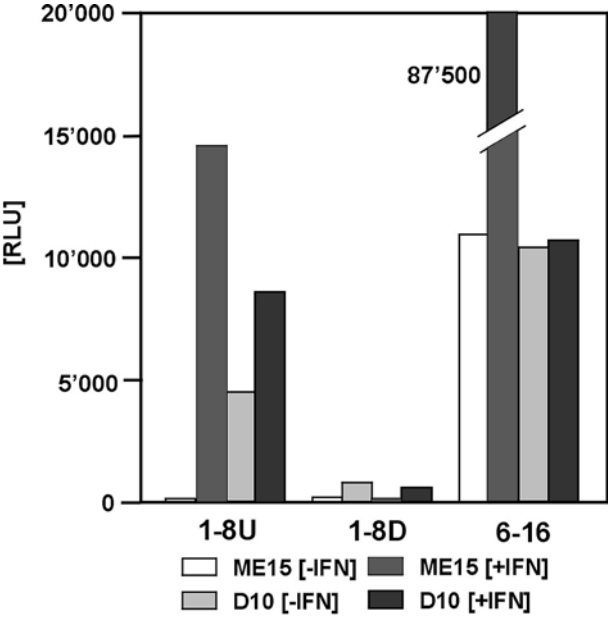

Figure 4. Reporter gene assays demonstrate that deregulated gene expression of 1-8U in the non-responder D10 is not due to promoter mutations. Vectors containing the promoters of 1-8U, 1-8D and 6-16 fused to the luciferase reporter gene were used for transient transfection of ME15 and D10 in the presence or absence of IFN-α. Luciferase activity was measured 48 h after the start of transfection. Bars represent the measured numbers of relative light units (RLU) for each condition. Luciferase activities under the control of the 1-8U, 1-8D and 6-16 promoters correlate closely with the expression pattern of the respective endogenous genes in the respective cell lines.

1-8U protein expression is sufficient to inhibit proliferation

Downregulation of the 1-8U gene in the mouse tumors and upregulation in human melanoma lines correlates indirectly with the cell division rate. The published data and our own work show only an indirect link between 1-8U expression and antiproliferative activity and little is known about the mechanism of action. The failure to obtain stable clones constitutively expressing 1-8U or 9-27 by others and ourselves provides indirect evidence that the 1-8U family proteins arrest cell division [10; R. Brem, unpublished results]. To overcome this problem, we first used a non-cytotoxic variant of the viral semliki forest (SFV) expression system to study overexpression of 1-8U in uninduced melanoma cells, which are efficiently infected by the virus [24]. Relative to mock-infected cells, infection of ME15 with SFV/1-8U results in efficient inhibition of proliferation 48 h after infection, while overexpression in D10 had no marked effect on proliferation, probably due to the pre-existing high levels of 1-8U in this cell line (fig. 5A). This result also provides a plausible explanation for the inherent lower growth rate of D10 compared to ME15 and in the absence of IFN-α (data not shown).

We next asked whether the activity of 1-8U is restricted to tumor cells and expressed the protein in EBNA cells using a modification of the original tetracycline-regulatable promoter system where all genetic elements required for tet-dependent expression are contained on a single plasmid [23, 26]. This expression system (pUC-combi) was applied previously in our laboratory for the stable overexpression of PC4, a transcriptional activator involved in responses to brain damage [32]. Culturing transfected EBNA cells in the presence of tetracycline has no impact on cell division, while removal and simultaneous induction of 1-8U expression results in a significant inhibition of proliferation relative to controls (fig. 5B). In this system, we also noticed that full inhibition of proliferation does not occur immediately but rather with a lag phase of 48 h. These data provide direct proof that recombinant overexpression of 1-8U is sufficient to inhibit proliferation of normal and tumor cells and qualify the gene as a tumor suppressor. Additionally, both the available human and mouse genomes contain several inactive 1-8U pseudogenes with defective promoters or missing initiation codons which is reminiscent of other experimentally confirmed tumor suppressor genes (data not shown).

