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# Synergistic activation of intercellular adhesion molecule 1 (ICAM-1) by TNF-α and IFN-γ is mediated by p65/p50 and p65/c-Rel and interferon-responsive factor Stat1α (p91) that can be activated by both IFN-γ and IFN-α

Andreas Jahnke[a], Judith P. Johnson[a,*]

[a]*Institute of Immunology, Goethestr. 31, 80336 Munich, Germany*

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**Abstract** Human ICAM-1 expression is up-regulated by IFN-γ and TNF-α and synergistically increased by a combination of both. Transient expression of ICAM-1/luciferase constructs led to definition of the regulatory regions mediating the cytokine response and showed that both are necessary for synergism. Immunochemical electromobility shift assays identified the TNF-α-dependent complexes that bound to the NF-κB like sequence at −187 as p65/p50 and p65/c-Rel. The interferon responsive region at −75 was bound by a Stat1α (p91) containing complex that was activated by both IFN-γ and IFN-α. Although both regions were required for synergism, no additional or enhanced binding complexes were observed.

*Key words:* Genetics; Cell adhesion molecule; Promoter region; Trans-activation; Regulatory-sequence; DNA-binding protein; Cytokine

## 1. Introduction

The glycoprotein intercellular adhesion molecule 1 (ICAM-1) serves as a counter-receptor for leukocyte β2 integrins and for the T cell mucin CD43 [1–4]. The interaction between ICAM-1 and its ligands is an important step in the development of the inflammatory response strengthening effector–target interactions and mediating leukocyte migration into the tissues [5,6]. While constitutive expression of ICAM-1 is restricted to a few cell types [1,7–10], ICAM-1 can be induced on essentially any cell type by IFN-γ and TNF-α [5,8,11–15]. Cytokines are often expressed in combination in vivo [16], and IFN-γ plus TNF-α can act synergistically to enhance ICAM-1 expression [5,13,14], as has been seen with MHC class I [17] and IL-8 [18]. We have determined the regions of the ICAM-1 promoter that are responsive to IFN-γ and TNF-α, and characterised the nuclear complexes binding to them. Following exposure to TNF-α the NF-κB-like responsive region RRBE (Rel-related protein binding region) is bound by two complexes, p50/p65 and a p65/c-Rel heterodimer. Stimulation with either IFN-γ or IFN-α led to binding to the interferon-responsive element of a protein complex shown to contain Stat1α (p91). Protein binding to both regions was required for the synergistic effect of TNF-α plus IFN-γ, however, additional or enhanced protein complexes were not observed.

## 2. Material and methods

### 2.1. Transient transfection and cyokine stimulation

Human melanoma cell line Mel JuSo was cultured as described [19]. Transient transfections were performed using the Transferrinfection system (Serva, Heidelberg, Germany). The constructs, transfection and luciferase assays have been described [12,19]. Six hours following transfection, the cells were washed with prewarmed medium and incubated for a further 24 h in fresh medium with or without cytokines. Recombinant human IFN-γ and IFN-α was obtained from Biogen, Cambridge, MA. Recombinant human TNF-α ($7.4 \times 10^6$ U/mg) was kindly provided by Dr. T. Subkowski, BASF, Ludwigshafen, Germany. Cells were treated with 100 U/ml IFN-γ, 1,000 U/ml IFN-α and 200 U/ml TNF-α where indicated.

### 2.2. Indirect immunofluorescence, flow cytometry and Northern blot analysis

Expression of ICAM-1 on the cell surface was determined by indirect immunfluorescence using the anti ICAM-1 mab P3.58BA-11 [20] or an isotype control upc10 (Sigma, St. Louis, MO). Total RNA was prepared using a modification of the caesium trifluoracetate method (CsTFA; Pharmacia, Freiburg, Germany). The sequence of the GAPDH-specific oligonucleotide was 5′ CCCTGGTGACCAGGCGGCCAATACGGCCAATCCGTTGACTCCGACTTTCCAC 3′ [21]. For further details see the legend to Fig. 1B.

### 2.3. TNF receptor binding assay

TNF-α was labelled with $^{125}$Iodine by the chloramine-T method [22]. Specific radioactivity was 20 μCi/μg for [$^{125}$I]TNF-α, and the recovery of biological activity was 74%. Mel JuSo cells were cultured in 24-well plates (Costar, Fernwald, Germany) with or without IFN-γ for 24 h to subconfluent state (about $7 \times 10^5$ cells/well). Specific binding of the radiolabelled TNF-α applied to the cells for 2 h at 4°C at a concentration of 0.5 nM, in the presence or absence of antibodies against TNF-R p75 and p55, was determined as described ([23] and references therein).

