The interferon-γ-mediated inhibition of lipoprotein lipase gene transcription in macrophages involves casein kinase 2- and phosphoinositide-3-kinase-mediated regulation of transcription factors Sp1 and Sp3

Sandra M. Harris, Elizabeth J. Harvey, Timothy R. Hughes, Dipak P. Ramji *

Cardiff School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, UK

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The mechanisms underlying transcriptional inhibition by interferon-γ (IFN-γ) are poorly understood despite the existence of a large number of genes that are regulated in this manner and the key role of this cytokine in inflammatory disorders such as atherosclerosis. We have previously identified a novel mechanism for transcriptional inhibition by IFN-γ that involves a reduction in the binding of transcription factors Sp1 and Sp3 to regulatory sequences in the lipoprotein lipase (LPL) gene. In the present study, we have investigated the signalling pathways that impact on the IFN-γ-mediated regulation of Sp1/Sp3 binding and LPL gene transcription in macrophages. The IFN-γ-mediated inhibition of LPL promoter activity was prevented by expression of dominant negative forms of casein kinase 2 (CK2) and protein kinase B (PKB), a key downstream component of the phosphoinositide-3-kinase (PI3K) pathway. IFN-γ activated both the catalytic subunits of CK2 without affecting their expression. CK2 interacted with both Sp1 and Sp3 and this association was increased by IFN-γ. Electrophoretic mobility shift assays showed that a CK2-mediated phosphorylation of either cellular extracts or recombinant Sp1 reduced binding to the regulatory region in the LPL gene. The action of PKB was potentially mediated through mammalian target for rapamycin proteins. Taken together, these results suggest a key role for CK2 and PI3K signalling pathways in the IFN-γ-mediated inhibition of macrophage LPL gene transcription through the regulation of Sp1/Sp3 binding.

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1. Introduction

The cytokine interferon-γ (IFN-γ) is a key regulator of the immune and inflammatory responses along with many other cellular actions during physiological and pathophysiological conditions [1]. For example, the cytokine plays a crucial role in the pathogenesis of atherosclerosis, an inflammatory disorder, by regulating the function and properties of all the cell types present in the vessel wall [2]. IFN-γ is expressed at high levels in atherosclerotic lesions and modulates foam cell formation, recruitment of leukocytes to the activated endothelium, and plaque stability [2]. The deficiency of either IFN-γ or its receptor limits the development of atherosclerosis in murine models of this disease whereas administration of the cytokine potentiates it [2–4].

The cellular actions of IFN-γ are characterized by dramatic changes in gene expression [12]. The mechanisms by which IFN-γ induces the expression of several genes have been extensively investigated, leading to the identification of the classical Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway [2]. However, several studies, including gene expression profiling of STAT1-deficient cells, have revealed the widespread use of additional pathways for IFN-γ action [5], the nature of which remains to be deciphered in detail. In addition, very little is currently understood about the mechanisms by which IFN-γ inhibits gene transcription despite the existence of a large number of genes that are regulated in this manner, several of which have a key role in atherosclerosis. For example, gene expression profiling has identified 660 genes whose expression in macrophages is inhibited by IFN-γ [6]. Among these is the lipoprotein lipase (LPL) gene, which catalyses the hydrolysis of the triacylglycerol component of chylomicrons and very low density lipoproteins, and has been linked to several pathophysiological conditions associated with perturbations in lipid metabolism and transport, including atherosclerosis, diabetes and obesity [7–9]. The IFN-γ-mediated suppression of LPL mRNA expression, protein levels and enzymatic activity has been observed in a range of macrophage sources from different species, including human monocyte-derived macrophages [7–9].

