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1α-Hydroxylase transactivation by γ-interferon in murine macrophages requires enhanced C/EBPβ expression and activation☆

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Abstract

γ-Interferon [γ-IFN] induction of macrophage 1α-hydroxylase mRNA and activity causes severe hypercalcemia in granulomatous disorders. These studies demonstrate transcriptional regulation. γ-IFN induces the activity of the murine 1α-hydroxylase [−1651; +22] promoter in the murine macrophage cell line Raw 264.7 only after a 24 h exposure. This slow kinetics is incompatible with classical γ-IFN-mediated transactivation. In fact, γ-IFN response mapped to the minimal [−85; +11] promoter, which lacks GAS or ISRE sites but contains a putative C/EBPβ site. C/EBPβ is a γ-IFN inducible gene and a novel mediator of γ-IFN-regulated transcription. As expected for a C/EBPβ-driven transcription, ectopic C/EBPβ expression was sufficient to increase 1α-hydroxylase activity, enhance minimal promoter activity and potentiate the induction of this promoter by γ-IFN. Importantly, the dominant negative C/EBPβ isoform antagonized C/EBPβ-transcriptional activity. γ-IFN induction of C/EBPβ expression is not sufficient for γ-IFN induction of minimal promoter activity. There is also a cell-specific induction of C/EBPβ-transcriptional activity by γ-IFN. In Raw cells, specific inhibition of γ-IFN induction of endogenous-C/EBPβ phosphorylation by MEKK1 markedly reduced basal promoter activity and the response to γ-IFN. We conclude that γ-IFN-induction of C/EBPβ expression and activation by phosphorylation contributes to γ-IFN-transcriptional control of 1α-hydroxylase expression in murine macrophages.

Keywords: C/EBPβ; γ-Interferon; 1α-Hydroxylase; Transcriptional control

1. Introduction

In normal physiological conditions, the kidney is the organ responsible for the production of most (if not all) of the circulating 1α,25-$(OH)_2D_3$, the hormonal form of Vitamin D, a potent calcitropic sterol. To maintain normal calcium homeostasis, renal 1α,25-$(OH)_2D_3$ production by 1α-hydroxylase, a mitochondrial cytochrome-P450, is tightly regulated by parathyroid hormone, calcium, phosphorus and 1α,25-$(OH)_2D_3$. 1α,25-$(OH)_2D_3$ controls its own levels in circulation by dual mechanisms. The sterol down-regulates its synthesis by renal 1α-hydroxylase and induces the expression of renal- and extrarenal 24-hydroxylase, the main enzyme inactivating 25-hydroxyvitamin D and 1α,25-$(OH)_2D_3$ [1,2].

In contrast to the tight regulation of renal 1α,25-$(OH)_2D_3$ synthesis, excessive 1α,25-$(OH)_2D_3$ production by activated macrophages causes severe hypercalcemia in several granulomatous disorders and chronic inflammation [3–6]. In those patients, 1α-hydroxylase activity is insensitive to feed-back inhibition by high serum calcium or 1α,25-$(OH)_2D_3$. Consistent with these in vivo observations, studies in vitro demonstrate that, contrary to its renal counterpart, macrophage 1α-hydroxylase activity is enhanced by increases in extracellular calcium [7] and unaffected by supraphysiological concentrations of 1α,25-$(OH)_2D_3$ [8]. The distinct responses to hypercalcemia of renal and macrophage 1α-hydroxylases suggest that 1α,25-$(OH)_2D_3$ production by activated macrophages is not aimed to maintain calcium homeostasis. A potential role in immune modulation of local 1α,25-$(OH)_2D_3$ synthesis by macrophages was suggested by the marked stimulation of 1α-hydroxylase activity by inflammatory stimuli such as lipopolysaccharide (LPS) [9], IFN-γ [10,11] and TNF-α [12]. The demonstration of a direct correlation between pleural levels of IFN-γ and 1α,25-$(OH)_2D_3$ production suggested the involvement of the cytokine in the abnormalities in 1,25D homeostasis [13]. In fact, exposure of normal monocytes, pulmonary alveolar macrophages or the monocytic cell line THP-1 to IFN-γ, markedly enhances macrophage 1α,25-$(OH)_2D_3$ production. γ-IFN potently antagonizes 1α,25-$(OH)_2D_3$ induction of 24-hydroxylase gene expression [10]. Direct binding of γ-IFN-activated Stat1 to the