Subcellular localization and function of 1-8U

The primary sequence of 1-8U contains two putative transmembrane domains. To explore the mechanism of 1-8U action, we established the cellular localization of 1-8U by immunofluorescence confocal microscopy using 1-8U-specific antiserum. Both in IFN-α-treated ME59/cl4 or untreated D10 cells, relatively high levels of

Table 1. Microarray analysis of IFN-α-modulated genes in ME59/cl4 and ME59/cl7 cells.

Accession number		cl4 A.D.	cl4 + IFN A.D.	cl7 A.D.	cl7 + IFN A. D.	CF cl4	CF cl7	cluster cl4	cluster cl7
Cluster 1 (normal induction)									
U72882	interferon-induced leucine zipper protein (ifp35)	20	3544	447	5727	176.20	11.82	up	up
X82200	staf50	111	6492	603	8792	57.67	13.59	up	up
M97936	stat1	401	20965	405	18619	51.24	44.99	up	up
M33882	p78	503	23574	1101	28554	45.85	24.93	up	up
X02612	cytochrome p(1)-450	187	4846	110	509	24.86	3.64	up	up
U53830	interferon regulatory factor 7a	231	5619	494	4214	23.38	7.53	up	up
M13755	interferon-induced 17-kDa/15-kDa	2328	31900	1987	25946	12.71	12.06	up	up
M30818	interferon-induced cellular resistance mediator mxb	261	3415	291	3466	12.08	10.91	up	up
AB000115	EST	1571	19829	1032	12888	11.62	11.49	up	up
M24594	interferon-inducible 56-kDa protein	2000	18844	1023	19287	8.42	17.85	up	up
M87434	2′5′ oligoadenylate synthetase	861	7590	1037	8203	7.81	6.91	up	up
J04080	complement component c1r mRNA, complete cds	1181	9401	1267	9420	6.96	6.44	up	up
AF008445	phospholipid scramblase	3130	18979	3926	17168	5.06	3.37	up	up
U47011	fgf8 gene (fibroblast growth factor 8 precursor)	20	107	20	151	4.32	6.55	up	up
X04602	interleukin bsf-2 (B-cell differentiation factor)	5900	28889	2313	13224	3.90	4.72	up	up
M55542	guanylate-binding protein isoform i (gbp-2)	1160	5223	967	3934	3.50	3.07	up	up
M14660	isg-54k gene (interferon-stimulated gene)	265	1129	20	297	3.25	13.84	up	up
U52513	rig-g	2114	8644	1041	8400	3.09	7.07	up	up
Cluster 2 (non-inducible in cl7)									
J04076	early growth response 2 protein	20	1678	20	48	82.91	1.39	up	~
X00038	h4 histone	20	839	20	20	40.95	0.00	up	~
M30703	amphiregulin	113	2893	109	286	24.67	1.62	up	~
AF005043	poly(ADP-ribose) glycohydrolase (hparg)	20	449	24	50	21.44	1.10	up	~
X15675	ptr7	20	410	20	20	19.50	0.00	up	~
U70663	zinc finger transcription factor hezf (ezf)	20	256	20	20	11.81	0.00	up	~
X54867	nkg2-a	20	253	20	64	11.64	2.18	up	~
X60992	T cell glycoprotein cd6	20	219	20	50	9.97	1.48	up	~
D16688	ltg9/mllt3	20	213	20	45	9.65	1.25	up	~
U37546	iap homolog c (mihc)	319	3067	668	1175	8.61	0.76	up	~
L22343	nuclear phosphoprotein	160	1498	415	1288	8.34	2.10	up	~
X17093	hla-f gene for leukocyte antigen f	1957	16130	4035	14609	7.24	2.62	up	~
HG4724	ATP-binding cassette	20	153	20	20	6.64	0.00	up	~
D12763	st2	614	4645	735	1212	6.56	0.65	up	~
L19871	transcription factor 3 (atf3)	733	5022	193	678	5.86	2.51	up	~
X57579	activin beta-a subunit	2747	16733	1500	4110	5.09	1.74	up	~
AB000584	TGF-beta superfamily protein	1049	6051	531	709	4.77	0.33	up	~
L07919	dlx-2 d	854	4765	616	764	4.58	0.24	up	~