### 2.4. Nuclear extracts and electromobility shift assays (EMSA)

Nuclear extracts were prepared as described [24]. Binding reactions were performed in a 20 μl reaction volume containing 20 mM HEPES, 1 mM $MgCl_2$, 50 mM KCl, 12% glycerol, 0.1 mM EDTA, 0.5 mM PMSF (phenylmethylsufonic acid), 0.5 mM DTT (dithiothreitol), 5 μg of nuclear protein and 1 μg poly[d(A-T)] (Boehringer, Mannheim, Germany) as unspecific competitor. After 10 min on ice 10 fmol $^{32}$P-labelled oligonucleotide and, where indicated, unlabelled competitor oligonucleotide in excess were added and incubation was continued for 1 h further. Samples were run on a 4% polyacrylamide gel (38:1) in 0.25 × TBE (prerun for 1 h) at 150 V for 2.5 h. The dried gels were exposed for autoradiography overnight.

*Corresponding author. Fax: (49) (89) 5160 2236.

*Abbreviations*: EMSA, electromobility shift assay; GAF, γ-activated factor; GAS, γ-activated sequence; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; IRE, interferon-responsive element; ISGF, interferon α-stimulated gene factor; RRBE, Rel-related protein binding element; TNF, tumor necrosis factor; TNF-R, TNF-α receptor.


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*2.5. Oligonucleotides*

Oligonucleotides were annealed and labelled with [α-$^{32}$P]dATP (Amersham, Braunschweig, Germany). Oligonucleotides with a 5′ overhang were labelled with Klenow polymerase (Boehringer, Mannheim, Germany), others with terminal deoxytransferase (Gibco-BRL, Gaithersburg, MD). The sequences of the oligonucleotides used are as follows (5′→3′; lower case letters indicate added cloning sites; the complementary sequence is not shown):

ICAM/NF-κB, cgggatccCTTGGAAATTCCGGAGC (−189 to −173)

ICAM/IRE, cgggatccGTTTCCGGGAAAGCAGCA (−77 to −60)

ICAM/SP1, cgggatccAGCACCGCCCCTTGG (−63 to −49)

ICAM/AP2, cgggatccCTTGGCCCCCAGGTG (−53 to −39)

HIV/NF-κB, AGCTTCAGAGGGGACTTTCCGAGAGG [25]

GAS, catgAGTTTCATATTACTCTAAATC [26]

IREGC, cgggatccGAAAGCGAAAGCGAAAGCGAAAGC [27].

*2.6. Immunochemical identification of nuclear factors (antibody EMSA)*

Nuclear extracts and oligonucleotides were incubated on ice for 15 min, antibodies (2 μl, 100 μg/μl) were added and the mixture incubated for a further 1 h on ice. The antibodies used (Santa Cruz Biotech., Santa Cruz, CA) were a monoclonal antibody directed against ISGF-3 p91, rabbit polyclonal anti-p65, anti-c-Rel and anti-p52 antiserum, an isotype control monoclonal anti-TNF-R p75 [23] and normal rabbit serum. Antiserum directed to p50 (Santa Cruz Biotech.) was used as an agarose-conjugate. In this case, nuclear extracts were incubated for 1 h on ice with 20 μl (20 μg IgG/μl agarose) anti-p50 or normal rabbit IgG-agarose, centrifuged, and the supernatant used in the binding reaction.

## 3. Results

The melanoma cell line Mel JuSo constitutively expresses ICAM-1 on its cell surface, as determined by indirect immunofluorescence and flow cytometry. Treatment for 24 h with either IFN-γ or TNF-α increased this expression, and a combination of both cytokines gave rise to a synergistic increase in ICAM-1 expression (Fig. 1A) that was associated with increased steady-state mRNA levels. Induction for 4 h with the cytokines either alone or in combination led to an increase in ICAM-1 mRNA as compared to the mRNA of the *GAPDH* gene (Fig. 1B). Densitometric analysis and comparison of ICAM-1 mRNA with that of GAPDH revealed an increase of ICAM-1 mRNA of approximately 10-fold with IFN-γ, 20-fold with TNF-α and 80-fold with a combination of both.

