Nuclear factor-κB motif and interferon-α-stimulated response element co-operate in the activation of guanylate-binding protein-1 expression by inflammatory cytokines in endothelial cells

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The large GTPase GBP-1 (guanylate-binding protein -1) is a major IFN-γ (interferon-γ)-induced protein with potent anti-angiogenic activity in endothelial cells. An ISRE (IFN-α-stimulated response element) is necessary and sufficient for the induction of GBP-1 expression by IFN-γ. Recently, we have shown that *in vivo* GBP-1 expression is strongly endothelial-cell-associated and is, in addition to IFN-γ, also activated by interleukin-1β and tumour necrosis factor-α, both *in vitro* and *in vivo* [Lubeseder-Martellato, Guenzi, Jörg, Töpolt, Naschberger, Kremmer, Zietz, Tschachler, Hutzler, Schwemmle et al. (2002) Am. J. Pathol. **161**, 1749–1759; Guenzi, Töpolt, Cornali, Lubeseder-Martellato, Jörg, Matzen, Zietz, Kremmer, Nappi, Schwemmle et al. (2001) EMBO J. **20**, 5568–5577]. In the present study, we identified a NF-κB (nuclear factor κB)-binding motif that, together with ISRE, is required for the induction of GBP-1 expression by interleukin-1β and tumour necrosis factor-α. Deactivation of the NF-κB motif reduced the additive effects of combinations of these cytokines with IFN-γ by more than 50 %. Importantly, NF-κB p50 rather than p65 activated the GBP-1 promoter. The NF-κB motif and ISRE were detected in an almost identical spatial organization, as in the GBP-1 promoter, in the promoter regions of various inflammation-associated genes. Therefore both motifs may constitute a co-operative inflammatory cytokine response module that regulates GBP-1 expression. Our findings may open new perspectives for the use of NF-κB inhibitors to support angiogenesis in inflammatory diseases including ischaemia.

Key words: guanylate-binding protein-1 (GBP-1), human umbilical-vein endothelial cells (HUVEC), inflammation, interleukin-1, nuclear factor κB (NF-κB), tumour necrosis factor.

INTRODUCTION

GBP-1 (guanylate-binding protein-1) is among the major IFN-γ (interferon-γ)-induced proteins in human cells [1]. GBP-1 belongs to a protein family with five different members in humans (GBP-1–GBP-5) [2,3] and in mice (mGBP-1–mGBP-5) [4–7]. Human GBP-1 has been shown to mediate an antiviral effect against vesicular stomatitis and encephalomyocarditis viruses [8]. Recently, we have shown that GBP-1 is an inflammatory response factor in endothelial cells [9–11]. First, GBP-1 characterizes endothelial cells activated by ICs (inflammatory cytokines) *in vitro* and *in vivo* [11]. Secondly, it mediates the inhibition of endothelial cell proliferation [9] and invasion by IC [10]. In agreement with these features, the expression of GBP-1 in endothelial cells is selectively induced by the ICs IFN-α/IFN-γ, IL-1α/IL-1-β (where IL stands for interleukin) and TNF-α (tumour necrosis factor-α), but not by other cytokines, chemokines or growth factors [11].

GBP-1 transcriptional response to IFNs has been characterized extensively [12–15]. An IFN-responsive region has been described as the GBP-1 promoter (position: -216 to $+19$ according to Lew et al. [12]). This sequence contained no consensus TATA or CAAT elements and was characterized by two overlapping elements ISRE (IFN-α-stimulated response element) and GAS (γ-IFN activation site) [12]. ISRE binds ISGF3 (IFN-stimulated gene factor 3), a heterotrimer consisting of STAT1 (signal transduction and activators of transcription 1), STAT2 and p48 (IRF-9, IFN regulatory factor-9) in response to IFN-α [16] and IRF-1 in response to IFN-γ [17]. GAS binds γ-IFN activation factor, a STAT1 homodimer in response to IFN-γ [15] and IFN-α activation factor in response to IFN-α [14]. Notably, in IFN-γ-treated fibroblasts, γ-IFN activation factor binding mediates the early transcriptional response (<1 h), whereas IRF-1 regulates the later phases of activation (>1 h) [15,18].

It is not known how IL-1 and TNF-α activate GBP-1 expression. IL-1β and TNF-α activate many different transcription factors including NF-κB (nuclear factor κB) [19,20]. NF-κB activation involves several structurally related proteins of the Rel/NF-κB family that associate as homo- or heterodimers. This family includes p65 (RelA), p50, cRel, p52 and RelB [21]. To regulate

Abbreviations used: BAY 11-7082, [(E)3-[(4-methylphenyl)sulphonyl]-2-propenenitrile]; C/EBP, CCAAT/enhancer-binding protein; EBM, endothelial cell basal medium; EMSA, electrophoretic mobility-shift assay; FBS, foetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBP-1, guanylate-binding protein-1; GFP, green fluorescent protein; IFN, interferon; GAS, γ-IFN activation site; HEK-293T cells, human embryonic kidney-293T cells; HUVEC, human umbilical-vein endothelial cells; IC, inflammatory cytokine; IL, interleukin; IRF-1, IFN regulatory factor-1; ISGF3, IFN-stimulated gene factor 3; ISRE, IFN-α-stimulated response element; IκB, inhibitory κB; MMP-1, matrix metalloproteinase-1; NF-κB, nuclear factor κB; Oct-1, octamer-binding protein-1; RANTES, regulated upon activation, normal T-cell expressed and secreted; STAT, signal transduction and activators of transcription; TNF, tumour necrosis factor; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane.

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gene expression, the respective dimers bind to a set of related 10 bp motifs (NF-κB-binding sites), which are present in the promoters of NF-κB-regulated genes [22]. In most cells, NF-κB transcription complexes are present in a latent, inactive state in the cytoplasm where they are bound to an IκB (inhibitory κB). Many stimuli can rapidly activate these transcription complexes by freeing them from their inhibitor and enabling them to translocate into the nucleus (reviewed in [23]). The most common form of NF-κB is a heterodimer of p65/p50 [23]. The expression of most NF-κB-regulated genes is activated by p65, whereas p50 modulates the expression levels. This is because, p65 but not p50, harbours a transactivation domain [22]. Rare exceptions to this mechanism have been observed. For example, expression of the C-reactive protein, a major inflammation-associated marker is activated via p50 and modulated via p65 [24,25]. In this case, it has been discussed that other proteins may be recruited into the binding complex that substitute the missing transactivation domain of p50.

In the present study, we detected a cRel (NF-κB)-binding motif in the GBP-1 promoter. We show that this motif is required for a transcriptional response to IL-1β and TNF-α and co-operates with ISRE in the regulation of GBP-1 expression in response to these cytokines. Interestingly, the cRel-binding motif was activated by NF-κB p50 and was detected in a conserved position relative to ISRE in the promoter regions of various inflammation-associated genes. The latter suggested that both elements may establish an IC-responsive promoter module.

EXPERIMENTAL

Cell culture and reagents

HUVEC (human umbilical-vein endothelial cells) were purchased from Cambrex (Verviers, Belgium) and used between passage numbers 4.0 and 6.0. Cells were grown in EBM (endothelial cell basal medium; Cambrex), supplemented with 5 % (v/v) FBS (foetal bovine serum) (EBM-full medium) and propagated in cell culture flasks (Nunc, Wiesbaden, Germany) coated with 1.5 % (w/v) bovine skin gelatin, type B (Sigma–Aldrich, Munich, Germany) in PBS. For all stimulation experiments, the cells were grown in low medium (EBM/0.5 % FBS without supplements). Cytokines were added in PBS containing 0.1 % BSA (Sigma–Aldrich) in varying concentrations and for different time periods as indicated in the Figures. PBS/0.1 % BSA was added as a negative control. Recombinant human IFN-γ, IL-1β and TNF-α were purchased from Roche (Mannheim, Germany). HEK-293T cells (human embryonic kidney-293T cells) were purchased from A.T.C.C. (no. 293T/17). Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany) supplemented with 10 % FBS (Invitrogen). All stimulations were performed in triplicate. Cells were routinely tested for mycoplasma contamination with a Mycoplasma detection kit (Roche) and were negative. PMA and Tos-Phe-CH_2Cl (tosyl-phenylalanylchloromethane, 'TPCK') were purchased from Sigma–Aldrich, BAY 11-7082 [(E)3-[(4-methylphenyl)sulphonyl]-2-propenenitrile] was from Calbiochem (La Jolla, CA, U.S.A.).