Table 1 (continued)

Accession number		cl4 A.D.	cl4 + IFN A.D.	cl7 A.D.	cl7 + IFN A. D.	CF cl4	CF cl7	cluster Cl4	cluster Cl7
Cluster 2 (non-inducible in cl7)									
D14520	gc box-binding protein bteb2	277	1519	182	344	4.48	0.89	up	~
U90551	histone 2a-like protein (h2a/l)	1405	7425	1292	2301	4.28	0.78	up	~
X91103	hr44 proteina	32	161	20	19	4.07	0.00	up	~
U66075	transcription factor hgata-6	373	1871	424	573	4.01	0.35	up	~
L15326	endoperoxide synthase type II	317	1567	363	861	3.94	1.37	up	~
U45878	inhibitor of apoptosis protein 1	616	2991	861	1741	3.86	1.02	up	~
X52541	early growth response protein 1 (hegr1)	1950	9366	1060	3712	3.80	2.50	up	~
L14430	UDP-glucose pyrophosphorylase	20	96	20	20	3.79	0.00	up	~
V01512	c-fos	687	3225	549	661	3.69	0.20	up	~
HG4668	trmef2	236	1003	417	444	3.26	0.06	up	~
U16261	mda-7	637	2681	1232	1488	3.21	0.21	up	~
X66867	max gene	263	1082	588	971	3.12	0.65	up	~
M27968	basic fibroblast growth factor (fgf)	756	3095	1388	1916	3.09	0.38	up	~
Cluster 3 (deregulated in cl7)									
X67325	isg12 (p27)	20	32586	9052	29752	999.90	2.29	up	~
M25756	1-8U	20	1969	1771	2983	98.45	0.68	up	~
X57985	gl105 gene (histone h2b)	21	1135	217	326	52.19	0.50	up	~
D88422	cystatin a	20	919	133	341	44.96	1.57	up	~
D12676	lysosomal sialoglycoprotein	20	617	233	740	29.85	2.17	up	~
X57206	1d-myo-inositol-trisphosphate 3-kinase b	20	506	783	481	24.31	–0.63	up	~
U37143	cytochrome p450 monooxygenase cyp2j2	20	390	193	165	18.50	–0.17	up	~
K03008	gamma-g2-psi	20	374	302	391	17.70	0.29	up	~
U72661	ninjurin1	20	303	442	432	14.16	–0.02	up	~
U68385	meis1-related protein 2 (mrg2)	24	312	635	492	12.26	–0.29	up	~
U80456	transcription factor sim2	20	251	162	195	11.55	0.20	up	~
U08049	myelin protein-22 (pmp22)	20	232	132	187	10.59	0.41	up	~
U18383	nuclear respiratory factor 1 (nrf-1)	20	232	143	165	10.58	0.16	up	~
Z80787	h4/j	39	281	191	205	6.21	0.07	up	~
M35198	integrin b-6	40	257	262	202	5.41	–0.30	up	~
X91504	arp1	717	3584	3384	2872	4.00	–0.18	up	~
U66077	daz	54	260	231	164	3.85	–0.41	up	~

Cells were cultured in the presence or absence of 1000 U/ml IFN-α for 48 h prior to RNA extraction. Cytokine-modulated gene expression was assayed using high-density oligonucleotide microarrays. The expression levels for each gene were calculated as normalized average difference (A.D. , expressed in arbitrary units) of fluorescence intensity relative to the hybridization signal of mismatch oligonucleotide probe sets. A threshold of 20 A.D. units was assigned to any gene with expression below 20 in order to maintain realistic change factors. Genes displaying reproducible change factors (CF) of at least threefold were included in the analysis and clustered according to their mode of regulation (up, upregulated; down, downregulated; ~, unmodulated). Cluster 1 contains genes upregulated in both lines. Cluster 2 comprises genes induced in ME/cl4 only. Cluster 3 includes deregulated genes. Genes are defined as deregulated if constitutive expression in D10 was at least threefold higher than in ME15 and no significant induction was observed in D10 after IFN treatment.