We have previously investigated several aspects of IFN-γ signalling in macrophages [9–11], including the mechanisms underlying transcriptional inhibition using LPL as a model gene [9]. Our studies identified a novel mechanism for transcriptional inhibition by IFN-γ whereby the cytokine decreases the binding of Sp1 and Sp3 to three conserved recognition sequences in the regulatory region of the LPL gene [9]. The purpose of this study was to investigate the signalling pathways underlying inhibition of LPL gene transcription via Sp1 and Sp3.
2. Materials and methods

2.1. Materials

Cell lines (mouse macrophage J774.2, human monocytic U937) were from the European Collection of Animal Cell Cultures. Antibodies were from Santa Cruz Biotechnology (casein kinase 2 (CK2)-α and α′) and Upstate Biotechnology (Sp1 and Sp3). The protein kinase B (PKB) activity assay kit was from Cell Signalling Technology. Recombinant IFN-γ and tumour necrosis factor-α (TNF-α) were from Peprotech, and recombinant Sp1 and purified CK2 enzyme were from Promega.

2.2. Cell culture

Cells were maintained in Dulbecco’s modified Eagle’s medium (J744.2) or RPMI 1640 (U937) supplemented with heat-inactivated (56 °C, 30 min) foetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% (v/v) CO2 in air. Treatment of J774.2 macrophages with IFN-γ (1000 U/ml) or the different pharmacological inhibitors was performed as previously described [9–14]. U937 cells were used for all transfection assays where differentiation was initiated using 1 µM phorbol-12-myristate-13-acetate (PMA) and monitored by morphological analysis [9,15].

2.3. Transient transfection and reporter gene assays

Transfection of U937 cells with an LPL promoter-luciferase DNA construct (−31 to +187) and CMV-β-galactosidase (internal control for transfection efficiency) was carried out using Superfect™ (Qiagen) as previously described [9,15]. In experiments involving the use of dominant negative (DN) constructs, the cells were initially transfected with the DN expression plasmid or control vector and incubated for 8 h before transfection of the cells with the LPL promoter-luciferase and CMV-β-galactosidase plasmids. The DN plasmids used were pcDNA3 HA-PKB AAA from Dr. B. Hemmings (Friedrich Miescher Institute for Biomedical Research, Basel) and pSG-CK2α-K68A from Drs. E. M. Chambaz and C. Cochet (INSERM, Grenoble). DN PKB specifies for an inactive form of PKBα with a mutation to alanine of lysine 179 in the ATP-binding site and threonine 308 and serine 473 that must be phosphorylated in the active kinase. DN CK2 codes for a kinase inactive mutant with a lysine to alanine change at residue 68 within the ATP-binding domain.

2.4. Western blot analysis

Whole cell extracts were prepared in buffers containing phosphatase and protease inhibitors and used for SDS-PAGE and western blot analysis as previously described [9–13,15].

2.5. Immunoprecipitations and CK2 activity assays

Whole cell extracts (150–300 µg) were prepared using buffers containing phosphatase and protease inhibitors and subjected to immunoprecipitation and in vitro kinase assays using the β-casein substrate as previously described [10]. For CK2 assays with Sp1, 0.5 µg of recombinant protein was used as a substrate instead of β-casein. For co-immunoprecipitation assays, the immunoprecipitated proteins were eluted using 0.1 M glycine (pH 2.5) and subjected to SDS-PAGE and western blot analysis.

2.6. Electrophoretic mobility shift assays (EMSA)

EMSA were carried out using whole cell extracts and radiolabelled oligonucleotides against the Sp1 binding sites in the LPL gene as previously described [9,15].

3. Results

3.1. Role for CK2 in the IFN-γ-mediated inhibition of LPL gene transcription

The murine J774.2 cell line is a useful model system for investigating the mechanisms underlying IFN-γ regulated macrophage gene expression because of demonstrated conservation of responses with primary cultures, including the action of this cytokine on LPL [9–14]. These cells were therefore employed to delineate the signalling pathways underlying inhibition of LPL gene transcription by IFN-γ. Our previous published studies showed that the IFN-γ-mediated reduction of Sp1/Sp3 binding to its recognition sequence in the LPL gene could be attenuated by incubation of the cells with 10 µM and 40 µM of the CK2 inhibitor apigenin [9,16–19]. Subsequent studies also found attenuation with 20 µM apigenin (data not shown). To corroborate that CK2 was indeed involved in the IFN-γ-mediated suppression of LPL gene transcription, the action of a plasmid construct specifying for a DN form of CK2-α [20] on LPL promoter activity in transfected macrophages was analysed. This DN construct has been used in a number of studies to demonstrate a key role of CK2 in specific responses [11,20]. Because J774.2 macrophages are difficult to transfect with exogenous DNA at high efficiency and as the action of IFN-γ on LPL gene expression is conserved in a range of macrophage sources, including primary cultures, along with other cellular systems (e.g. renal mesangial cells) [7–9,21], the human monocytic U937 cell line was employed for all transfection experiments. Indeed, the U937 cell line has been used extensively to delineate the regulatory sequences required for the regulation of gene transcription in macrophages [9,15]. The IFN-γ-mediated suppression of LPL promoter activity observed when the cells were transfected with the control pSG5 vector only was abrogated in cells expressing DN CK2 (Fig. 1). The action of IFN-γ on CK2 synthesis and activity was therefore investigated.