Promoter-reporter plasmids

A 3757 bp fragment of the non-coding 5′-region of the human *gbp-1* gene (position: -1778 to $+1979$, nomenclature according to Lew et al. [12]) was amplified by PCR (forward primer 5′-CTGGTACCCCGGCTCCCCTTATTTC-3′ and reverse primer, 5′-GCGATGTCCAGGCTGTTCCCTTGTC-3′) from the PAC-clone RP5-837D10 (The Sanger Centre, London, U.K.). The plasmids pro3757-GBP-1, pro1762-GBP-1 and pro237-GBP-1 were constructed by inserting the 3757 bp fragment or parts of it with 1762 bp (position: -1778 to -17) and 237 bp (position: -218 to $+19$) fragments into the multiple cloning site of the pGL3-Basic vector (Promega, Mannheim, Germany) in front of the *firefly* luciferase indicator gene. The sequence of the inserted fragments was confirmed by sequence analysis (SequiServe, Vaterstetten, Germany). The ISRE (5′-ACTTTCAGTTTCAT-3′, position: -129 to -116) and cRel (5′-GGAAATCCCA-3′, position: -62 to -53) binding sites of pro237-GBP-1 were inactivated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) to generate ΔISRE (5′-AC**AA**TCA**T**TTTCAT-3′), ΔcRel (5′-G**T**A**T**ATCCCA-3′) and the respective ΔISRE/cRel double mutant. Sequence analysis with MatInspector Professional (Genomatix, Munich, Germany, see below) confirmed that these mutations concurred with a complete loss of the respective binding site, did not introduce additional binding sites and affect only the ISRE and cRel (NF-κB)-binding sites in the 237 bp fragment. All constructs containing p65, p50 and IRF-1 were gifts from J. Hiscott (McGill University, Quebec, Canada) [26]. The consensus NF-κB promoter plasmid (pNF-κB-EGFP) contained four tandem repeats of consensus NF-κB-binding sites linked to a thymidine kinase minimal promoter and GFP (green fluorescent protein) as reporter. This plasmid was obtained by exchange of the destabilized GFP variant d2EGFP in pNF-κB-d2EGFP (BD Biosciences/ClonTech, Heidelberg, Germany) by a stable EGFP (enhanced GFP).

Cell transfection

HUVEC were seeded in 6-well plates (Corning, Wiesbaden, Germany) with a density of 6×10^4 cells/well, 24 h before transfection. Cells were washed in PBS and a mixture of 2 μg of plasmid DNA, 100 μl of EBM and 20 μl of Superfect transfection reagent (Qiagen, Hilden, Germany), which was preincubated at room temperature (21–25 °C) for 10 min was added together with 0.6 ml of EBM-full medium. After an incubation period of 2 h at 37 °C, the cells were washed with PBS and incubated for 24 h in EBM-full medium before stimulation. HEK-293T cells were seeded in 12-well plates (Corning) with a density of 3×10^5 cells/well, 24 h before transfection. The cells were transfected with a total of 2 μg of plasmid DNA using Effectene transfection reagent (Qiagen) according to the manufacturer's instructions with a ratio DNA/Effectene = 1:10. Cells were washed with PBS 2.5 h post-transfection and resuspended in Dulbecco's modified Eagle's medium/10 % FBS. GFP expression was determined by fluorescence microscopy using an Axiovert 25 microscope (Zeiss, Jena, Germany) connected to a Coolpix 995 digital camera (Nikon, Düsseldorf, Germany).

Luciferase reporter gene assay

Cells were harvested with 200 μl 1× passive lysis buffer (luciferase reporter assay system; Promega) according to the manufacturer's instructions. Expression of *firefly* luciferase was determined quantitatively using an Orion Microplate luminometer (Berthold Detection Systems, Pforzheim, Germany) employing the luciferase assay reagent (Promega) as a substrate. The obtained values were normalized according to their total protein content determined by the D_C Protein Assay (Bio-Rad Laboratories, Munich, Germany). Results are shown as the means for fold induction with respect to the negative control and are representative of three independent experiments with three experimental points each, unless otherwise indicated.

Western-blot analysis

Western-blot analysis of GBP-1 was performed as described previously [9,11]. The blots were incubated with a rat monoclonal anti-GBP-1 antibody (1B1 [11], 1:500), a mouse monoclonal anti-MMP-1 (matrix metalloproteinase-1, 1:500; MAB901, R&D Systems, Abingdon, Oxfordshire, U.K.) and a mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) serum (1:20 000; Chemicon, Hofheim, Germany). After incubation for 1 h at room temperature, the primary antibodies were removed and the blots were incubated for 45 min with goat anti-rat (Dianova, Hamburg, Germany) and sheep anti-mouse (Amersham Biosciences, Freiburg, Germany) IgG antibodies coupled with horseradish peroxidase (1:5000). All stimulations employed in Western-blot analysis were performed at least in triplicate. One representative gel of these stimulations is shown.

EMSA (electrophoretic mobility-shift assay) analysis

HUVEC were stimulated with IFN-γ (100 units/ml), IL-1β (200 units/ml), TNF-α (300 units/ml) or PBS/0.1 % BSA (negative control) alone or in combination in a low medium for 2 h. Nuclear protein was isolated using high salt extraction [27,28]. For EMSA, 5 μg of protein was incubated with 4×10^4 c.p.m. of ^{32}P-labelled oligonucleotide coding for the region of interest [27,28]. Wild-type and mutant ISRE and cRel EMSA probes had identical sequences as described above (see the Promoter-reporter plasmids subsection) for ISRE, cRel, ΔISRE and ΔcRel-binding sites. All other oligonucleotides and transcription factor-specific antibody reagents were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The oligonucleotides were GAS/ISRE consensus (sc-2537), GAS/ISRE mutant (sc-2538), STAT1 p84/p91 consensus (sc-2573), STAT1 p84/p91 mutant (sc-2574), NF-κB consensus (sc-2505), NF-κB mutant (sc-2511), IRF-1 heterologous (sc-2575; this oligonucleotide can be purchased under the name IRF-1 consensus, in agreement with Santa Cruz Biotechnology it has to be stated that this IRF-1 oligonucleotide does not contain an IRF-1 consensus motif but a specific IRF-1 motif of the IRF-2 promoter region [29]) and IRF-1 heterologous mutant (sc-2576). Supershift antibodies were IRF-1 (sc-497), IRF-3 (sc-9082X), IRF-7 (sc-9083X), ISGF3γ/p48 (sc-496X), p65 (sc-109), p50 (sc-114), cRel (sc-70), RelB (sc-226X), p52 (kindly provided by P. J. Nelson) and STAT1α p91 (sc-345X). All experiments and stimulations were performed at least in triplicate. One representative EMSA gel of each assay is shown.

Bioinformatics tools and promoter modelling

All software employed was part of the Genomatix Suite/GEMS Launcher software package (http://www.genomatix.gsf.de). Potential control elements and transcription factor binding sites were analysed using MatInspector Professional software [30]. The MatInspector program assigns a core similarity of unity when the highest conserved bases (generally 4) of a matrix ('core sequence') match exactly in the sequence. The matrix similarity takes into account all bases over the matrix length. The MatInspector applies individually optimized matrix thresholds to ensure selection of high-quality binding sites all over the library of binding-site matrices. The task 'Definition of common frameworks' was used to define automatically potential promoter modules by comparison of the human and the mouse promoters. The task 'Definition of models' [31] was then applied to refine promoter modules based on information about the individual elements involved, their strand orientation, their sequential order and the distances between the elements. The promoter modules generated were compared with the database using ElDorado software. Thresholds of 90 % match (i.e. 90 % model score) were set to identify similar promoter modules in other genes. Deletion mutants of the ISRE and cRel-transcription factor binding sites were designed by using SequenceShaper software. SequenceShaper determines mutations that will modify the desired binding site (e.g. deleting it) without affecting other known binding sites, thus minimizing the regulatory side effects of the mutations.

Statistical analysis

Student's t test was used to determine differences between the means and the corresponding control values with $P < 0.05$ considered to be statistically significant. SPSS (version 11.5) software for Windows was employed to perform the calculations.