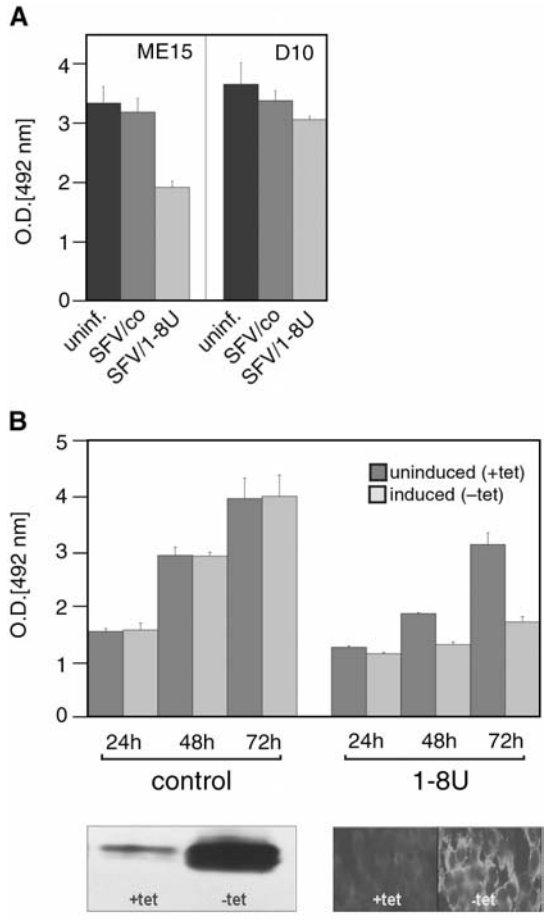

Figure 5. Ectopic expression of 1-8U inhibits proliferation of IFN-α sensitive cells. (*A*) SFV-mediated expression of 1-8U inhibits cell growth of IFN-α-sensitive ME15 cells but not of IFN-α-resistant D10 cells. Cells were infected with SFV/1-8U or the control virus (SFV/co) and cell numbers were assayed 48 h post infection. Cell numbers were also measured for uninfected (uninf.) cells. (*B*) Tet-regulatable expression of 1-8U allowed determination of the effect of 1-8U expression on proliferation of EBNA cell transfectants. 1-8U is expressed in the absence of tet. The control cell line carries an insertless vector and is not inhibited upon withdrawal of tet. Induction of 1-8U was verified by Western blot analysis (lower left panel) and immunohistochemistry using a polyclonal antibody against 1-8U and a FITC-coupled secondary antibody (lower right panel).

1-8U were detected in a distinct, punctate and vesicle-like distribution, most densely clustered in the perinuclear space, with no significant staining of the nucleus. This microscopic localization suggestive of membrane association of 1-8U was directly confirmed by differential centrifugation of cell homogenates where 1-8U was detectable in membrane fractions but entirely absent from the fraction containing the cytosolic, water-soluble proteins of IFN-α-induced ME59/cl4 cells (fig. 6A). Note that the subcellular location of 1-8U in ME59/cl7 and ME15 was essentially the same as shown above and that the distribution of 1-8U in the resistant lines did not change upon IFN-α treatment (data not shown).

Using marker antibodies to the endoplasmatic reticulum, the trans-Golgi network or the cell surface protein caveolin, we could not detect a significant colocalization of 1-8U with these markers. A significant colocalization was obtained with MHC class I (HLA-A, -B, -C) proteins, which are expressed in all melanoma cells and upregulated by IFN-α in sensitive lines only [3]. MHC class I proteins form the complex that presents antigens to cytotoxic T cells [33]. Note the presence of both signals on the plasma membrane and in vesicular structures (fig. 6B).