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DNA binding domain of the Vitamin D receptor (VDR) prevents 1α,25-$(OH)_2D_3$/VDR transactivation of this gene. Reduced-24-hydroxylase expression is sufficient to account for excessive 1α,25-$(OH)_2D_3$ production causing high serum levels of 1α,25-$(OH)_2D_3$. However, γ-IFN increases 1α-hydroxylase mRNA levels in the human monocytic cell line THP1, in both the monocyte and macrophage phenotypes [11], and also in freshly isolated murine macrophages and in the murine macrophage cell line P388D1 [14]. The present studies address the molecular mechanisms underlying γ-IFN induction of 1α-hydroxylase mRNA levels in the murine macrophage cell line Raw 264.7.

2. Materials and methods

2.1. Plasmids, antibodies

All reporter constructs utilized in this study were described previously [15] and were kindly provided by Dr. Hector DeLuca (University of Wisconsin, Madison). The β-galactosidase expression plasmid was obtained from Michael Rauchman (Washington University). Wild type rat C/EBPβ expression plasmid and its dominant negative isoform, cloned in a pSCT vector, originally described by Descombes and Schibler [16] were kindly provided by Dr. Kilberg (University of Florida, Gainesville). Wild type and T235A mutant of human C/EBPβ, cloned in a pCMV vector were a gift of Dr. Kalvakolanu (University of Texas). Rabbit anti C/EBPβ was purchased from Santa Cruz Biotechnologies.

2.2. Cell culture

Raw 264.7 (ATCC) were cultured in DMEM medium (Gibco BRL) containing 10% fetal bovine serum (FBS) (Fisher), 1 mM Na pyruvate and incubated at 37 °C in a 5% CO_2 atmosphere. 2f cells were cultured under similar conditions except for the lack of Na pyruvate in the culture media.

2.3. Transient transfection and treatment

Raw cells (3×10^5) or 2×10^5 2f cells were seeded per well in six-well plates. After an overnight incubation, cells were transfected using Superfect (Qiagen) following the manufacturer's protocol. In Raw cells, 2 μg of the indicated reporter plasmid, 0.1 μg of β-galactosidase expression plasmid and the dose of the expression vectors [C/EBPβ (LAP), its dominant negative mutant (LIP), or its point mutation at threonine 235] indicated in each experimental protocol were co-transfected per well. In 2f cells, eliciting higher transfection efficiency, the concentration of transfected plasmids was reduced to 0.5 and 0.01 μg for the indicated luciferase reporter and β-galactosidase, respectively, or 0.05 μg for the indicated-expression vector(s). The total amount of transfected DNA was kept constant by adding pGEM DNA, if required. After an overnight incubation, cells were treated with vehicle (control) or the indicated concentration of IFN-γ (50–500 IU/ml; Sigma, St. Louis) for Raw cells, or 500 IU/ml of the cytokine in 2f cells and incubated in 2% FBS DMEM media for 24 h.

2.4. Inhibition of γ-IFN phosphorylation of C/EBPβ

Prior to a 24 h-exposure to γ-IFN, transfected cells were pretreated for 90 min with 10 or 30 μM the specific MEK inhibitor U0126 (Promega). Media was then removed and replaced by fresh 2% FBS-DMEM. Cell viability was not compromised by the pulse of inhibitor, as judged by the lack of changes in β-galactosidase activity and preserved cell morphology under light microscopy.

2.5. Luciferase and β-galactosidase activities

After 24 h of treatment, cells were lysed and Luciferase and β-galactosidase activities were measured using Luciferase Reporter System (Promega) and Galacto-Light (Applied Biosystems), respectively, following the manufacturer's instructions. Results represent the average (±S.E.M.) of triplicate determinations per experimental condition from at least two independent experiments.