RESULTS

A 237 bp fragment of the GBP-1 promoter mediates the induction of GBP-1 expression in response to IL-1β, TNF-α and IFN-γ

To determine which region of the GBP-1 promoter is susceptible to activation by all three IC, fragments of different lengths of the immediate 5′-upstream non-coding region were cloned in front of the *firefly luciferase* gene as an indicator of gene expression. A fragment designated as pro3757-GBP-1 contained 3757 bp (position: -1778 to $+1979$, positions according to Lew et al. [12]) upstream of the start codon of GBP-1 (Figure 1A, left panel). This fragment included the first intronic sequence of the *gbp-1* gene as well as the ISRE and GAS elements that have been shown to regulate GBP-1 expression in response to IFN-α and IFN-γ [12]. In addition, two smaller fragments were cloned: pro1762-GBP-1 (position: -1778 to -17, Figure 1A, left panel) did not contain the intronic sequence and the major transcription start site (position: $+1$; cf. Figure 2A). pro237-GBP-1 (position: -218 to $+19$, Figure 1A, left panel) corresponded to the minimal IFN-responsive promoter fragment (position: -216 to $+19$).

Each of these promoter fragments could be activated by stimulation (5 h) with IFN-γ (100 units/ml), IL-1β (200 units/ml) and TNF-α (300 units/ml) in primary HUVEC (Figure 1A, right panel). For these concentrations, the activation of each fragment as compared with its basic activity was maximal (results not shown). Activation of pro237-GBP-1 was 3–4-fold with each cytokine (Figure 1A, white bars), indicating that pro237-GBP-1 is sufficient to mediate GBP-1 expression in response to all IC.

GBP-1 protein expression (Figure 1B, left panel), similar to pro237-GBP-1 activity (Figure 1A, white bars), was induced by all IC in these cells. However, GBP-1 protein was more strongly induced by IFN-γ as compared with IL-1β and TNF-α (Figure 1B, right panel).

GBP-1 expression in response to combined ICs indicates the involvement of an additional transcription-factor-binding site

IL-1β and TNF-α activate several different transcription factors, including NF-κB [32,33], Oct-1 (octamer-binding protein-1) [34], C/EBP (CCAAT/enhancer-binding protein) [19] and IRF-1 [20]. Sequence analysis of pro237-GBP-1 identified potential binding sites for all of these transcription factors (Figure 2A). To investigate whether IL-1β and TNF-α act exclusively via the ISRE site or engage an additional site, combination effects of IC on pro237-GBP-1 activity were analysed at concentrations that

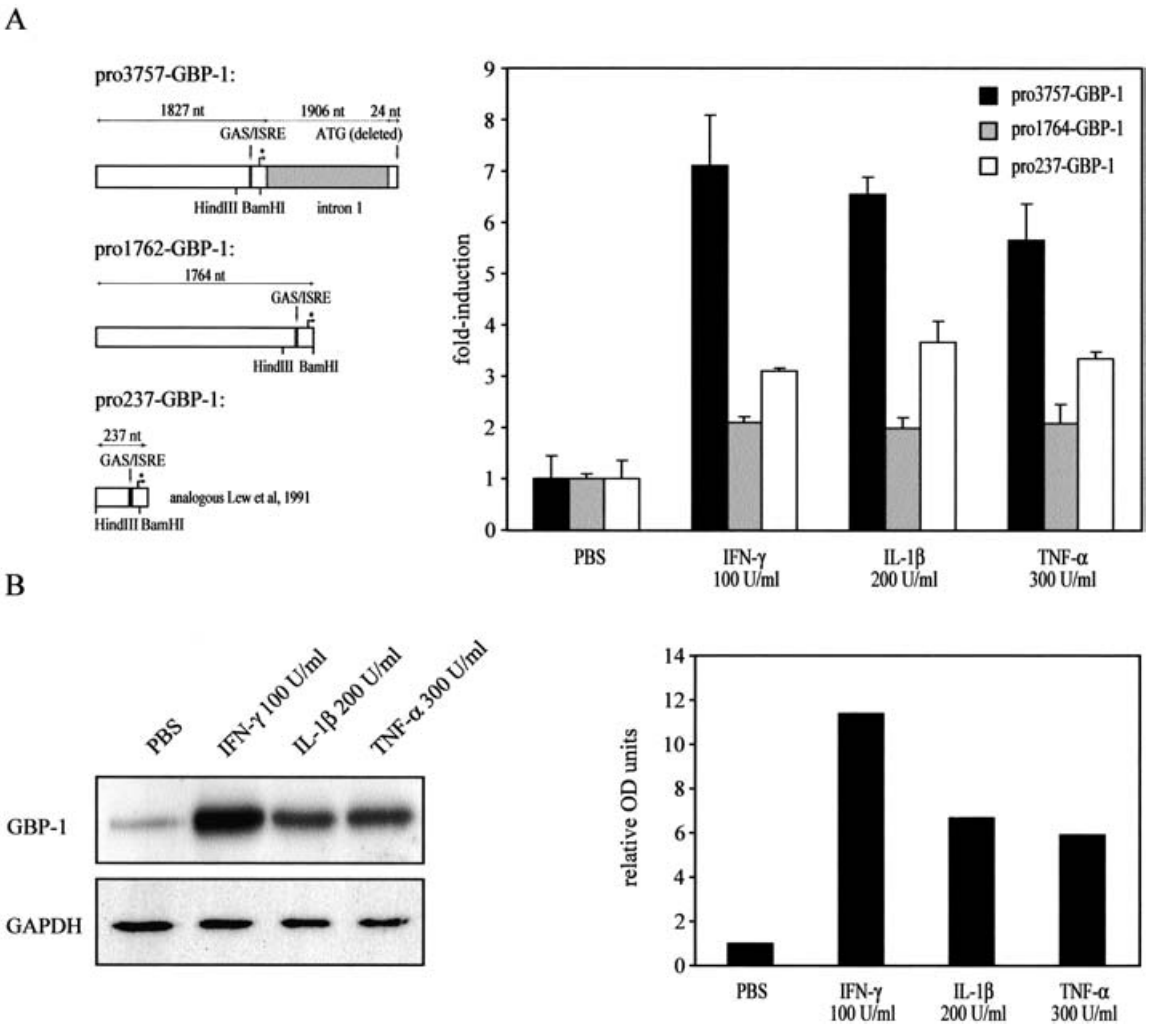

Figure 1 Activation of GBP-1 promoter fragments by IL-1β, TNF-α and IFN-γ in HUVEC

(**A**) Schematic representation (left panel) and promoter activity (right panel) of pro3757-GBP-1, pro1762-GBP-1 and pro237-GBP-1 in HUVEC, 5 h after stimulation with 100 units/ml IFN-γ, 200 units/ml IL-1β, 300 units/ml TNF-α and PBS/0.1 % BSA as detected by luciferase measurement of transfected cells. Results were adjusted to the total amount of protein and are depicted as the mean values for fold induction. *, A region with four transcription start sites (cf. Figure 2A). (**B**) Left panel, Western-blot analysis of GBP-1 protein in HUVEC transfected as in (**A**) 24 h after stimulation. GAPDH demonstrated that similar amounts of protein were used; right panel, quantitative evaluation of band intensities. GBP-1 protein levels were normalized to GAPDH and are depicted in the graph as fold induction compared with unstimulated control cells (PBS).

induced suboptimal promoter activity (2 units/ml IL-1β, 5 units/ml TNF-α and 5 units/ml IFN-γ; Figure 2B). Combinations of IFN-γ with either IL-1β or TNF-α increased promoter activity significantly ($P < 0.02$) when compared with combinations of IL-1β and TNF-α (Figure 2B). This indicated that an additional binding site, different from ISRE, may be involved in the regulation of GBP-1 expression in response to IL-1β and TNF-α. In addition, an increase in promoter activity was observed when single cytokines were applied consecutively. Pretreatment (30 min) of HUVEC with 5 units/ml IFN-γ significantly increased the response of pro237-GBP-1 to 2 units/ml IL-1β or 5 units/ml TNF-α (Figure 2C). This indicated that IFN-γ may render the promoter more sensitive for the second stimulus.

NF-κB is involved in GBP-1 promoter activation by IL-1β and TNF-α

To investigate which transcription-factor-binding site may cooperate with ISRE in the regulation of GBP-1 expression, a promoter module analysis was performed (Table 1). By this analysis, we investigated which transcription-factor-binding site(s) may appear at the same distance to ISRE, as in the GBP-1 promoter, also in other promoters. A potential cRel (NF-κB)-binding site was detected 65 bp downstream of the ISRE site in the GBP-1 promoter and in an identical sequential order with only slight variations in the distance (60–67 bp) in 14 different human genes and in the promoter region of mouse GBP-1 (Table 1). This conserved module-like organization was selectively detected for the ISRE and the cRel-binding site but not for combinations of ISRE with the Oct-1 or the C/EBP-binding site. In addition, in five of the human genes [CD1 B antigen, G-protein-coupled receptor 31, H2A histone family (member J), forkhead box D2 and phosphatidylinositol glycan (class K)], the cRel and ISRE sites were organized in the same orientation as in the human GBP-1 promoter (Table 1).