We examined the ultrastructural localization of 1-8U in more detail by immunoelectron microscopy. Antibodies to 1-8U detect the protein in large vesicles, which appear as bright, round-shaped structures (fig. 6C, left panel). Magnification of the vesicles revealed that these organelles are complex with multiple internal vesicles, which are structurally similar to exosomes, a relatively new class of organelles involved in the export of proteins to the extracellular space (fig. 6C, right panel).

Discussion

We have shown that differential expression of 1-8U in resistant and sensitive human melanoma lines correlates directly with inhibitory effects of IFN-α and that recombinant expression of this gene is sufficient to inhibit proliferation in sensitive cell lines.

IFNs are known to induce tumor suppressor genes and directly inhibit cell proliferation [34]. By using an annexin/propidium iodide assay, we found that 1-8U-induced inhibition of growth is independent of apoptosis (data not shown). Little is known about 1-8U function because most studies have focused on 9-27, the gene encoding the IFN-inducible Leu13 antigen [35–37]. Consistent with the 1-8U data, activation of 9-27 inhibits proliferation of leukocytes which is not that surprising given the overall 95% primary sequence identity between the two proteins [10, 29]. Furthermore, IFN-α treatment of human melanoma xenografts in SCID mice strongly inhibits tumor development together with an upregulation of 1-8U [C. Krepler, unpublished data]. Strikingly, only five additional IFN-inducible genes are actively transcribed 4 weeks after tumor implantation and cytokine treatment. Conversely, a transition from a slow to fast cell division rate (i.e. transformation, tumor progression, metastasis) is usually associated with suppression of 1-8 family gene transcription, another feature of bona fide tumor suppressor genes [12, 13, 27].

The human melanoma line D10, which constitutively expresses high levels of 1-8U, does not respond to the growth inhibitory effects of IFN but shows a significantly reduced cell division rate compared to unstimulated ME15 cells (data not shown). This explains our observation that infec-