2.6. Measurement of 1α-hydroxylase activity

The rate of conversion of 25-hydroxycholecalciferol to 1α,25-dihydroxyvitamin D_3 was measured as previously described [10,17]. Raw cells in the resting state, or activated by exposure to 100 IU/ml of recombinant murine γ-IFN for 24 h, or transfected with either the C/EBPβ expression vector (0.1 μg per 3×10^5 cells) or empty vector were studied. Transfections were performed in 10 cm-well plates. After an overnight incubation, untransfected and transfected cells were scraped and seeded in six-well plates at a concentration of 10^6 cells/well. 1α-Hydroxylase activity was measured after a 24 h incubation. Reactions were initiated by the addition of 25-hydroxy [26(27)-methyl^{3}H]-cholecalciferol. After a 2 h incubation at 37 °C, reactions were stopped by addition of 1 ml of acetonitrile. Radioinert (100 ng of) 1α,25-$(OH)_2D_3$ in 20 μl of ethanol were added to monitor recovery. The ^{3}[H]-1,25-$(OH)_2D_3$ synthesized was purified by C_{18}-cartridge solid phase extraction followed by straight phase HPLC isolation of the 1,25-$(OH)_2D_3$ fraction using 4% isopropanol in methylene chloride and quantified as previously described [10,17].

3. Results

The demonstration that freshly isolated murine macrophages and the murine macrophage cell line P388D1 respond to γ-IFN inducing 1α-hydroxylase mRNA [14], led us

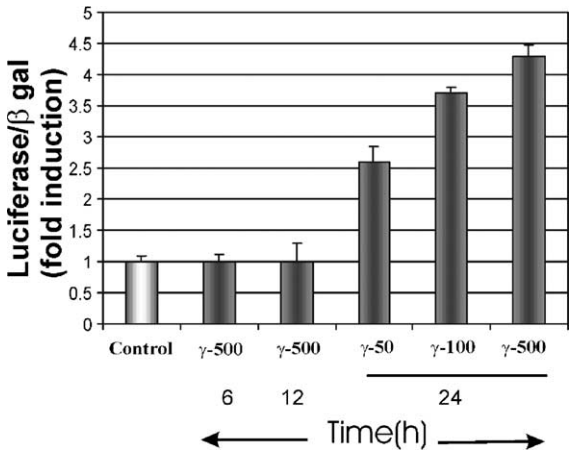

Fig. 1. Time course and dose response for γ-IFN regulation of murine-1α-hydroxylase promoter activity. Luciferase and β-galactosidase activities in Raw cells transfected with a luciferase reporter driven by the murine 1α-hydroxylase [−1651; +22] promoter as specified in methods. Twenty-four hours after transfection, cells were treated with 0 or the indicated concentration of murine recombinant γ-IFN for 6, 12 or 24 h. Results represent the mean ± S.E.M. of triplicate determinations per experimental condition from three independent experiments.

to test the potential involvement of transcriptional mechanisms. To this end we utilized the murine macrophage cell line Raw 264.7. Raw cells expressed 1α-hydroxylase activity and responded to γ-IFN with a three-fold induction of 1α-hydroxylase activity (Fig. 4B). The regulation by γ-IFN of the activity of a luciferase-reporter plasmid, driven by the murine-1α-hydroxylase promoter [1651 + 22], was examined. Fig. 1 shows a dose dependent induction of 1α-hydroxylase-promoter activity by γ-IFN. These results demonstrate that γ-IFN regulation of 1α-hydroxylase mRNA levels is partially mediated by transcriptional mechanisms.

Importantly, this induction only occurred after a 24 h exposure to the cytokine. Either a 6 or a 12 h exposure to γ-IFN were insufficient to induce 1α-hydroxylase-gene transcription. This time course recapitulates the slow increases in 1α-hydroxylase mRNA levels induced by the cytokine in murine macrophages [14]. However, they argue against a classic Stat1-mediated mechanism since γ-IFN/Stat1-induced transactivation occurs within 5–6 h after exposure to γ-IFN [18].