NF-κB inhibitors such as BAY 11-7082 (blocks phosphorylation and subsequent degradation of IκB-α [35]) and Tos-Phe-CH_2Cl (inhibits proteases of the proteasome that degrade IκB-proteins [36]) blocked pro237-GBP-1 activity (Figures 3A

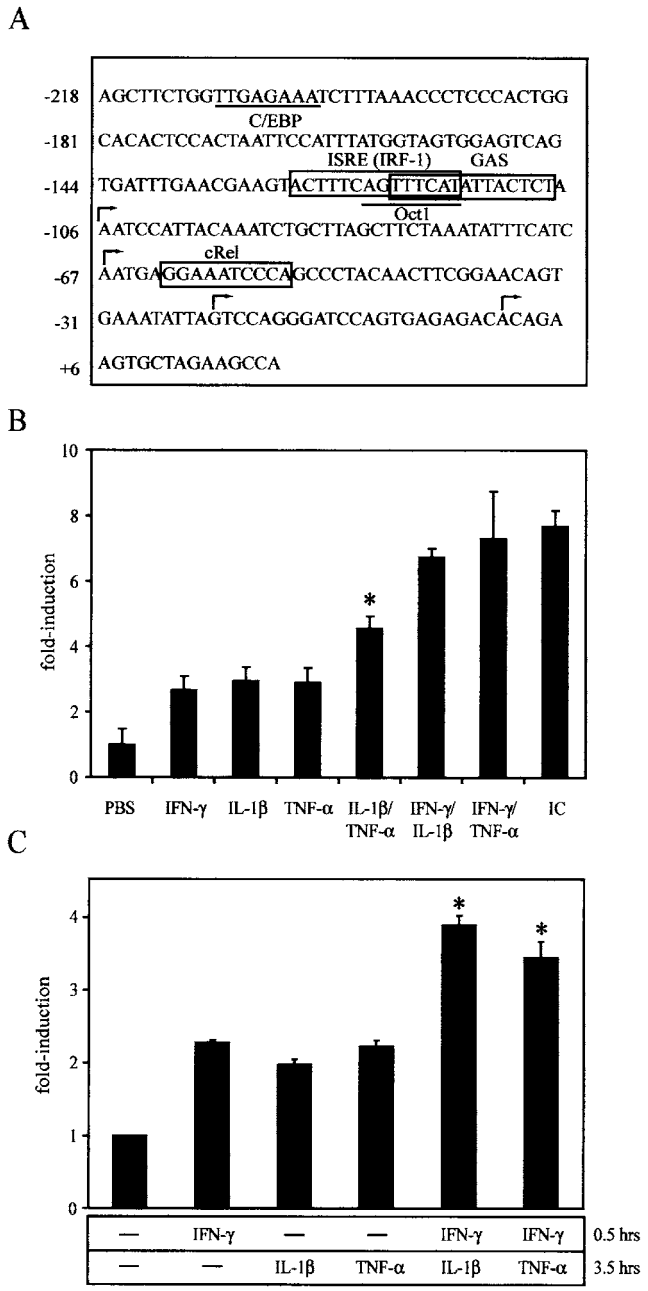

Figure 2 GBP-1 promoter activity in the presence of combined IC

(**A**) Sequence and regulatory elements of pro237-GBP-1 [12]: ISRE, GAS and the cRel (NF-κB)-binding sites are framed, potential C/EBP and Oct-1-binding sites are underlined. Transcription start sites are marked with arrows. (**B**) pro237-GBP-1 activity in HUVEC, 5 h after stimulation with PBS/0.1 % BSA, 5 units/ml IFN-γ, 2 units/ml IL-1β, 5 units/ml TNF-α alone and in combination. Results were adjusted to total protein amount and are depicted as mean values for fold induction (*$P < 0.02$ against all other combinations). (**C**) pro237-GBP-1 activity in HUVEC after a pretreatment with ICs. HUVEC were transfected and subsequently incubated with or without 5 units/ml IFN-γ for 0.5 h. The medium was changed and the cells were either treated or untreated with 2 units/ml IL-1β or 5 units/ml TNF-α for another 3.5 h. Promoter activity in cells that were consecutively stimulated was significantly higher (*$P < 0.05$) when compared with cells that were only treated once.

and 3B, grey bars) in the presence of IL-1β and TNF-α at the levels of unstimulated control cells (Figures 3A and 3B; PBS). In contrast, IFN-γ-induced promoter activity was only slightly affected by these inhibitors (Figures 3A and 3B, black bars). This supported that NF-κB may be involved in GBP-1 promoter activation in response to IL-1β and TNF-α.

However, neither GBP-1 protein expression (Figure 4A, upper panel) nor pro237-GBP-1 activity (Figure 4B) was activated by increasing concentrations of PMA (0.05–0.2 mM), a potent activator of NF-κB. In contrast, the expression of MMP-1 (Figure 4A, lower panel) that served as a positive control was clearly activated under these conditions. This indicated that the induction of GBP-1 expression by IC may be due to NF-κB activation in a specific manner.

In the next step, the effect of overexpressed NF-κB proteins (p65 and p50) alone and in combination with IRF-1 on pro237-GBP-1 activity was investigated. HEK-293T cells were used for these experiments because these cells can be co-transfected with a high efficiency when compared with HUVEC. IRF-1 and surprisingly p50 activated pro237-GBP-1 (Figure 4C). In contrast, p65 had an inhibitory effect (Figure 4C). Simultaneously, in the presence of p50, p65 and IRF-1, pro237-GBP-1 was clearly activated (Figure 4C).

The biological activity of overexpressed p65 and p50 was controlled in another NF-κB reporter system. A plasmid with tandem consensus NF-κB-binding sites in front of the GFP-cDNA was co-transfected with p65 and p50 expression plasmids in HEK-293T cells (Figure 4D). The consensus NF-κB promoter was strongly activated by p65 and only very weakly by p50 (Figure 4D). These results confirmed the functional integrity of the overexpressed p50 and p65 proteins and demonstrated that the GBP-1 promoter reacts uniquely with NF-κB.

cRel (NF-κB) motif together with ISRE is required for a transcriptional response of the *gbp-1* gene to IL-1β and TNF-α

To determine the specific role of the ISRE and the cRel sites in GBP-1 expression in response to IL-1β, TNF-α and IFN-γ, deletion mutants of the respective sites were generated. The ISRE site was mutated by changing the binding motif from 5′-ACTTTCAGTTTCAT-3′ (cf. Figure 2A) to 5′-ACAATCA-TTTTCAT-3′ (ΔISRE promoter), the cRel site was inactivated by changing the motif from 5′-GGAAATCCCA-3′ (cf. Figure 2A) to 5′-GTATATCCCA-3′ (ΔcRel-promoter) and in a third construct both transcription factor binding sites were mutated (ΔISRE/ΔcRel promoter). Binding-site analysis using the Sequence-Shaper software indicated that these mutations did not introduce new binding sites nor did they affect other potential binding sites present in the pro237-GBP-1 sequence.

None of the IC alone, in concentrations that induced maximal response of the wild-type promoter, could activate the ΔISRE promoter or the ΔISRE/ΔcRel promoter in HUVEC (Figure 5A, ΔISRE). The ΔcRel promoter was not activated by IL-1β and TNF-α (Figure 5A, ΔRel, black and grey bars), but was significantly induced ($P < 0.01$) by IFN-γ, although at a lower level when compared with the wild-type promoter (Figure 5A, ΔRel, white bar). This showed that the transcriptional response to IL-1β and TNF-α requires both the ISRE and the cRel element, whereas the response to IFN-γ requires only the ISRE element but may be enhanced by the cRel element.

At combined application of these cytokines in suboptimal concentrations, all combinations with IFN-γ (Figure 5B, wild-type, black, dark grey and white bars) stimulated pro237-GBP-1 at least 2.7-fold higher when compared with combined IL-1β and TNF-α (Figure 5B, wild-type, light grey bar) which was in agreement with earlier findings (cf. Figure 2B). None of these IC combinations activated the ΔISRE promoter or the ΔISRE/ΔcRel promoter (Figure 5B, ΔISRE, ΔISRE/Rel). In contrast, the promoter with the ΔcRel mutation was activated by all IC

Table 1 Presence of the cRel/ISRE promoter module in the regulatory region of different genes

A promoter module analysis was performed using ElDorado and GEMS Launcher (Genomatix Suite). The GBP-1 promoter module was defined as follows: IRF-1 element with a minimal core similarity of 0.75 and matrix similarity of 0.85; cRel (NF-κB) element with a minimal core similarity of 0.75 and matrix similarity of 0.96; distance range 60–70 bp. Distances are given from the beginning of an element to the beginning of the next element. Initially, a total number of 81 matches of the module in 101 667 sequences of the GenBank®/EMBL database was obtained. Those sequences that were specifically residing in the regulatory regions of functional genes are listed. Conserved positions of nucleotides in the transcription-factor-binding sites are depicted with bold letters.