3.2. The action of IFN-γ on the synthesis and activity of CK2

CK2 exists as a tetramer with two main catalytic (α and α′) and two regulatory β subunits as well as free catalytic subunits [10,22]. In order to investigate whether IFN-γ affects the synthesis of CK2, the levels of the α and α′ polypeptides in J774.2 cells treated with IFN-γ were determined by western blot analysis. Sp1 and Sp3 were also analysed for comparative purposes (Sp1 can also act as a loading control as IFN-γ has no effect on its expression [9]). Fig. 2A shows that

![Fig. 1.](image)
IFN-γ does not affect the steady state levels of CK2-α and -α′ polypeptides. In contrast, consistent with our previous studies [9], IFN-γ reduced the polypeptide levels of Sp3 but not Sp1 (Fig. 2A).

Our previous studies on the action of IFN-γ on CK2 activation, which was restricted to the α isoform and a single time point (3 h) [10], showed a dramatic increase in activity following stimulation of the cells with this cytokine. Preliminary time course experiments showed that the activity of both CK2 catalytic subunits was induced within 1 h of incubation of the cells with IFN-γ, peaked at 3 h and was sustained, albeit at reduced levels, for 12–20 h (data not shown). In order to confirm the inhibitory action of apigenin, its effect on the IFN-γ-induced activity of both the catalytic subunits at 3 h was determined. Fig. 2B shows that, consistent with previous studies [e.g. [10,16–19]], apigenin indeed abolishes CK2 activity of both subunits without affecting the expression of the polypeptides.

3.3. CK2 associates with, and phosphorylates, Sp1

Our subsequent studies focused on the interaction and action of CK2 on Sp1 because preliminary co-transfection assays showed that Sp1, but not Sp3, was the main activator of the LPL promoter (data not shown). We investigated the interaction of CK2 subunits with Sp1 by co-immunoprecipitation assays. STAT1 was included for comparative purposes. Thus, Sp1 or STAT1 was immunoprecipitated from extracts of J774.2 macrophages that had either been left untreated or stimulated with IFN-γ for 3 h, a period corresponding to dramatic activation of CK2. The immunoprecipitated protein was then subjected to western blot analysis with antibodies against isoform-specific antibodies as indicated. The result shown is representative of at least three independent experiments.

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subunits of CK2 and that this association was increased in cells treated with IFN-γ. A similar profile was observed when the analysis was performed with the Sp3 antibody (Supplementary material, Fig. 1). In contrast, no interaction was seen between STAT1 and the CK2 polypeptides (Fig. 3A).

The kinase assays presented in Fig. 2B used the classical β-casein as a substrate. The assay was therefore repeated with recombinant human Sp1 (rhSp1) produced in Sf9 cells using a baculovirus expression system (Promega). The activity of CK2-α or -α′ in terms of their ability to phosphorylate rhSp1 was also induced by IFN-γ (Fig. 3B). Two polypeptide species of 82 kDa and 57 kDa for rhSp1 were observed in this assay, which migrated at an apparently lower molecular weight, due to the lesser extent of glycosylation in Sf9 cells than in mammalian cells (Promega).