To map the γ-IFN response element on the 1α-hydroxylase promoter, several deletion-constructs upstream of a luciferase reporter gene in the GL2 basic vector were used. All these constructs, generated at Dr. DeLuca's laboratory, have been described previously [15].

Fig. 2 shows that two internal deletion constructs, one the 1037× with basal promoter activity and the other BDC, which lacks a repressor sequence at [−1126 to 1037], had no effect on the response to γ-IFN. Similarly, three 5′ deletion constructs that eliminated putative AP1 sites, potential targets for γ-IFN activation, did not affect the response to γ-IFN. Thus, the putative γ-IFN responsive element maps to the minimal 1α-hydroxylase promoter. Importantly, neither the pGL2 basic nor the pGL2 promoter driven by a minimal promoter of the potent SV40 vector, responded to γ-IFN.

This minimal promoter (Fig. 3) contains no γ-IFN-consensus sequence (neither GAS nor ISRE) but a putative C/EBPβ binding site, overlapping the transcriptional start

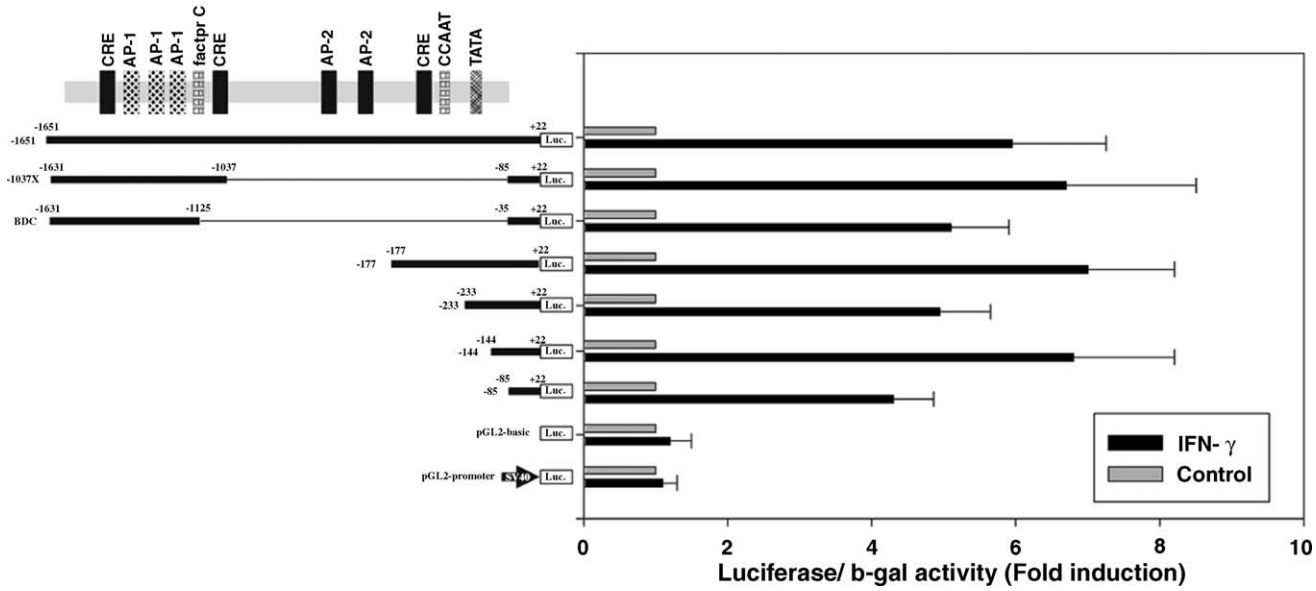

Fig. 2. Mapping the γ-IFN responsive sequence in the murine 1α-hydroxylase promoter. Luciferase and β-galactosidase activities in Raw cells transfected with a luciferase reporter, driven by the internal or 5′ deletion construct indicated in the left panel, as specified in methods. Twenty-four hours after transfection, cells were treated with 0 or 500 IU/ml of murine γ-IFN for 24 h. Results represent the mean ± S.E.M. of triplicate determinations per experimental condition from at least two independent experiments.