Promoter region	IRF-1 site (strand orientation)	cRel site (strand orientation)	Distance (bp)	Model score (%)	Functional role
Human GBP-1	TG**AAA**CT**GAAA**GT (−)	T**GG**GATT**TCC** (−)	65	93.3	
CD1 B antigen	GGG**TAA**GA**GAAA**CTC (−)	G**GG**CTT**TCC** (−)	61	94.2	
G-protein-coupled receptor 31	GT**GAA**GC**GAAA**CA (−)	G**GG**CTT**TCC** (−)	60	91.5	Participates in janus kinase/STAT pathway [46]
Kell blood group	GC**AAA**AT**GAAA**CA (+)	AG**GG**ACT**TTCC**A (−)	61	95.9	
H2A histone family (member J)	GG**AAA**CA**GAAA**AC (−)	C**GG**GTT**TCC** (−)	66	93.0	
Phospholipase C (γ2)	CCA**AAG**AA**GAAA**CTG (−)	**GG**GAA**TTCC**C (+)	64	92.8	Intracellular signal transduction enzyme (activated by IFN [50])
Small inducible cytokine A5 (RANTES)	GA**AAA**CT**GAAA**TA (+)	G**GG**AGT**TCC** (+)	68	94.9	Chemokine inducible by IFN-γ, IL-1β and TNF-α [39]
Niemann–Pick disease (type C1)	GA**AAA**GG**GAAA**GC (−)	G**GG**GAC**TTCC** (+)	65	96.1	
Lysosome-associated membrane protein 3	GA**GAA**AC**GAAA**CC (+)	C**GG**AGT**TCC** (−)	64	91.1	Present in endothelial cells in endosomes [49]
RNA helicase RIG-I	GA**AAA**TC**GAAA**GT (+)	G**GG**ATT**TCC** (+)	68	96.0	Induced by lipopolysaccharide in endothelial cells [47]
Forkhead box D2	AA**AAA**GA**GAAA**TC (−)	T**GG**AAT**TCC** (−)	64	93.2	Involved in cell growth and development [48]
Phosphatidylinositol glycan (class K)	CC**AAA**CT**GAAA**AT (−)	C**GG**GTT**TCC** (−)	64	93.4	
Zinc finger protein 297	AA**AAA**GA**GAAA**AG (+)	**GG**GGA**TACC**C (+)	67	91.1	
5-Hydroxytryptamine receptor	GA**AAA**TG**GAAA**TG (+)	T**GG**AAT**TCC** (+)	66	92.7	
CD69 antigen	TT**AAA**GA**GAAA**CA (−)	T**GG**GTT**TCC** (+)	62	92.3	
Mouse GBP-1	AA**AAA**AG**GAAA**GA (+)	T**GG**AGT**TCC** (−)	61	91.1	

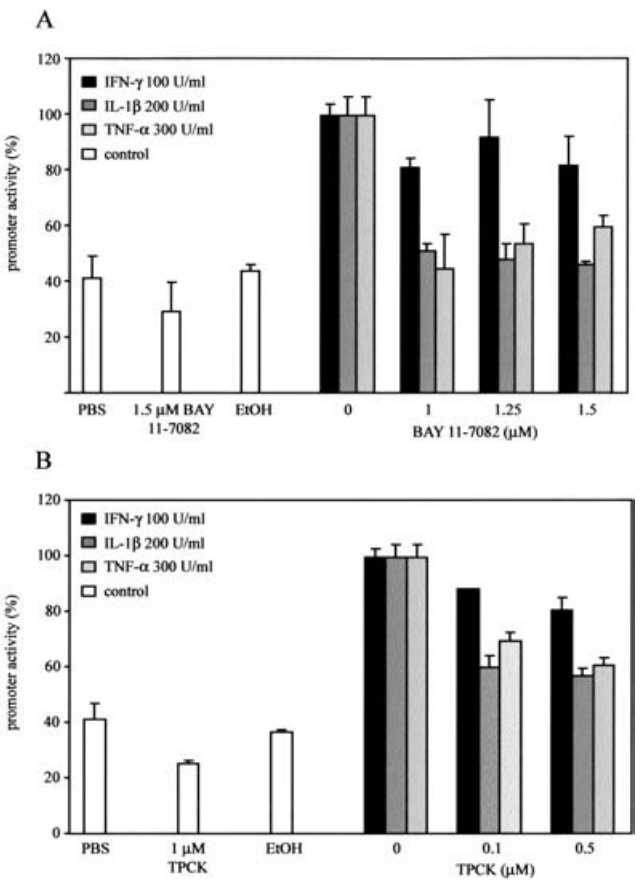

Figure 3 Sensitivity of GBP-1 promoter activity to inhibitors of NF-κB

pro237-GBP-1 activity in HUVEC that were pretreated (2 h) with (**A**) BAY 11-7082 (1–1.5 μM) or (**B**) Tos-Phe-CH_2Cl (0.1–0.5 μM) were subsequently stimulated (5 h) with 100 units/ml IFN-γ, 200 units/ml IL-1β, 300 units/ml TNF-α and PBS/0.1 % BSA (PBS). Luciferase activity was determined and results were adjusted to total protein amount. The means of the relative promoter activity are shown in comparison with its activity in the absence of the NF-κB inhibitors (0).

combinations containing IFN-γ (Figure 5B, ΔcRel, black, dark grey and white bars). However, in all of these cases, the promoter activity was clearly below 50 % of the wild-type promoter activity [ΔcRel/wild-type$^{\mathrm{IFN-\gamma/IL-1\beta}}$ = 29.1 % (Figure 5B, black bars), ΔcRel/wild-type$^{\mathrm{IFN-\gamma/TNF-\alpha}}$ = 34.1 % (Figure 5B, dark grey bars) and ΔcRel/wild-type$^{\mathrm{IFN-\gamma/IL-1\beta/TNF-\alpha}}$ = 43.5 % (Figure 5B, white bars)]. This indicated that the cRel site is also important for the activation of GBP-1 expression in response to IFN-γ in combination with IL-1β and TNF-α.

IL-1β, TNF-α and IFN-γ induce binding of IRF-1 to the ISRE element within the GBP-1 promoter

EMSA was used to investigate the binding of transcription factors to the ISRE and cRel element in IC-activated HUVEC. Cells were stimulated with IC in concentrations (100 units/ml IFN-γ, 200 units/ml IL-1β and 300 units/ml TNF-α) that induced maximal promoter activity (cf. Figure 1A, right panel). Specific stimulation-dependent complexes with ISRE (Figure 6A, specific complex in this Figure and in all the Figures indicated by an arrow) and the cRel-binding site (Figure 7A) were strongest 2 h after stimulation. In the experiment with the ISRE oligonucleotide, an additional band with a higher molecular mass was observed, which was only very slightly affected by the stimulation (Figures 6A and 6B). Therefore this complex was regarded as stimulation-independent and was not investigated further.