3.4. CK2-mediated phosphorylation of Sp1 is associated with decreased DNA binding

To further investigate the potential impact of CK2-mediated phosphorylation of Sp1 on DNA binding activity, experiments were carried out using purified CK2. Firstly, the effect of CK2-mediated phosphorylation of extracts from untreated macrophages on Sp1/Sp3 binding was analysed by EMSA. The probe used corresponded to the +36/+90 region of the LPL gene [15] and contained all three Sp1/Sp3 binding sites. Consistent with our previous studies [9,15], three DNA-protein complexes were produced (C1 to C3; Fig. 4A). Antibody interference assays have shown that complex C1 is composed of Sp1 whereas complexes C2 and C3 consist mainly of Sp3 [9,15]. Phosphorylation of extracts from untreated cells with two different concentrations of CK2 reduced the binding of Sp1/Sp3 to levels observed in IFN-γ-treated cells (Fig. 4A). Similar results were obtained when probes containing regions +9/+49 (single Sp1/Sp3 binding site) and +46/+90 (two Sp1/Sp3 binding sites) [9] were employed (Supplementary material, Fig. 2). Furthermore, the binding of rhSp1 to the +9/+49 and the +46/+90 regions was decreased by phosphorylation with purified CK2 (Fig. 4B).

3.5. The phosphoinositide-3-kinase (PI3K) pathway is also involved in the regulation of LPL gene transcription through Sp1/Sp3

Our previous studies showed that the PI3K inhibitor wortmannin prevented, at least in part, the IFN-γ-mediated inhibition of LPL enzymatic activity and mRNA expression in J774.2 macrophages [14]. More recently, we have shown that PI3K, along with CK2, is involved in the classical JAK-STAT pathway of IFN-γ signalling through the regulation of STAT1 phosphorylation at serine 727, which is necessary for maximal transcriptional activity [11]. PKB (also called Akt) is a key downstream mediator of PI3K activation [23]. The action of IFN-γ on PKB activity in J774.2 macrophages was therefore investigated using an in vitro kinase assay in which its ability to phosphorylate glycogen synthase kinase 3 (GSK3) was analysed. The levels of phospho-GSK3β were included for comparative purposes. PKB was immunoprecipitated from whole cell extracts and subjected to a kinase assay in which its ability to phosphorylate a classical GSK3β substrate was measured (Fig. 5). The results are representative of three independent experiments.

In Fig. 6, the effect of DN PKB on the IFN-γ-mediated decrease in LPL promoter activity. U937 cells were transfected with the control pcDNA3 vector or DN PKB followed by the LPL promoter-luciferase construct (−31/+187 in the pGL2 Basic-luciferase plasmid) [15]. Cells were then differentiated for 12 h with PMA (1 µM) and either left untreated (pcDNA or DN PKB) or incubated for 12 h with IFN-γ (pcDNA + IFN or DN PKB + IFN). Luciferase activity was normalized to total protein concentration and is expressed as Relative Luciferase Activity. The data shown are mean±SD from three independent experiments each carried out in triplicate. * represents significant prevention of the IFN-γ-mediated suppression of relative luciferase activity observed in cells transfected with the pcDNA3 vector (P<0.05).

In Fig. 7, the potential role of the different signalling pathways in the IFN-γ-mediated decrease in Sp1/Sp3 binding. J774.2 macrophages were either left untreated (−) or incubated for 20 h with IFN-γ (+) in the absence or the presence of the indicated inhibitors: A. LY294002 (0.05 µM, 0.5 µM and 5 µM); B. SB415286 (5 µM, 25 µM and 50 µM); C. AG490 (5 µM, 50 µM and 150 µM); and D. rapamycin (100 nM, 500 nM and 1 µM). Whole cell extracts were subjected to EMSA using radiolabelled oligonucleotides that recognise the +9/+49 and the +46/+90 regions of the LPL gene. The results shown are representative of two to three independent experimental series.
kinase (GSK)-3α/3β, is measured. Consistent with previous studies in other cellular systems [24–26], IFN-γ activated PKB in J774.2 macrophages (Fig. 5). In order to ascertain whether PKB affects LPL gene transcription, the effect of DN PKB on LPL promoter activity in transfected cells was analysed. Fig. 6 shows that the IFN-γ-mediated decrease in LPL promoter activity could be prevented by expression of DN PKB.