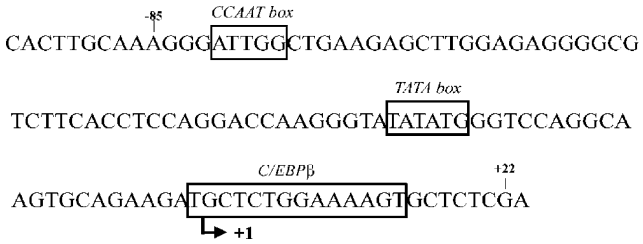

Fig. 3. DNA sequence of the minimal [−85; +22] 1α-hydroxylase promoter.

site. C/EBPβ, a γ-IFN inducible gene, is the transcription factor for γ-IFN-mediated transactivation of p48 [19,20], a gene central for macrophage responses to interferons [21]. Importantly, γ-IFN/C/EBPβ transactivation of p48 follows a slow kinetics, similar to that observed for γ-IFN induction of 1α-hydroxylase mRNA levels [14] and promoter activity in murine macrophages.

To directly evaluate the contribution of C/EBPβ to γ-IFN induction of 1-hydroxylase gene transcription, Raw cells were co-transfected with the minimal promoter [−85; +22] and an expression vector for either C/EBPβ or its truncated isoform, LIP [16]. LIP lacks C/EBPβ-transactivation domain and functions as a dominant negative C/EBPβ. Fig. 4A shows that ectopic C/EBPβ expression resulted in marked increases in minimal promoter activity. In contrast, the dominant negative isoform, LIP, suppressed basal promoter activity. Furthermore, simultaneous co-expression of LIP with C/EBPβ in a 1:1 ratio reversed C/EBPβ induction of promoter activity to basal levels.

Further support for a role for C/EBPβ in 1α-hydroxylase-gene expression came from the demonstration that ectopic C/EBPβ expression markedly increased 1α-hydroxylase activity over that expressed in cells transfected with the vector alone (Fig. 4B). Transfection itself increased 1α-hydroxylase activity over that expressed in the same batch of untransfected cells, possibly due to macrophage activation.

There is evidence in the literature that γ-IFN induces C/EBPβ expression in Raw cells [19]. Under our experimental conditions, exposure of Raw cells to 100 U/ml of γ-IFN for 24 h markedly enhanced C/EBPβ content (Fig. 5A). This induction in endogenous C/EBPβ expression in response to γ-IFN could account for the increase in basal promoter activity and also for the additive effects of γ-IFN to the enhancement in minimal promoter activity induced by ectopic C/EBPβ (Fig. 5B). Similar to its effects on basal promoter activity, in three independent experiments ectopic expression of 0.1 μg of LIP reduced the response to γ-IFN with a similar potency to that elicited in inhibiting basal-promoter activity. Taken together these results support the contribution of C/EBPβ transcriptional activity to γ-IFN induction of 1α-hydroxylase-gene expression. However, similar studies in the human fibroblastic cell line 2f showed an intact response to γ-IFN in the induction of C/EBPβ expression, but these cells did not enhance 1α-hydroxylase-promoter activity in response to γ-IFN (Fig. 6). These results suggest that γ-IFN induction of C/EBPβ protein expression is not sufficient for transactivation of the 1α-hydroxylase gene.