In order to determine the regions responsible for the synergistic activation of ICAM-1, its 5′ upstream region from −1352 to +1 (the downstream transcription start site was set to +1 [28]) was placed in front of the promoterless luciferase reporter gene construct pXP-2 [29]. Mel JuSo cells were transiently transfected with plasmids containing the 5′ end and internal deletions for 6 h, washed once and then exposed to IFN-γ, TNF-α or both for 24 h. The luciferase activity of the induced cells was compared to that of the untreated cells and the results are shown in Fig. 2 as fold induction for each of the plasmids used. The promoterless construct pXP-2 was used as a negative control and showed no inducibility by either cytokine. All constructs containing between 1352 and 339 bp of the ICAM-1 upstream sequence showed a 6-fold induction with

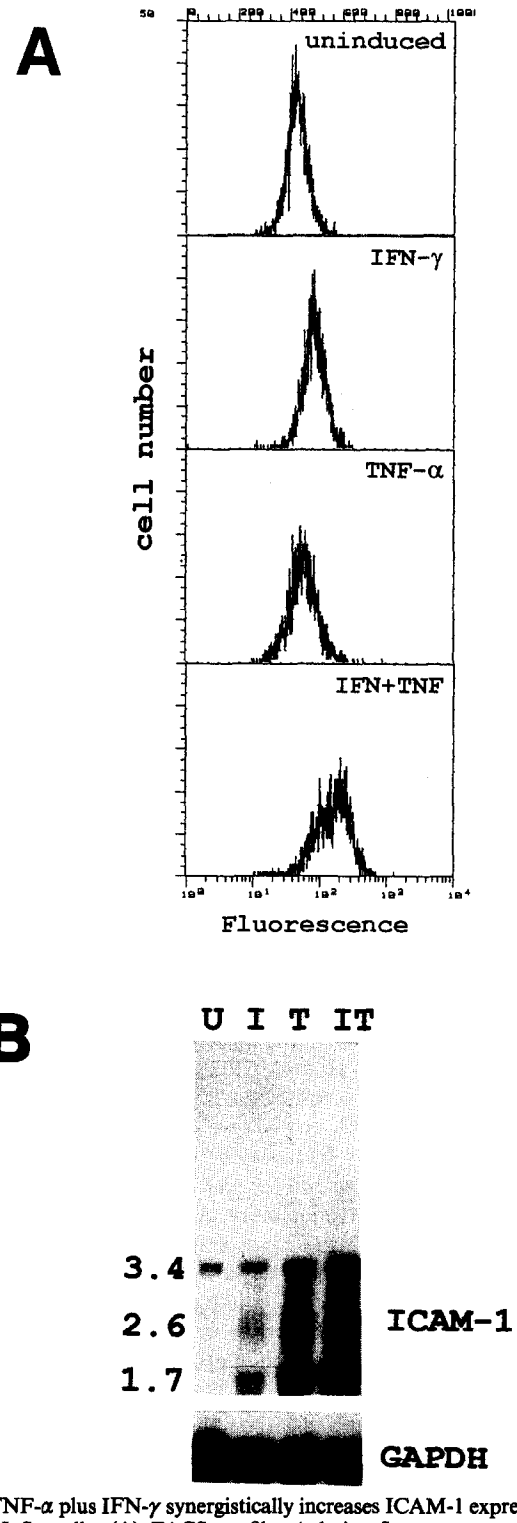


Fig. 1. TNF-α plus IFN-γ synergistically increases ICAM-1 expression in Mel JuSo cells. (A) FACS profiles (relative fluorescence vs. cell number) for Mel JuSo cells stained with monoclonal antibody P3.58BA-11 directed against ICAM-1. The cells were either left untreated or were treated with indicated cytokines for 24 h. The fluorescence of isotype control antibody upc10 was subtracted from the ICAM-1 graphs. (B) Northern blot of ICAM-1. 20 μg total Mel JuSo RNA treated as indicated (U, uninduced; I, IFN-γ; T, TNF-α; IT, both cytokines) for 4 h were electrophoresed, blotted and hybridised with an ICAM-1 cDNA 690 bp *Pst*I fragment followed by an oligonucleotide specific for the *GAPDH* gene. The size (kb) of the three observed ICAM-1 mRNAs are indicated.