To determine whether the PI3K pathway was also involved in the cytokine-mediated reduction of Sp1/Sp3 binding to its recognition sequence in the LPL gene, the effect of pre-treatment of the cells with three different concentrations of the PI3K inhibitor LY294002 on the IFN-γ-mediated decrease in Sp1/Sp3 binding was analysed by EMSA. Representative, comparative experiments were also carried out with SB415286, an inhibitor of GSK, as we found that IFN-γ had no effect on phosphorylation-mediated activation of GSK-3αβ or serine 9 (data not shown). Fig. 7A shows that the presence of LY294002 attenuated the IFN-γ-mediated reduction of Sp1/Sp3 binding to the LPL gene. Such an effect was not observed with the GSK inhibitor SB415286 (Fig. 7B). Our previous studies showed that the IFN-γ-mediated activation of PKB could be attenuated by inhibition of the PI3K pathway and JAK2 but not by CK2 [11]. Indeed, co-immunoprecipitation assays demonstrated a constitutive interaction between JAK2 and the p85 subunit of PI3K (data not shown). Consistent with such an interaction, inclusion of the JAK2 inhibitor, AG490, at three different concentrations, prevented the IFN-γ-mediated suppression of Sp1/Sp3 binding (Fig. 7C).

PKB-mediated phosphorylation of rSp1 had no effect on DNA binding (data not shown), thereby suggesting that a downstream kinase(s) was potentially mediating the actions of PKB on Sp1/Sp3 binding. The mammalian targets of rapamycin (mTOR) proteins represent an important class of downstream targets of PKB, which have been implicated in the control of several cellular functions [27]. The action of the mTOR inhibitor rapamycin was therefore analysed. As shown in Fig. 7D, rapamycin attenuated the decrease in Sp1/Sp3 binding.

3.6. The synergism between IFN-γ and TNF-α on LPL gene transcription is not mediated at the level of Sp1/Sp3 DNA binding

Interactions between cytokines in the regulation of macrophage function are relatively common and our previous studies showed a synergistic action of IFN-γ and TNF-α on the expression of LPL mRNA, protein and enzymatic activity [13]. Because this synergism was mediated at the level of gene transcription [28], we wondered whether this occurred at the level of Sp1/Sp3 binding to the LPL gene. Indeed, a recent study has shown that the suppression of sodium–hydrogen exchanger-3 expression by both IFN-γ and TNF-α is mediated through decreased binding of Sp1 and Sp3 [29]. In addition, the TNF-α-mediated downregulation of murine growth hormone receptor expression has been shown to be due to inhibition of Sp1 and Sp3 binding [30]. EMSA were therefore performed using extracts from J774.2 macrophages that had either been left untreated or exposed to TNF-α, IFN-γ, or both TNF-α and IFN-γ, and radiolabelled oligonucleotides corresponding to the +9/+49 or +46/+90 region of the LPL gene. Several different concentrations of TNF-α, which were active in suppressing another response in the laboratory (C/EBP-α promoter activity in Hep3B cells), did not reduce binding by Sp1/Sp3 (Fig. 8A). In addition, the IFN-γ-mediated reduction in the binding of Sp1/Sp3 was not enhanced by TNF-α (Fig. 8B). The concentrations of these two cytokines used corresponded to those that produced a marked synergism at the level of LPL mRNA expression and enzymatic activity [15].

4. Discussion

The cytokine IFN-γ is a key regulator of the immune and inflammatory response and plays a crucial role in the pathogenesis of atherosclerosis [2]. The cellular actions of IFN-γ are associated with both the activation and the inhibition of gene transcription [2,5,6,9–11]. However, the mechanisms underlying the IFN-γ-mediated inhibition of gene transcription are poorly understood despite 25% of the macrophage transcriptome being regulated in this manner [6]. Our previous studies using LPL as a model gene identified a novel pathway involving the IFN-γ-mediated decrease in Sp1/Sp3 binding to regulatory sequences in the gene [9]. The studies presented here provide insights into the signalling pathways underlying such regulation and reveal potentially key roles for CK2 and the PI3K/PKB/mTOR pathways in the inhibition of gene transcription by IFN-γ.