The specific stimulation-dependent ISRE complex was most prominent in IFN-γ-treated cells and was present in lower amounts in nuclear extracts of cells treated with IL-1β and TNF-α (Figure 6B). The specificity of this band was confirmed by competition experiments (Figure 6C). An unlabelled wild-type ISRE oligonucleotide and a GAS/ISRE consensus oligonucleotide inhibited the appearance of this complex in IL-1β- (Figure 6C), TNF-α- and IFN-γ-stimulated cells (results not shown), whereas a consensus STAT1 oligonucleotide, a consensus NF-κB oligonucleotide and the respective mutated oligonucleotides had no effect (Figure 6C). An heterologous IRF-1 element competed with the specific complex to some extent but not completely (Figure 6C, wild-type IRF-1 heterologous). Supershift analysis

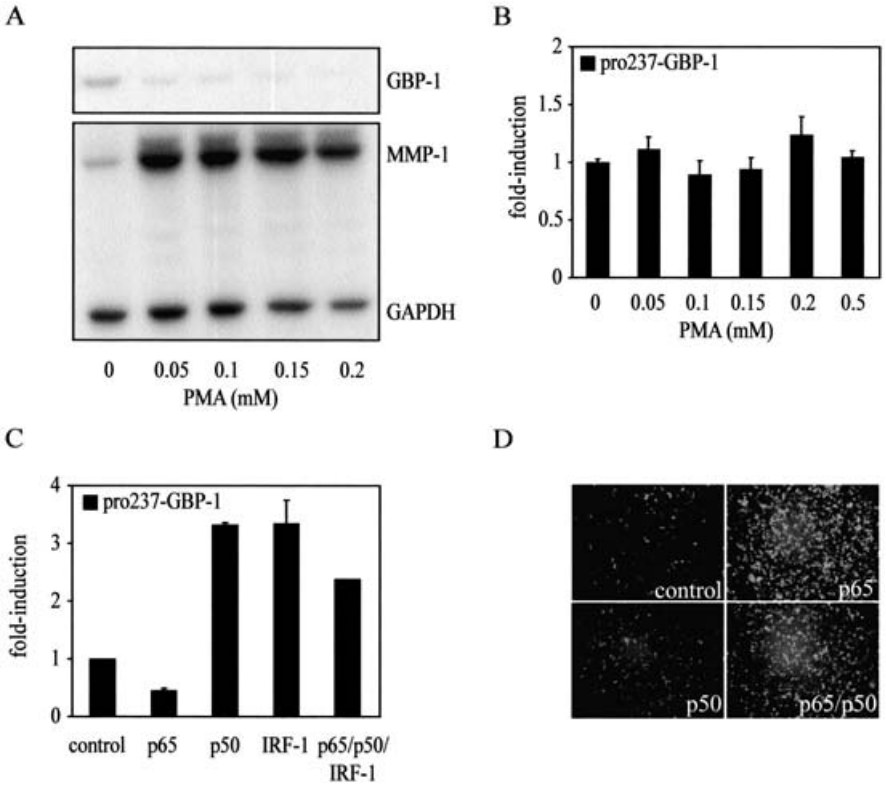

Figure 4 Activation of the GBP-1 promoter by NF-κB p50 but not PMA

GBP-1 protein expression and pro237-GBP-1 activity in HUVEC stimulated with increasing concentrations (0.05–0.5 mM) of PMA. (**A**) Western-blot analysis of GBP-1 24 h after cell stimulation with PMA. Detection of MMP-1 was used as a control for PMA activity and GAPDH protein as an internal standard showing that similar amounts of protein were loaded. (**B**) pro237-GBP-1 activity was analysed by luciferase measurement, 5 h after stimulation with PMA. Results were normalized to total protein amount and are presented in terms of fold induction (means $\pm$ S.D.) as compared with control cells (0). (**C**) HEK-293T cells were co-transfected with pro237-GBP-1 and with expression plasmids of p65, p50 and IRF-1 as indicated. Activation of the GBP-1 promoter was analysed by luciferase measurement, 22 h after transfection. Values were adjusted to total protein content and the results are expressed in terms of fold induction in comparison with the negative control (control). (**D**) HEK-293T cells were co-transfected with a NF-κB reporter plasmid (pNF-κB-EGFP) and p65, p50 expression plasmids or the respective empty control vector as indicated. GFP expression was determined 20 h post-transfection by fluorescence microscopy.

with antibodies against different members of the IRF-1 family (IRF-1, IRF-3, IRF-7 and p48/ISGF3γ), NF-κB family (p65 and p50) and STAT family (STAT1) demonstrated that IRF-1 bound to the ISRE element in IL-1β (Figure 6D), TNF-α- and IFN-γ-stimulated HUVEC (results not shown). STAT1, p65 and p50 did not bind to the ISRE element under these conditions (Figure 6D) as well as other members of the STAT family including STAT2 and STAT3 (results not shown). Simultaneous stimulation with combinations of cytokines did not induce the formation of additional complexes (Figure 6E).

IL-1β and TNF-α induce binding of p65/p50 heterodimers to the cRel (NF-κB) site of the GBP-1 promoter

To investigate which factor(s) may bind to the cRel binding site, wild-type and mutant cRel oligonucleotides were incubated with nuclear extracts of HUVEC stimulated with IL-1β, TNF-α and IFN-γ. Two sequence-specific and stimulation-dependent complexes appeared selectively in IL-1β- and TNF-α-stimulated HUVEC but not in IFN-γ-stimulated cells (Figure 7B). The formation of these specific complexes was competed in IL-1β- and TNF-α-stimulated cells with an unlabelled wild-type cRel oligonucleotide and an NF-κB consensus oligonucleotide but not by the mutant cRel oligonucleotide (Figure 7C). A commercially available consensus mutant NF-κB oligonucleotide revealed a significant competition effect, which suggested residual binding activity of this mutant (Figure 7C). Using supershift analysis, the specific complexes were retarded with an anti-p50-antibody and an anti-p65-antibody in IL-1β- (Figure 7D) and TNF-α- (Figure 7E) stimulated HUVEC. This demonstrated that a p65/p50 heterodimer binds to the cRel sequence in IL-1β- and TNF-α-stimulated cells. IRF-1 did not bind to the cRel motif (Figure 7E). Simultaneous stimulation with combinations of cytokines did not induce the formation of additional complexes (Figure 7F).

DISCUSSION

It has been shown that ISRE is necessary for GBP-1 expression in response to IFN-γ [12]. In the present study, we investigated which promoter elements can cause up-regulation of GBP-1 expression in endothelial cells that are exposed to IL-1β and TNF-α. We detected a cRel (NF-κB)-binding motif in the promoter region of GBP-1. This motif together with ISRE was required for a transcriptional response to IL-1β and TNF-α, indicating co-operative activity of both motifs.

Several pieces of evidence suggested that ISRE is involved in the activation of GBP-1 expression in endothelial cells by IL-1β and TNF-α: (i) mutational deactivation of this element abrogated

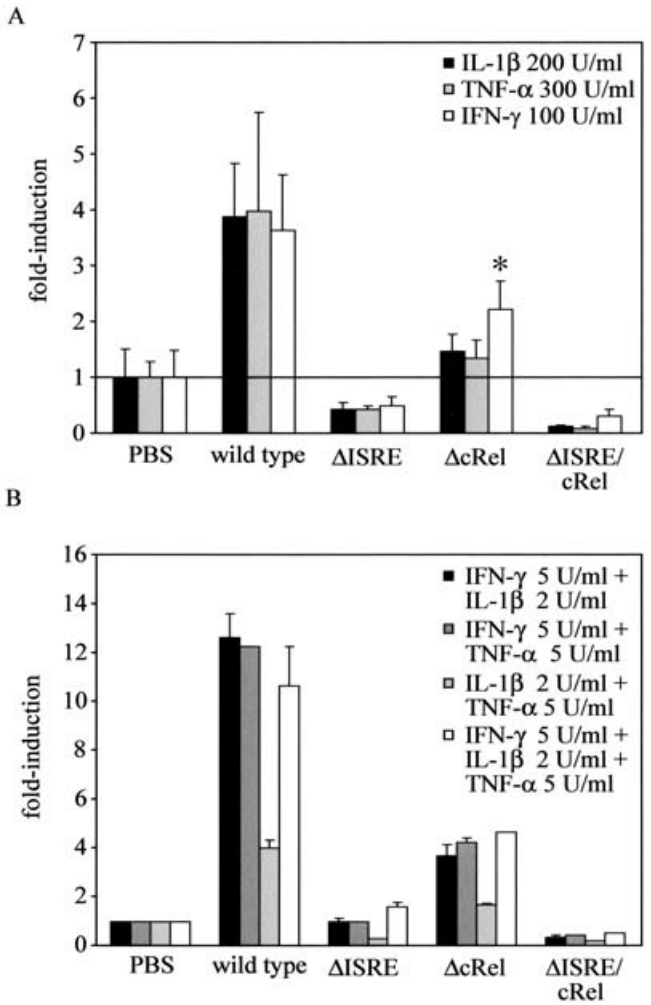

Figure 5 Co-operation of the ISRE and the cRel-transcription-factor-binding sites in the transcriptional response of pro237-GBP-1 to IC

(**A**) Activity of pro237-GBP-1 (wild-type), ISRE (ΔISRE), cRel (ΔcRel) and double (ΔISRE/cRel) mutants in HUVEC stimulated (5 h) with IL-1β (black bars), TNF-α (grey bars) or IFN-γ (white bars). Promoter activity of the ΔcRel mutant in response to IFN-γ was significantly higher (*$P < 0.01$) when compared with IL-1β or TNF-α. (**B**) Activity of pro237-GBP-1 mutants in the presence of combined IC. IC concentrations were adjusted at a suboptimal level that induced comparable promoter activities. The following IC combinations were used: IFN-γ and IL-1β (black bars); IFN-γ and TNF-α (dark grey bars); IL-1β and TNF-α (light grey bars); IFN-γ, IL-1β and TNF-α (white bars). Results were normalized to the total amount of protein and are expressed as the means $\pm$ S.D. for fold induction.

the promoter activity of pro237-GBP-1 in response to both cytokines and the GAS sequence was not altered in this mutant, indicating that it cannot complement for the lack of ISRE; (ii) both cytokines induced, in endothelial cells, a stimulation-dependent protein–DNA complex of nuclear proteins binding to ISRE as shown by EMSA; and (iii) supershift analysis demonstrated that IRF-1 is a component of this complex. All of these results were also observed in IFN-γ-treated endothelial cells, indicating that activation of ISRE by IRF-1 is necessary for a transcriptional response to all three cytokines.