γ-IFN enhances C/EBPβ-transcriptional activity at the p48 promoter through induction of a MEKK1-mediated phosphorylation [22]. Based on this finding and the lack of γ-IFN induction of minimal promoter activity in 2f cells, we examined whether a similar C/EBPβ-activation mechanism was required for γ-IFN/C/EBPβ transactivation of 1α-hydroxylase. To this end, the response to γ-IFN was tested in Raw cells transfected with a mutant human C/EBPβ T235A [23], which lacks the phosphorylation site required for γ-IFN/MEKK1-induced activation. In two independent experiments, this mutant C/EBPβ retained the ability to induce promoter activity 15.7 ± 1.7-fold over untransfected cells. However, there was no additional increase in minimal promoter activity in response to γ-IFN (not shown). Surprisingly, however, the increased in basal promoter activity induced by the mutant C/EBPβ was 2.4 ± 0.3-fold above that of wild type human C/EBPβ. The

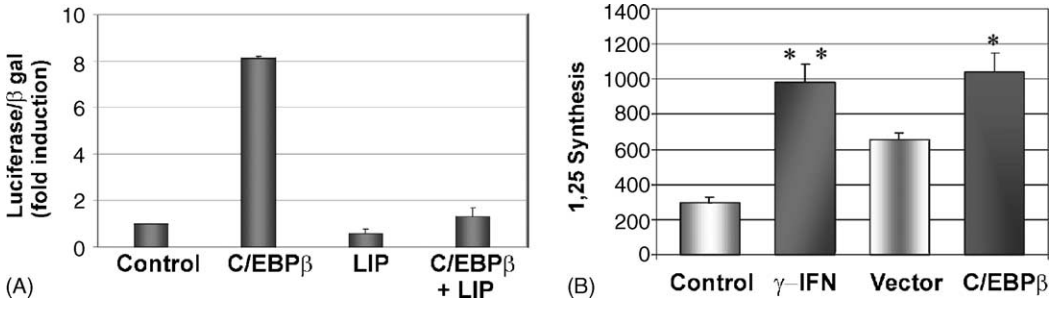

Fig. 4. Ectopic C/EBPβ expression enhances [−85; +22] promoter activity (A) and 1α-hydroxylase activity (B). (A) Luciferase and β-galactosidase activities in Raw cells co-transfected with the murine 1α-hydroxylase [−85; +22] promoter as specified in methods and 0.1 μg of the expression vectors for rat C/EBPβ or is dominant negative isoform, LIP. Twenty-four hours after transfection, cells were treated with 0 or 100 IU/ml of murine γ-IFN for 24 h. Results represent the mean ± S.E.M. of triplicate determinations per experimental condition from three independent experiments. (B) 1,25-$(OH)_2D_3$ synthesis in 2 h (dpm/10^6 cells) by untransfected Raw cells, either untreated (control) or exposed to 100 IU/ml γ-IFN for 24 h, and in cells transfected with 0.1 μg of rat C/EBPβ expression vector (C/EBPβ) or vector alone (vector) (see Section 2). Results represent the mean ± S.E.M. of quatruplicate determinations per experimental condition: (*) and (**) indicates $P < 0.05$ and 0.01 using unpaired *t*-tests.

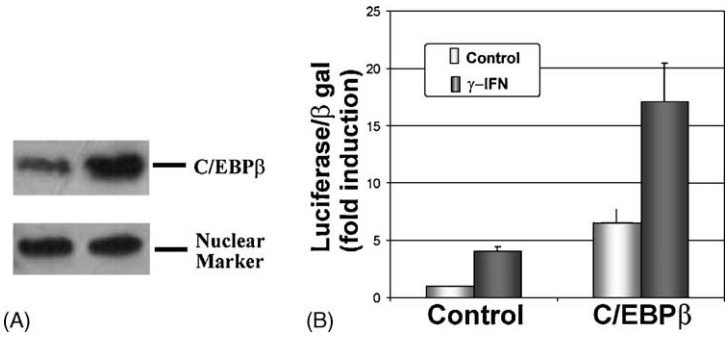

Fig. 5. γ-IFN induction of C/EBPβ expression (A) and minimal [−85; +22] promoter activity (B) in Raw 264.7 cells. (A) Representative Western blot analysis of C/EBPβ expression in untreated Raw cells (control) and in cells exposed to 100 IU/ml of γ-IFN for 24 h. (B) Luciferase and β-galactosidase activities in Raw cells co-transfected with the murine 1α-hydroxylase [−85; +22] promoter as specified in methods and 0.1 μg of the expression vectors for rat C/EBPβ. Twenty-four hours after transfection, cells were treated with 0 or 100 IU/ml of murine γ-IFN for 24 h. Results represent the mean ± S.E.M. of triplicate determinations per experimental condition from four independent experiments.