CK2 phosphorylates serine or threonine residues in acidic domains with (S/T)XX(D/E) being the canonical motif [22]. The constitutive activity of CK2 is stimulated further by several extracellular mediators [10,22]. A number of recent studies have suggested a potentially important role for CK2 in the control of the inflammatory response [22]. For example, inhibition of CK2 has been shown to reduce pain-related behaviors in murine models of acute and chronic inflammatory pain [31]. In addition, in a rat model of glomerulonephritis, which similar to atherosclerosis is an inflammatory disorder, the progression of the disease could be prevented by administration of either antisense oligodeoxynucleotides against CK2 or inhibitors such as apigenin [32]. CK2 is indeed a major regulator of nuclear factor-κB (NF-κB), a key transcription factor in signalling by pro-inflammatory cytokines such as interleukin-1 and TNF-α [22,33]. Our recent studies have revealed a potentially important role for CK2 in IFN-γ signalling [10,11]. Thus, the IFN-γ-induced expression of the inducible cAMP early repressor (ICER), a transcriptional inhibitor, was mediated via stimulation of CK2-α activity leading to the activation of transcription...
Factors belonging to the cAMP response element binding protein family, which bind to regulatory sequences in the ICER gene promoter [10]. More recently, we have shown a key role for CK2 in the IFN-γ-induced expression of several genes implicated in atherosclerosis that are known targets of the classical JAK-STAT pathway [11]. CK2 regulates the phosphorylation of STAT1 at serine 727 in macrophages [11]. We have now extended these findings to indicate a role of CK2 in transcriptional suppression by IFN-γ-mediated via phosphorylation of Sp1 (and possibly Sp3), which leads to decreased binding to regulatory sequences in the LPL gene. Transcriptional inhibition that is potentially mediated by a CK2-mediated phosphorylation of Sp1 has also been suggested for the flow-dependent suppression of endothelial Toll-like receptor 2 expression [34]. Indeed, CK2-mediated phosphorylation of the carboxyl terminus of Sp1 is associated with a decrease in its DNA binding activity [35].

An increasing body of evidence supports a role for the PI3K pathway in IFN-γ signalling and our previous studies have revealed a key function in the regulation of a large number of genes implicated in atherosclerosis [11]. Deletion of the PI3Ky gene has been demonstrated to attenuate atherosclerosis in apolipoprotein E-deficient mice, a mouse model for this disease [36]. A large number of genes regulated by IFN-γ through PI3K signalling are known targets for the JAK/STAT pathway [11]. Indeed, the action of PI3K in these cases appears to be mediated, at least in part, through the regulation of STAT1 serine 727 phosphorylation [11]. The studies presented here extend the role of PI3K in IFN-γ signalling to transcriptional suppression by this cytokine through Sp1/Sp3. A potential role for mTOR was identified as pre-treatment of the cells with rapamycin attenuated the IFN-γ-mediated reduction in Sp1/Sp3 binding. The PI3K/mTOR pathway has previously been implicated in the insulin-mediated increase in LPL activity in adipocytes [37], and our studies extend this to transcriptional inhibition through Sp1/Sp3. The precise mechanisms underlying such regulation remain to be determined but could include direct phosphorylation-mediated reduction of Sp1 binding. In addition, as mTOR is also associated with translational suppression [24,27], it could potentially be involved in the reduction of Sp3 polypeptide levels observed when the cells are treated with IFN-γ. Interestingly, a recent study investigating the mechanisms underlying the PI3K-mediated suppression of PMA-induced expression of p21WAF1/Cip1 showed that blocking of the PI3K/mTOR pathway was associated with increased binding by Sp1 [38]. Such a mechanism might therefore be common in transcriptional inhibition through Sp1/Sp3.

In conclusion, our studies provide novel insights into the role and mechanism of action of CK2 and PI3K signalling in the IFN-γ-mediated suppression of LPL gene transcription. Although IFN-γ regulates gene transcription mainly through the JAK-STAT pathway, several studies have indicated the existence of alternative pathways. The CK2- and PI3K-mediated regulation of Sp1/Sp3 binding could represent one such mechanism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellsig.2008.08.016.

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