For the experiments discussed above, cytokine concentrations, which induced maximal promoter activity were used. Combination effects of cytokines were investigated with submaximal concentrations, in which each cytokine alone induced roughly half-maximal promoter activity. Under these conditions, the GBP-1 promoter was activated significantly stronger when IFN-γ was combined with IL-1β or TNF-α as compared with combined IL-1β and TNF-α. A similar additive/synergistic effect of TNF-α and IFN-γ has been observed on the promoter activity of several inflammation-associated genes such as ICAM-1 (intercellular cell-adhesion molecule-1 [37]), iNOS (inducible nitric oxide synthase [33]) or the chemokine RANTES/CCL5 (where RANTES stands for regulated upon activation, normal T-cell expressed and secreted) [38,39]. The additive/synergistic activity of both cytokines has been attributed to the fact that IFN-γ and TNF-α utilize distinct signalling pathways that may activate different promoter elements [38]. In analogy, this suggested that an additional binding site, different from ISRE, may be involved in the response of GBP-1 expression to IL-1β and TNF-α.

In fact, we obtained several pieces of evidence that a cRel site may co-operate with ISRE in the regulation of GBP-1 expression: (i) the NF-κB inhibitors BAY 11-7082 and Tos-Phe-CH_2Cl inhibited the transcriptional response to IL-1β and TNF-α; (ii) promoter constructs with a defective cRel site did not respond to any of the two cytokines; and (iii) both cytokines activated the binding of the NF-κB proteins p65 and p50 to the cRel site.

Surprisingly, overexpression of single NF-κB proteins demonstrated that the GBP-1 promoter in contrast with a standard NF-κB promoter was activated by p50 but not by p65 (cf. Figure 4). In agreement with our findings, this has also been observed with the promoter of the C-reactive protein [24,25]. It has been suggested that p50 may activate this promoter via interaction with C/EBPβ [24]. In addition, it has been reported that bcl-3 can interact with p50 and may provide a transactivation domain that is missing in p50 [40]. In agreement with this, our experiments also indicated that a third protein may bind to the cRel motif in IL-1β- and TNF-α-stimulated endothelial cells. EMSA with the cRel oligonucleotide revealed two protein–DNA complexes with different molecular mass, both of which contained p65 and p50 (Figures 7A–7F). These two complexes may either contain p50 and p65 in different stoichiometric ratios and/or an additional factor(s) such as C/EBPβ or bcl-3.

ISRE and NF-κB-binding sites have been shown to co-operate in the transcriptional regulation of other inflammation-associated genes such as human IFN-β [41], HLA-B (human leucocyte antigen-B) [41] and mouse IP-10 (10 kDa IFN-γ-inducible protein) [42]. Therefore we investigated whether ISRE and the cRel motif may co-operate in the GBP-1 transcriptional response to combinations of IC. Activity analysis of a GBP-1 promoter with a deactivated ISRE showed that this element is necessary for any response to IC. Deactivation of the cRel motif also had a significant impact and reduced the effects of all combinations of IC by $>50\%$ (Figure 5B). This clearly indicated that this site co-operates with ISRE in the regulation of IC-induced GBP-1 expression.

Several mechanisms may regulate the co-operation of both motifs in the GBP-1 promoter. We can ignore that in endothelial cells the co-operative effect is mediated by direct interaction of IRF-1 and NF-κB, which has been observed in embryonal carcinoma and neuroblastoma cells [43,44]. Supershift analyses clearly revealed that NF-κB is not present in the IRF-1–ISRE complex (Figure 6D) and that IRF-1 is not present in p65–p50–cRel (NF-κB) complexes of IL-1β-stimulated endothelial cells (Figure 7E). In addition, in EMSA, no additional complexes appeared with ISRE or the cRel oligonucleotides when the cells were stimulated with combined or single cytokines. This indicated that the same factors may be involved in the co-operative interaction of both binding sites in response to IL-1β and TNF-α alone as well as to combinations of IC.