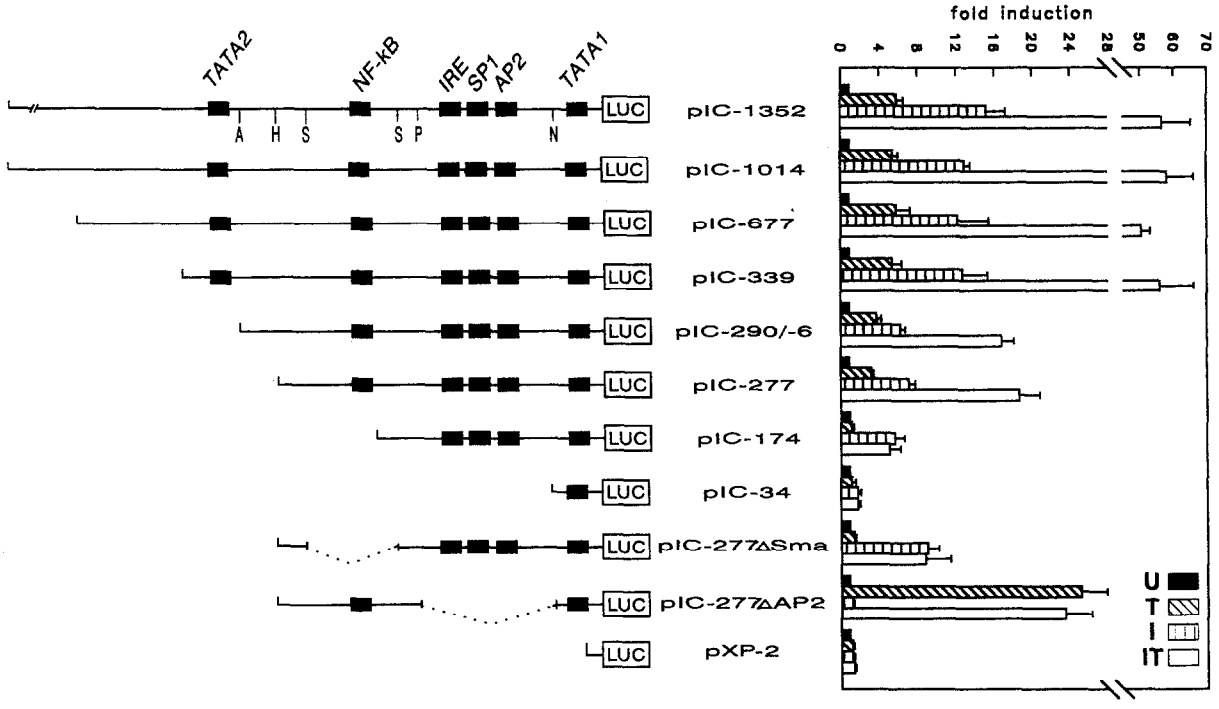


Fig. 2. The NF-κB and the IRE sites are necessary for a synergistic response to TNF-α and IFN-γ in Mel JuSo. The right side indicates the fold induction of the transiently transfected constructs under the influence of TNF-α (T, hatched bars), IFN-γ (I, striped bars) and IFN-γ plus TNF-α (IT, open bars) for 24 h. The luciferase activity of the uninduced cells (U, filled bars) was set at 1. The left side shows a scheme of the ICAM-1 5 region cloned in front of a luciferase gene (LUC). TATA1 and -2 represent the downstream and upstream TATA box of the ICAM-1 promoter, respectively. IRE, interferon responsive element; NF-kB, NF-κB binding site; AP2 and SP1, binding sites for transcription factors AP-2 and Sp1. A, *Acc*I; H, *Hin*dIII; S, *Sma*I; P, *Pst*I; N, *Nco*I.

TNF-α (hatched bars), 14-fold induction with IFN-γ (striped bars) and a 55-fold induction with both cytokines (open bars). Deletion to position −277 reduced overall inducibility (perhaps a reflection of the higher constitutive expression of these constructs [19]), but still showed synergistic induction with IFN-γ plus TNF-α. The shortest construct, pIC-34, containing only the downstream TATA box, showed no inducibility with either cytokine. Deletion of the region between −277 and −174 abolished the TNF-α inducibility as well as the synergistic effect but did not affect IFN-γ inducibility. An internal deletion in pIC-277, which removed the *Sma*I fragment between −227 and −136, also abolished the TNF-α response. Thus the element mainly responsible for TNF-α induction lies between position −227 and −174. Interestingly the inducibility of pIC-277ΔSma by IFN-γ was higher than that of pIC-174 or pIC-277, although the constitutive level of pIC-277ΔSma was identical to that of pIC-277. Sequence comparison of the region between −227 and −174 revealed good homology to a NF-κB consensus motif at bp −187 to −178.

An internal deletion in pIC-277 between position −107 and −34 revealed that this region is responsible for interferon inducibility. Construct pIC-277ΔAP2 showed no inducibility after treatment with IFN-γ, but still was induced with TNF-α. The combination of IFN-γ plus TNF-α had no additional effect on the induction. Plasmid pIC-277ΔAP2 (that showed low constitutive expression) led to a much higher inducibility with TNF-α than did pIC-277, the construct without internal deletion. Sequence analysis of the region from −107 to −34 revealed homology between −75 and −66 and published sequences of elements responsible for the IFN-γ response (called here IRE for interferon-responsive element).