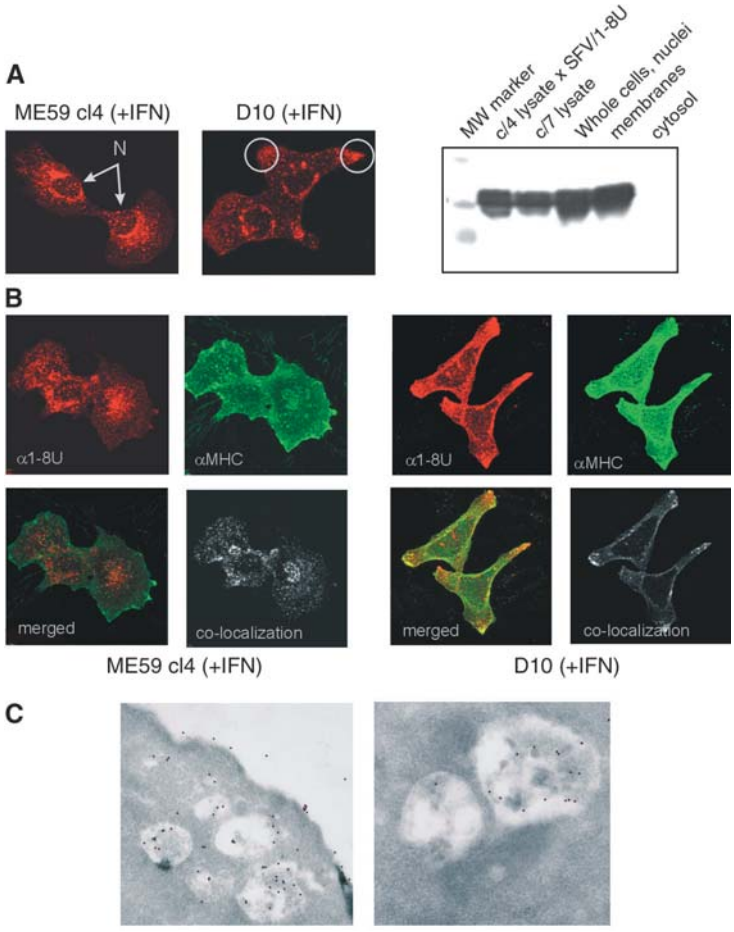

Figure 6. (*A*) The intracellular localization of 1-8U in melanoma cells was determined by immunofluorescence confocal microscopy using a specific antibody against 1-8U and a Cy3-coupled secondary antibody (two left panels). Membrane association of 1-8U was shown by Western blot analysis of fractions of IFN-treated, homogenized ME59/cl4 cells (right panel). (*B*) Double labeling shows that 1-8U and MHC class I proteins colocalize. Staining of 1-8U was as in (*A*), MHC class I proteins were labeled with a specific primary and Cy2-conjugated secondary antibody. Colocalization maps were generated using the Bitplane Colocalization software. Colocalization appears in white. (*C*) Immunoelectron microscopy detects 1-8U in large vesicles and in the vicinity of the cell membrane in ME59/cl4 cells (left panel). These vesicles contain smaller subcompartments (right panel). As secondary antibody, a gold-conjugated mouse anti-rabbit IgG was used. In all experiments, cells were treated with 1000 U/ml IFN-α for 48 h prior to proceeding.

tion of D10 with SFV/1-8U does not further arrest proliferation contrary to the sensitive line ME15, probably due to saturation of the receptor complex (fig. 7).

Insensitivity to the growth inhibitory effect of the cytokine has been linked to defects in signal transduction. Cells with defective receptor-associated tyrosine kinases JAK1 and TYK2, defective transcription factors STAT1 or STAT2 or cells without surface receptors do not respond to IFN stimulation [38–40]. ME59/cl7 is an IFN-resistant clone selected by exposure of a heterogenous melanoma cell population to high doses of cytokine. In general, this cell line does not show disabled IFN signaling, because a significant portion of IFN response genes remain fully inducible (table 1). A previous microarray-based comparison of IFN-inducible genes using the nonclonal lines ME15 and D10 gave a very similar result to that obtained here (fig. 5) [3].

A detailed analysis of the protein content and tyrosine phosphorylation status of the JAKs and STATs involved in IFN-α signaling in D10 revealed a defect in STAT3 phosphorylation as the only detectable abnormality [18]. Serine phosphorylation of STAT1 is necessary for growth inhibitory effects of IFN-α [40], and an involvement of STAT3 in this process is possible, but has not been proven experimentally [18, 41]. The view that unknown epigenetic or trans-acting factors rather than mutations of individual IFN-inducible genes mediate IFN resistance in D10 is supported by reporter gene assays with wild-type 1-8U, 1-8D and 6-16 promoters. 1-8D, for example, is uninducible in ME15 due to an ISRE base pair transition but still deregulated and active in D10 like its functional counterpart 1-8U (fig. 4). The 6-16 promoter on the other hand is inducible in ME15 but remains inactive in D10 after IFN stimulation.