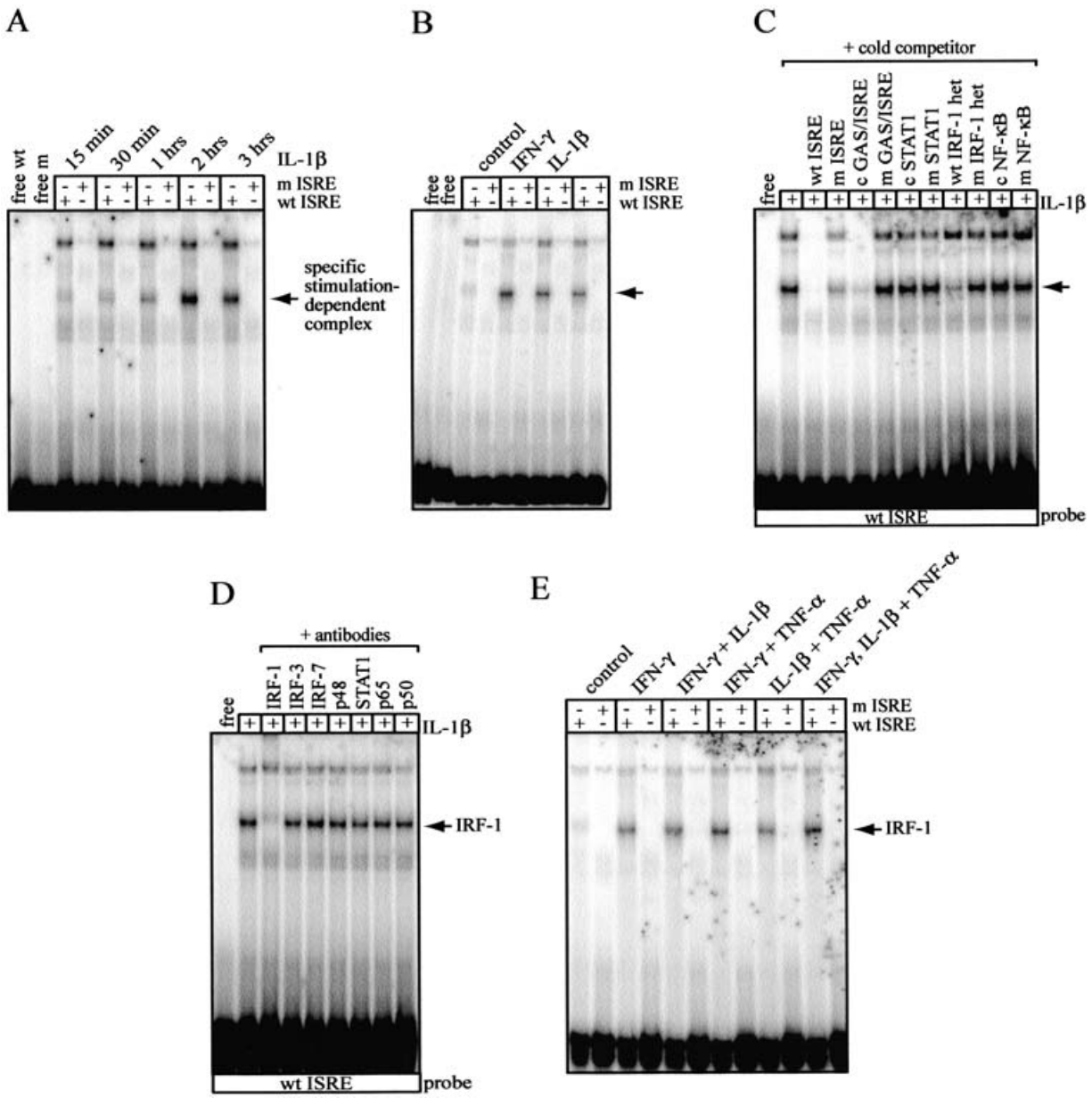

Figure 6 Binding of IRF-1 to ISRE in IL-1β-, TNF-α- and IFN-γ-stimulated HUVEC

(**A**) EMSA analysis with wild-type and mutant ISRE oligonucleotides (wt ISRE and m ISRE) as probes and nuclear extracts from HUVEC stimulated with 200 units/ml IL-1β for different time periods. In this figure and in all the figures, a specific stimulation-dependent complex is indicated by an arrow. (**B**) Analysis as in (**A**), HUVEC were stimulated with PBS/0.1 % BSA (control), 100 units/ml IFN-γ, 200 units/ml IL-1β and 300 units/ml TNF-α for 2 h. (**C**) EMSA competition experiment with wt ISRE oligonucleotide as probe and nuclear extracts from IL-1β-stimulated HUVEC. As unlabelled competitors, 20 ng of the following oligonucleotides were added: wt ISRE, m ISRE, c (consensus) GAS/ISRE, m GAS/ISRE, c STAT1, m STAT1, wt IRF-1 het (heterologous), m IRF-1 het, c NF-κB and m NF-κB. (**D**) EMSA supershift experiment with wt ISRE oligonucleotide as probe and nuclear extracts from HUVEC stimulated (+) with IL-1β (200 units/ml); antibodies against IRF-1, IRF-3, IRF-7, p48 (ISGF3), STAT1, p65 and p50 were added to the binding reactions before incubation with the probe. (**E**) EMSA analysis with wt ISRE and m ISRE oligonucleotides as probes and nuclear extracts from HUVEC stimulated for 2 h with PBS/0.1 % BSA (control), 100 units/ml IFN-γ, 200 units/ml IL-1β and 300 units/ml TNF-α alone and in combination.

We believe it is of special interest that the ISRE and the cRel-binding sites were detected in an identical sequential order as in the human GBP-1 promoter with only slight variations in the distance (60–67 bp) in the promoter region of the mouse GBP-1 gene and of 14 different human genes (Table 1). Fixed sequential order, distinct distance and co-operative activity of transcription-factor-binding sites are the characteristic features of promoter modules [31,45]. It may be noted that many of the genes where this module-like composition of ISRE and the cRel-binding site were detected are involved in inflammation (G-protein-coupled receptor [46], RNA helicase RIG-I (retinoic acid-inducible gene-I) [47] and forkhead box D2 [48]) are known to be expressed in endothelial cells {lysosome-associated membrane protein 3 [49], phospholipase C (γ2 [50]) and RNA helicase RIG-I [47]} or are similar to GBP-1 stimulated by IL-1β, TNF-α and IFN-γ (RANTES [39,51]). In this framework, ISRE and the

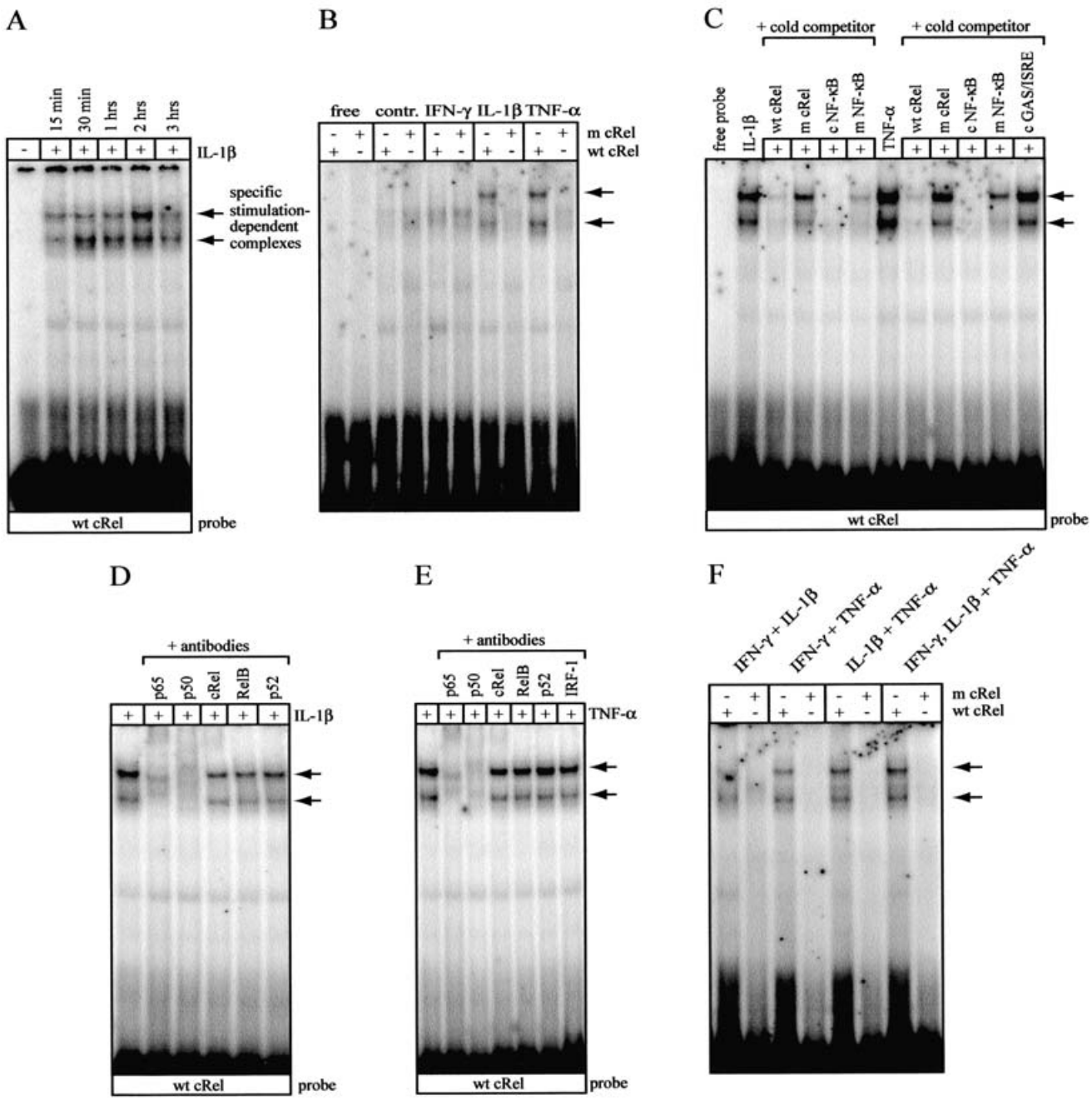

Figure 7 Binding of p65/p50 to the cRel site in IL-1β- and TNF-α-stimulated HUVEC

(**A**) EMSA analysis with wt (wild-type) cRel oligonucleotide as probe and nuclear extracts from HUVEC stimulated with 200 units/ml IL-1β for different time periods. (**B**) Analysis as in (**A**) with wt cRel and m (mutant) cRel oligonucleotides as probes and nuclear extracts from HUVEC stimulated (2 h) with PBS/0.1 % BSA (control), 100 units/ml IFN-γ, 200 units/ml IL-1β and 300 units/ml TNF-α. (**C**) EMSA competition experiments with the wt cRel oligonucleotide as a probe and nuclear extracts from 200 units/ml IL-1β- and 300 units/ml TNF-α-stimulated HUVEC. As unlabelled competitors, 20 ng of the following oligonucleotides were added: wt cRel, m cRel, c NF-κB, m NF-κB and c GAS/ISRE. EMSA supershift analysis with the wt cRel oligonucleotide as a probe and nuclear extracts from HUVEC stimulated (2 h) with (**D**) 200 units/ml IL-1β and (**E**) 300 units/ml TNF-α. The indicated specific antibodies were added before the probe. (**F**) EMSA analysis with wt cRel and m Rel oligonucleotides as probes and nuclear extracts from HUVEC stimulated for 2 h with PBS/0.1 % BSA (control), 100 units/ml IFN-γ, 200 units/ml IL-1β and 300 units/ml TNF-α alone and in combination.

cRel-binding site identified here may constitute an IC-response module that regulates GBP-1 expression in response to IFN-γ, IL-1β and TNF-α in endothelial cells. Due to the highly anti-angiogenic activity of GBP-1 in endothelial cells, our findings may open new perspectives for the use of inhibitors of NF-κB to support angiogenesis in inflammatory diseases including ischaemia.

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