In order to exclude the possibility that the synergism observed could be due to an increase in TNF-α receptor expression under the influence of IFN-γ we analysed the binding of radioactively labelled TNF-α to Mel JuSo cells treated for 24 h either with IFN-γ (100 U/ml) or only with medium as a control. A less than 10% increase in binding of TNF-α was observed after IFN-γ treatment, indicating that the increase in the TNF receptor is not responsible for the synergism observed in ICAM-1 expression (data not shown). In addition, exposure of the cells to TNF-α alone followed by washing and exposure to IFN-γ 3 h later still led to synergistic induction of reporter gene constructs, confirming that synergism does not depend on an increase in TNF-R.

Electromobility shift assays (EMSAs) were performed using the putative ICAM-1 NF-κB motif and nuclear extracts obtained from Mel JuSo cells treated for 15 min with IFN-γ, TNF-α, both cytokines or medium alone (Fig. 3A). Exposure of the cells to TNF-α either alone (lane 4) or in combination with IFN-γ (lane 5) led to the appearance of one new band (lower complex, LC) and a dramatic increase in the amount of a second complex (upper complex, UC). Competition with unlabelled oligonucleotides suggested that these two complexes reflect the binding of NF-κB. Both bands were inhibited by the HIV/NF-κB binding sequence (lanes 9–11) as well as by the unlabelled putative ICAM-1 NF-κB binding sequence (lanes 6–8) but were not influenced by the presence of ICAM-1 Sp1 (lane 12) AP-2 or binding sites (lane 13). A third weak band