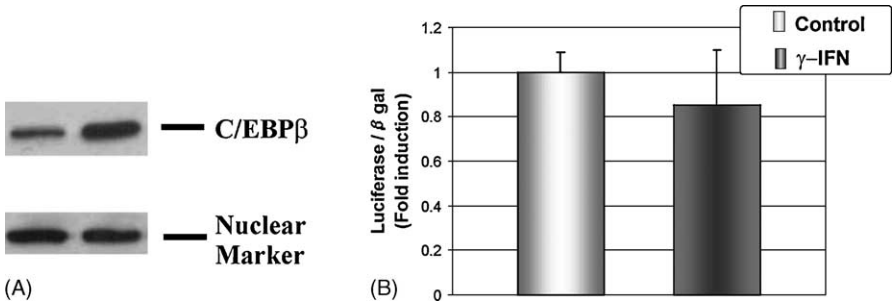

Fig. 6. γ-IFN induces C/EBPβ expression (A) but not minimal [−85; +22] promoter activity (B) in 2f cells. (A) Representative Western blot analysis of C/EBPβ expression in untreated 2f (control) and in cells exposed to 100 IU/ml of γ-IFN for 24 h. (B) Luciferase and β-galactosidase activities in 2f cells co-transfected with the murine 1α-hydroxylase [−85; +22] promoter as specified in methods. Twenty-four hours after transfection, cells were treated with 0 or 500 IU/ml of murine γ-IFN for 24 h. Results represent the mean ± S.E.M. of triplicate determinations per experimental condition from two independent experiments.

differential induction of minimal promoter activity by the wild type- and mutant-C/EBPβ expression vectors could not be attributed to higher expression of the mutant protein. Both expression vectors induced basal promoter activity similarly in the same batch of Raw cells, transfected simultaneously with the whole [−1651; +22]-1α-hydroylase reporter (not shown).

C/EBPβ transcriptional function is dynamically modulated by multiple phosphorylation sites in response to a single physiological regulator [24]. These findings and the conflicting results on the regulation of basal and γ-IFN-regulated minimal-promoter activity by ectopic-C/EBPβ T235A expression led us to directly address the requirement of γ-IFN phosphorylation of endogenous C/EBPβ beta through MEKK1 for the induction of minimal 1α-hydroxylase-promoter activity. Raw cells were exposed for 90 min to the highly specific MEK inhibitor U0126 [25] prior to the 24 h exposure to γ-IFN. Fig. 7 shows that prevention of C/EBPβ phosphorylation by U0126 reduced basal promoter activity in a dose dependent manner. The inhibitor also caused a parallel reduction in γ-IFN-induction of promoter activity. However, the response to γ-IFN was not completely abolished. These results suggest that γ-IFN induced phosphorylation of C/EBPβ through MEKK1 is one component of a more complex transcriptional control of 1α-hydroxylase expression by γ-IFN.