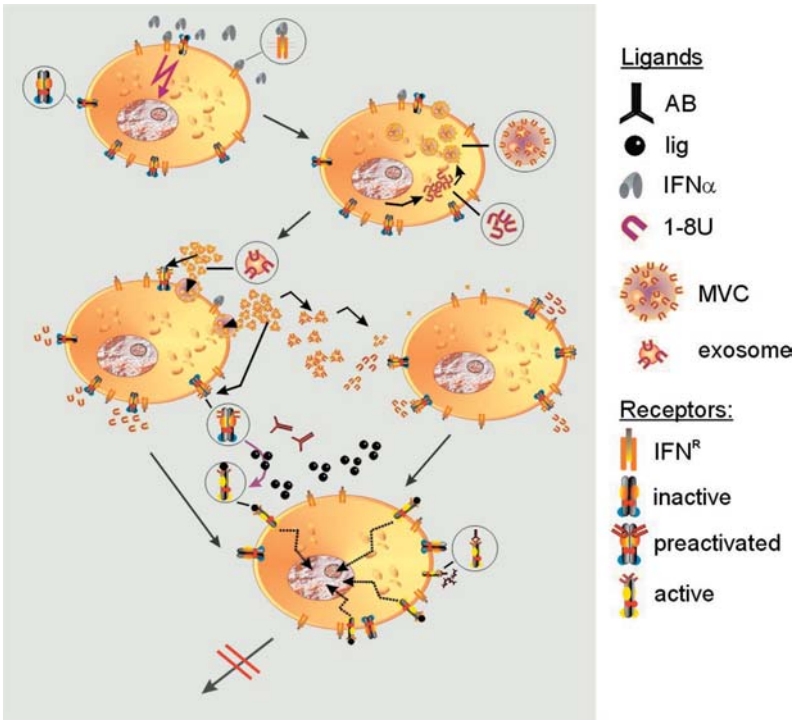

Figure 7. Proposed model for IFN-α-inducible inhibition of proliferation mediated by 1-8U. Top to bottom: INF-α stimulation induces gene expression of 1-8U and other response genes. Following synthesis, 1-8U is transferred to multivesicular complexes (MVC) and assembled into exosome-like particles. Upon transport to the cell surface, fusion of MVCs with the plasma membrane allows assembly of 1-8U with a pre-existing 1-8U receptor complex (1-8U^{RC}) on the cell surface. Released exosome-like particles or free 1-8U can also travel to non-stimulated cells where receptor assembly occurs. A putative ligand or antibodies bind to the pre-activated complex, which induces dimerization and transduction of the antiproliferative signal to the nucleus. In IFN-resistant cells, constitutive 1-8U expression pre-activates all receptors and recombinant expression of 1-8U has no further impact on the proliferation rate. The model also accounts for induction of anti-proliferative activity induced by antibodies to the 1-8U homolog 9-27 (AB) and explains the transmission of antiproliferative activity from IFN induced cells to non-treated cells present in the same culture. Down-regluation of 1-8U would abolish any 1-8U^{RC} signaling and inhibition of proliferation. The hydrophobic, activated receptor complexes may enable adhesion of neighboring cells.

Microarray transcript imaging of ME59/cl4 and ME59/cl7 did identify additional genes with 6-16-like regulation, including egr-2, amphiregulin and gata-6, which can act as negative regulators of proliferation [42–45]. However, none of these genes has ISREs in the control region and activation probably occurs through transcription factors, which are under direct IFN control. Apart from 1-8U, additional genes are deregulated in ME59/cl7. p27 is an IFN-inducible gene of unknown function. sim-2 and mrg-2 encode transcription factors which have not been associated with IFN action until now. Their dysregulation in IFN-resistant cells may cause constitutive activation of otherwise silent genes. Overexpression of deregulated genes in IFN-responsive cells followed by microarray analysis may reveal the function and identify downstream target genes of these inducible transcription factors.

The association of 1-8U protein with the plasma cell membrane and multivesicular compartments that contain exosome-like organelles together with the observed abundance after IFN induction are unusual properties of a cell cycle control protein. Exosomes are small membrane vesicles generated by inward budding of multivesicular compartment membranes and are secreted as a consequence of fusion of these large organelles with the plasma membrane [46]. They occur in many cell types including various tumors, antigen-presenting cells as well as mast and B cells [47–50]. Depending on the cell type, exosomes contain a discrete set of proteins including MHC class I molecules and tetraspanins [46]. One of the tetraspanins found in exosomes is CD81, which has been described to be part of the 9-27 multimeric complex on the surface of B cells [51, 52].

Preliminary results show that 1-8U is present in exosome preparations from D10 melanoma cells together with the tetraspanins CD81 and CD82 and MHC class I molecules which is in line with our observation that 1-8U and MHC class I proteins colocalize. The rate of release of exosomes from untreated ME15 and D10 is similar and does not increase upon IFN-α stimulation [T. Roehn, unpublished data]. Also noteworthy in this context is that a membrane fraction enriched for 17-kDa proteins is able

to induce inhibition of tumor cell growth, conceivably by providing 1-8U to the tetraspanin complex followed by signal transduction [53]. Accordingly, exosomes released from IFN-α-treated cells may make contact with other cells and deliver their content. This could also explain the observed transfer of antiproliferative activity from IFN-α-stimulated to native cells when cocultured [54].