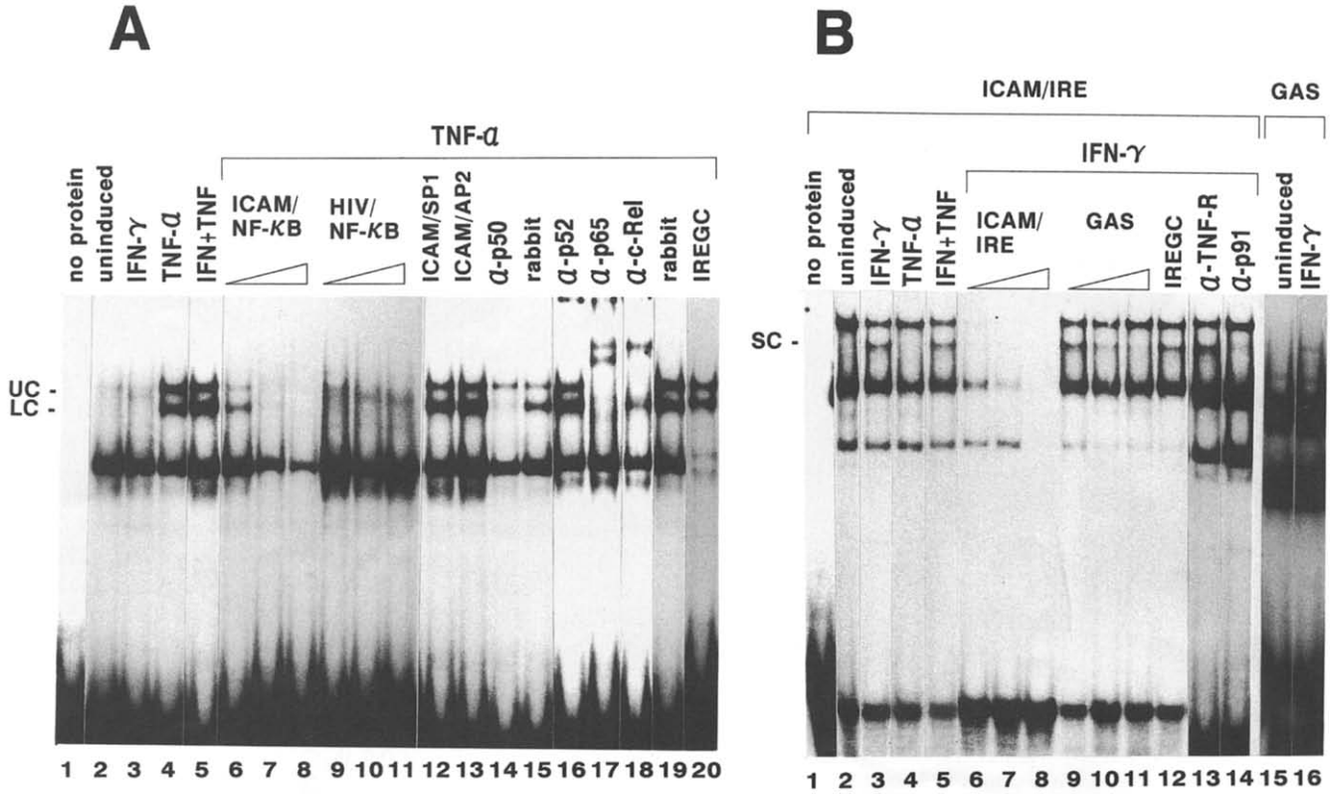


Fig. 3. EMSAs with oligonucleotides containing the ICAM-1 NF-κB or IRE sites and immunochemical identification of the cytokine specific complexes. (A) ICAM/NF-κB oligonucleotide with extracts of cells left untreated or treated with IFN-γ, TNF-α or both cytokines for 15 min (lanes 2–5). Unlabelled competitor ICAM/NF-κB (lanes 6–8) and HIV/NF-κB (lane 9–11) was added in increasing concentrations (10-, 50-, 250-fold excess), while ICAM/SP1, ICAM/AP2 and IREGC were added at 250-fold excess (lanes 12,13,20). Antibodies (lanes 14–19) were used as described in section 2. UC and LC are the upper and lower complexes, respectively. (B) Oligonucleotides ICAM/IRE (lanes 1–14) and GAS (lanes 15,16) generate an IFN-γ induction specific complex (SC). Competition of ICAM/IRE with itself (lanes 6–8) or GAS (lanes 9–11) with increasing excess (10-, 50-, 250-fold). IREGC 250-fold (lane 12). Antibody p91 and isotype control (lanes 13,14) used as described in section 2.

migrating slightly faster than UC was sometimes observed with extracts of both interferon-induced and uninduced cells (lanes 2 and 3). While competition with unlabelled HIV/NF-κB oligonucleotide readily inhibited the TNF-α-specific bands (UC and LC), this diffuse band was not affected. Thus this band seems to be cytokine independent and NF-κB unspecific. Two fast migrating bands were observed with all extracts and were independent of cytokine treatment. These complexes apparently reflect binding to the filled-in *Bam*HI cloning sites present in the ICAM-1/NF-κB oligonucleotide, as they are inhibited by an unrelated filled-in competitor oligonucleotide (IREGC, lane 20).

Specific antibodies were used to determine the composition of the observed complexes. Exposure of the nuclear extracts of TNF-α-treated cells to insolubule anti-p50 reduced the intensity of LC without affecting the upper band (lane 14). Control rabbit IgG had no effect on either band (lane 15). The addition of anti-p65 supershifted both LC and UC (lane 17), indicating that both complexes contain p65. An antibody directed against c-Rel supershifted only UC (lane 18). Rabbit IgG (lane 19) and an antibody against p52 (lane 16) had no effect on either complex. These results indicate that LC consists of a p65/p50 (NF-κB) heterodimer while UC consists of a p65/c-Rel heterodimer.

Following exposure to IFN-γ either alone or in combination with TNF-α, a single new specific complex (SC) was observed (Fig. 3B, lanes 3 and 5). This band was not detected with cell extracts of uninduced or TNF-α-treated cells (lanes 2 and 4) and was inhibited by unlabelled ICAM/IRE (lanes 6–8) and to a lesser extent by unlabelled GAS oligonucleotide (lanes 9–11) containing the *GBP* gene consensus binding site for the GAF [30]. A related oligonucleotide, IREGC, containing 4 repeats of the GAAAGC motif shown to be responsible for IFN-α/γ and virus inducibility [31], did not compete with this complex (lane 12). Additional bands were observed with nuclear extracts of Mel JuSo cells independently of cytokine exposure, indicating that these complexes are not involved in the IFN-γ response, although the fastest migrating band could be out-competed with unlabelled and not filled-in GAS and IREGC oligonucleotides (lanes 9–12).

Treatment with a monoclonal antibody raised against the ISGF-3 p91 protein (= Stat1α = Stat91) depleted SC (lane 14), indicating that this complex contains p91 or an antigenically related protein. An isotype control antibody against p75 TNF receptor had no influence on the observed complexes (lane 13).

EMSAs performed with labelled GAS oligonucleotide (shown to bind p91; GAF[26]) revealed, in extracts of IFN-γ-induced cells, the formation of a band migrating similarly to the p91-containing complex (lanes 15 and16), suggesting identity with both complexes.

ICAM-1 expression in Mel JuSo cells can also be up-regulated by IFN-α. We therefore examined whether IFN-α (1000 U/ml, 15 min incubation) could also induce a protein binding