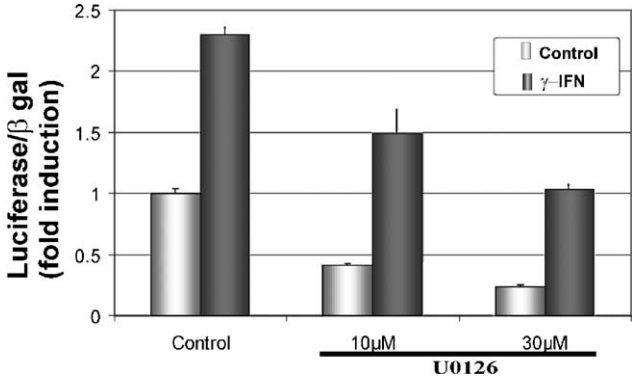

Fig. 7. Specific MEKK1 inhibition reduces endogenous C/EBPβ transcriptional activity and the response to γ-IFN in Raw 264.7. Luciferase and β-galactosidase activities in Raw cells co-transfected with the murine 1α-hydroxylase [−85; +22] promoter as specified in methods. Twenty-four hours after transfection, cells were treated with 0, 10 or 30 μM U0126 in DMSO for 90 min prior to exposure to 0 or 100 IU/ml of murine γ-IFN for 24 h. Results represent the mean ± S.E.M. of triplicate determinations per experimental condition from two independent experiments.

4. Discussion

These studies, designed to characterized the mechanisms underlying γ-IFN induction of 1α-hydroxylase expression, demonstrate the contribution of a C/EBPβ driven-transcriptional activation of this gene. γ-IFN induction of C/EBPβ expression and phosphorylation through MEKK1 contributes to increase 1α-hydroxylase gene expression and, consequently, 1α,25-$(OH)_2D_3$ production in murine macrophages.

Similar to the reports in freshly isolated murine macrophages and the murine macrophage cell line P388D1 [14], Raw 264.7 cells expressed 1α-hydroxylase activity and responded normally to γ-IFN by inducing 1α,25-$(OH)_2D_3$ production. Dose response studies demonstrated that γ-IFN induction of 1α-hydroxylase activity in Raw cells could be partially accounted for transcriptional activation of the 1α-hydroxylase gene. Importantly, the slow kinetics for γ-IFN induction of 1α-hydroxylase promoter activity (24 h exposure) in our studies, and that observed for the increases in mRNA levels of the enzyme in vivo in response to the cytokine in murine macrophages [14], are incompatible with direct Stat1-transactivation through the classical γ-INF activation sequences (GAS or ISRE). In fact, the response to γ-IFN mapped to the minimal-1α-hydroxylase promoter, which lacks GAS or ISRE sites.

The presence of a putative C/EBPβ site in this promoter raised the interesting possibility of a mechanism similar to that for γ-IFN-transactivation of p48, a gene central in the response to α- and β-interferons [19]. C/EBPβ is a γ-IFN-inducible gene. Furthermore, similar to 1α-hydroxylase, C/EBPβ expression increases as a result of macrophage differentiation [26].

As expected for a potential C/EBPβ-mediated mechanism, ectopic C/EBPβ expression was sufficient to increase 1α-hydroxylase activity in Raw cells. In these cells, C/EBPβ also transactivates the minimal-1α-hydroxylase promoter. Whereas ectopic C/EBPβ expression markedly enhanced basal promoter activity, the truncated, dominant negative isoform LIP decreased basal promoter activity and antagonized both the induction of promoter activity by ectopic C/EBPβ expression and the response to γ-IFN. γ-IFN-induced increases in C/EBPβ expression [20], however, are not sufficient for cells to respond to γ-IFN with an enhanced transactivation of the 1α-hydroxylase gene.

Cell-specific activation of C/EBPβ-transcriptional activity could contribute to the distinct effects of the cytokine between macrophages and the fibroblastic cell line 2f, which elicits an intact response to γ-IFN in the transactivation of p48 [19].

γ-IFN induction of MEKK1 mediated phosphorylation is certainly a contributor to increase C/EBPβ-transcriptional activity at the 1α-hydroxylase promoter as judged by the reduction in basal promoter activity and the response to γ-IFN in the presence of the MEK inhibitor U0126.

Multiple phosphorylation sites in the C/EBPβ molecule account for its versatility in DNA-binding affinity and transcriptional activity [24]. It is therefore possible that phosphorylation sites other than the consensus MAPK site, activated by MEKK1, also contribute to enhance 1α-hydroxylase gene transcription in response to γ-IFN.

Studies in macrophages from the C/EBPβ knock out mice provide a unique tool to identify the actual contribution of C/EBPβ to γ-IFN transactivation of 1α-hydroxylase.

Acknowledgements

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