An additional property of 1-8 family proteins is their involvement in mediating adhesion in connection with normal cellular processes. Activation of the 9-27 complex on leukocytes not only results in growth arrest, but also in homotypic adhesion of B and T cells [55, 56]. The rat homolog *rat8* triggers cell-cell contact formation during the development of the mammary gland [57]. Expression of the mouse 1-8U equivalent *fragilis* in germ cells is apparently important during embryogenesis and the development of primary tissues. Expression of this gene marks the onset of germ cell competence, supposedly by demarcating putative germ cells from somatic neighbors through homotypic adhesion. Expression of *fragilis* also correlates with an increase in the cell cycle time in nascent germ cells while surrounding somatic cells continue to divide rapidly [58].

The bovine IFN-τ-inducible homolog of 1-8U is induced during embryogenesis and is apparently responsible for adhesion of the embryo to uterine tissue [59]. We are currently testing whether bovine 1-8U has trans-species activity and inhibits proliferation of human or mouse cells.

A model summarising the known functions of 1-8 family proteins is shown in figure 7. We propose that 1-8U in cultured cells is exported in multivesicular compartments together with other proteins (e.g. CD81, CD82, MHC) to the cell surface where it is assembled into tetraspanin receptors, which transmit antiproliferative signals to the nucleus induced by an orphan ligand present in the culture medium or on the surface of neighboring cells. A portion of the 1-8U-containing exosomes can bind to non-stimulated cells where transmission of the antiproliferative signal occurs. Once all surface receptors contain 1-8U, no further signal enhancement can be achieved, for example by recombinant overexpression of 1-8U in D10. This can explain the correlation between high constitutive expression of 1-8U and IFN resistance and the inherent lower proliferation rate of resistant melanoma cell lines such as D10 relative to M15 (see Appendix).

Absence of 1-8U in mast cell tumors due to downregulation would eliminate the antiproliferative signal resulting in rapid proliferation. 1-8U induction by IFN reactivates the receptor and the antiproliferative signal is transmitted to the nucleus. The same mechanism may explain the antiproliferative activity of a 17-kDa enriched membrane fraction added to the culture medium, which may potentially contain 9-27 [53].

At the same time, these surface structures on 1-8U-positive, slowly dividing cells may enable cell-cell contacts and adhesion resulting in the formation of primitive tissue cell layers as observed for *rat8* or *fragilis* [57, 58]. In tumors, IFN-α treatment would cause adhesion of the malignant cells to the primary tumor, which would prevent release of metastasizing cells followed by penetration into other tissues and secondary tumor development. In summary, IFN-inducible genes are apparently not only involved in host defense but rather participate in normal developmental processes. Future studies may identify additional members of this class of genes using a combination of functional and genomic experimental approaches.

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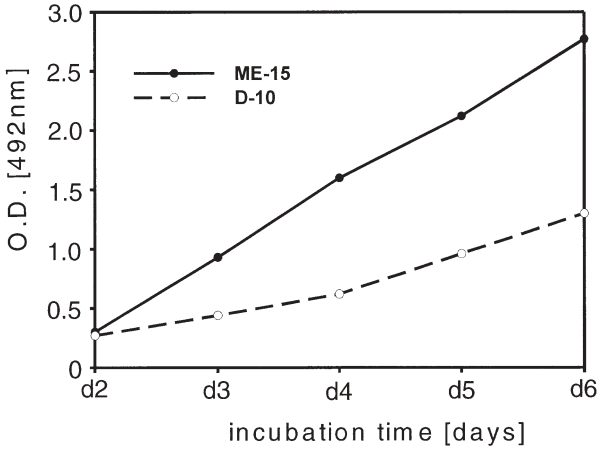
Appendix

Lower inherent growth of the IFN-α-resistant cell line D10 compared to ME15.



Cells were grown in the absence of IFN-α. Cell numbers were measured every 24 h using the commercial assay described in Materials and methods.

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