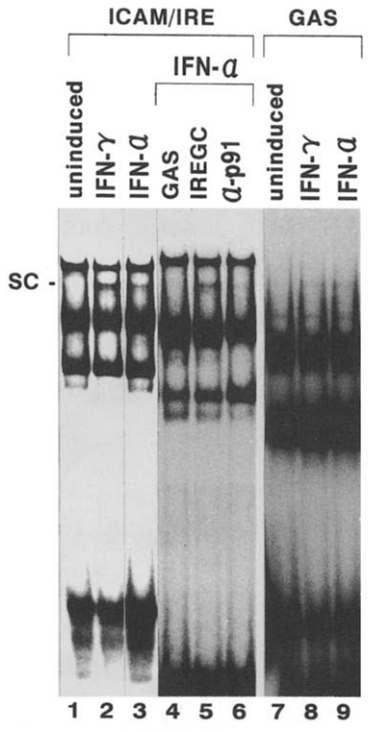


Fig. 4. Induction with IFN-α led to a specific complex (SC) identical to that observed after IFN-γ treatment. Mel JuSo cells were left untreated or were induced with IFN-γ (lanes 1,2 and 7,8) or IFN-α (lanes 3–6,9) for 15 min. Competitor GAS and IREGC were added at 250-fold excess (lanes 4,5), anti p91 (lane 6) was used as described in section 2.

to the ICAM/IRE and GAS oligonucleotides. Nuclear extracts of induced Mel JuSo cells incubated with either oligonucleotide led to a complex with identical migration characteristics to that observed after treatment with IFN-γ (Fig. 4, lanes 2,3 and 8,9). Competition analysis showed that the GAS oligonucleotide was also a potent competitor of the IFN-α-induced complex (lane 4), while IREGC did not compete even at a 250-fold excess (lane 5). Using the anti-p91 antibody the bound protein was identified as a p91-containing complex (lane 6).

Nuclear extracts prepared from Mel JuSo cells exposed to IFN-γ and TNF-α formed the same complexes with ICAM/NF-κB and ICAM/IRE oligonucleotides and with similar intensities as observed with TNF-α and IFN-γ alone (Fig. 3A, lanes 4 vs. 5; Fig. 3B, lanes 3 vs. 5). To rule out oligonucleotide binding-induced changes in protein conformation, 10 fmol of unlabelled ICAM/NF-κB was added to the ICAM/IRE binding experiments and unlabelled ICAM/IRE to the ICAM/NF-κB binding experiments. No additional or enhanced bands were observed in the nuclear extracts from cells exposed to both IFN-γ and TNF-α (data not shown). In order to exclude the possibility that the observed synergistic induction of ICAM-1 was mediated by NF-κB and the AP-2 or Sp1 binding site close to the IRE element, oligonucleotides ICAM/AP2 and ICAM/SP1 were tested for interaction with nuclear extract of cells treated with either cytokine alone or a combination of interferon and TNF. Neither sequence revealed a cytokine-specific or enhanced complex, indicating that these regions play no role in TNF-α- or IFN-γ-mediated ICAM-1 induction (unpublished observation).

## 4. Discussion

Exposure of a variety of cell types to IFN-γ or TNF-α results in increased surface ICAM-1 expression which is synergistic in the presence of both cytokines [5,13,14]. Surface expression correlates with steady-state mRNA levels, indicating that the regulation of ICAM-1 expression by cytokines occurs at the transcriptional level [11,13,15]. Using transient transfection of reporter gene constructs, containing 5′ end and internal deletions of the region upstream of the ICAM-1 gene, the regions responsible for these responses have been identified, and using EMSAs the protein complexes binding to these regions have been characterised.

The position of the TNF-α responsive element in Mel JuSo (bp −227 to −174) is identical to that observed in other cells [12,31], indicating a general TNF-α induction pathway concerning ICAM-1. The TNF-α-responsive region contains an NF-κB-like motif and, as has recently been reported in TNF-α-treated U937 cells, 2 specifically binding complexes (LC, UC; Fig. 3A) were observed in Mel JuSo cells. Cells that were cytokine treated for 3 h instead of 15 min generated the same bands, although they were weaker, indicating a low stability of the induced complexes (data not shown).

Antibodies directed against various Rel-related proteins were used to characterise the proteins binding to this region, and identified the lower complex as p65/p50 (NF-κB protein) and the upper one as p65/c-Rel heterodimer. An element in the human urokinase gene (uPA) binding both p65/p50 and p65/c-Rel, termed RRBE (Rel-related protein-binding element) [32], shows homology to the ICAM-1 sequence (uPA, GGGAAAGTAC; ICAM-1, TGGAAATTCC). The p65/c-Rel complex has recently been shown to be a strong activator of transcriptional activity [33], and the results reported here indicate that this complex is involved in the up-regulation of ICAM-1 by TNF-α.

The interferon-responsive region in Mel JuSo was identified between position −107 and −34. This correlates with results of Look et al. [11] and Wawryk et al. [34] but stands in contrast to Voraberger et al. [31] who observed an interferon-response element upstream of position −1352. Using a longer construct, pIC-5800, we could not detect any further interferon-responsive region in Mel JuSo, suggesting that the ICAM-1 response to IFN-γ may be regulated differently in various cells.

The region mediating the IFN-γ induction in Mel JuSo cells contains a palindromic sequence, TTTCCGGGAAA, at bp −76 to −66, that is homologous to GAS sequences of other IFN-γ-responsive genes [35]. This sequence bound an IFN-γ induction specific nuclear complex (Fig. 3B, lane 3 vs. 2,4), confirming results of Look et al. [11] who showed that the same sequence mediates the IFN-γ response in epithelial cells. Using a monoclonal antibody that is not cross-reactive with p84 we could show that the specific band contains p91 (= Stat1α = Stat91 [36]), probably binding as a homodimer (GAF) [37].

The IFN-γ-induced complex observed in Mel JuSo cells could be inhibited by unlabelled GAS oligonucleotide (50-fold excess; Fig. 3B, lane 10) and was also detected when radiolabelled GAS was used for binding (lane 16). Using a 20-fold excess of unlabelled GAS oligonucleotide Look et al. could not inhibit this complex, although a 20-fold excess of the gammaresponse region (GRR) of the FcγRI gene was a potent competitor [11]. Sequence comparison between the binding

regions of ICAM/IRE (TTCCGGGAA), FcγRI/GRR (TTCCCAGAA [38]) and GBP/GAS (TTACTCTAA [26]) suggest that the GRR of the FcγRI gene could be a better competitor of the ICAM-1 binding complex. Furthermore other groups have shown cross-competition of the FcγRI GRR and the related ICSBP elements with 50- or 100-fold excess of GAS oligonucleotides [38,39]. These results indicate that ICAM/IRE, GAS and GRR are able to bind the same IFN-γ-induced complex(es), albeit with different binding affinities. A 250-fold excess of an oligonucleotide containing a 4-fold repeat of GAAAGC (one half side to the ICAM/IRE palindrome) did not compete for the specific complex, indicating that this protein needs the full palindrome for binding (lane 12 IREGC). Since MacDonald [27] could show that this site conferred IFN-α, γ and virus inducibility, it seems possible that this oligonucleotide actually binds a protein not identical with p91. Cells treated for 3 h with IFN-γ showed the same specific interferon band with nearly the same strength.

Treatment of cells with IFN-α led to the same band with oligonucleotides ICAM/IRE and GAS as was observed with IFN-γ (Fig. 4, lanes 2,3 and 8,9). This band was also depleted by unlabelled GAS oligonucleotide and was reduced by anti-p91. Thus IFN-α and IFN-γ seem to induce, at least in this in vitro assay, the same binding protein in Mel JuSo. Similar observations were made by Kanno et al. [39] with the murine ICSBP gene.

Exposure of transfected cells to both TNF-α and IFN-γ led to a synergistic increase in reporter gene expression (Fig. 2; 55-fold vs. 6- and 14-fold). Analysis of deletion constructs revealed that both elements, the NF-κB/RRBE site as well as the IRE/GAS site, are involved in this synergism. Deletion of either cytokine-responsive site led to a loss of synergistic induction. EMSAs with other putative transcription factor binding sites in the responsive regions excluded them as responsive sites, confirming the importance of the IRE/GAS and the NF-κB/RRBE sites.

IFN-γ and TNF-α lead to synergistic effects on the expression of a number of different genes [17,18]. Increase in TNF-α receptor expression by IFN-γ has only been observed in certain cases and seems to be cell-type dependent [18,40–43]. Exposure to IFN-γ had no effect on the TNF-R expression in Mel JuSo, suggesting that synergistic induction by TNF-α and IFN-γ is mediated by an element downstream of the cytokine receptors. In other cases, the synergistic induction of transcription is associated with augmented nuclear complex bands [18] or with the appearance of a new band due to adjacent binding of two transcription factors on one oligonucleotide [17]. Using purified proteins, a physical interaction of transcription factors has been shown, for example between NF-κB and members of the C/EBP family and with Sp1 [44–46], a phenomenon that could be responsible for cytokine-mediated synergism in gene expression.

Although we detected cytokine-specific complexes with oligonucleotides, no new bands or changes in band intensity were observed in the nuclear extracts of Mel JuSo cells treated with a combination of IFN-γ and TNF-α. This suggests either that the protein–protein interaction is too weak to be detectable in the absence of purified proteins or that the DNA structure may play a role in synergism. The use of a 126 bp PCR product from position −189 to −63, comprising both the NF-κB/RRBE and IRE/GAS sites, was unable to resolve this question since the large number of proteins binding to this fragment obscured any differences between cytokine-treated and untreated cells. Thus although we identified and characterised the elements necessary for the synergistic increase of ICAM-1 expression induced by TNF-α and IFN-γ and examined some possible ways of interaction, further experiments (perhaps with purified protein) are still necessary to determine the mechanism of synergism.

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