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Interferon-α inhibits chromogranin A promoter activity in neuroendocrine pancreatic cancer cells

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Abstract Interferon-α (IFN-α) treatment can suppress the hypersecretion syndrome associated with functional neuroendocrine tumors. Chromogranin A (CgA) is a matrix protein of neuroendocrine secretory vesicles and appears to be essential for an appropriate neuroendocrine secretory function. To test the hypothesis that IFN-α can directly interfere with CgA gene transcription, we performed transient transfection studies in pancreatic neuroendocrine tumor cells employing CgA-luciferase reporter gene constructs showing that IFN-α inhibited basal and protein kinase C-dependent CgA promoter activity. Using 5′-deletion constructs in combination with mutational analysis of the proximal CgA core promoter, a cyclic AMP response element (CRE) at −71 to −64 bp was identified as the IFN-α response element of the CgA gene. Furthermore, functional studies indicated that IFN-α exerts its effect on the CgA promoter via interference with CRE binding protein (CREB)/CREB binding protein (CBP)-dependent transactivation of the CgA-CRE.

Key words: Neuroendocrine tumor; Chromogranin A gene regulation; Hypersecretion syndrome; Interferon-α; cAMP-responsive element binding protein; cAMP-responsive element binding protein binding protein

1. Introduction

Patients with metastatic gastroenteropancreatic neuroendocrine tumor (NET) disease present with symptoms of a tumor-related hypersecretion syndrome, which is characterized by flushing, diarrhea, bronchial constriction and occasionally valvular heart disease and hypertension [1]. These symptoms are caused by bioactive molecules comprising regulatory peptides, serotonin and tachykinins, which are produced and secreted by neuroendocrine tumor cells [2]. Biotherapy with interferon-α (IFN-α) represents an established concept for the treatment of the hypersecretion syndrome, featuring less serious side effects than systemic chemotherapy [3–7]. Clinical studies demonstrated that IFN-α therapy can decrease expression of neuroendocrine tumor secretory products and that this effect depends on interference with pre-translational mechanisms of gene regulation [3,8]. In this context, chromogranin A (CgA), an essential matrix protein of neuroendocrine secretory granules, has been identified as a cellular target of IFN-α [3,8]. CgA is critically involved in the formation and condensation of secretory granules of neuroendocrine cells and in vitro studies demonstrated that diminished cellular CgA levels might result in perturbation of secretory functions [9–11]. In addition, molecular analysis of mouse CgA (mCgA) and human CgA genes revealed that a cAMP-responsive element binding protein (CREB) recognition motif located in the highly conserved proximal CgA core promoter is crucial for neuroendocrine-specific as well as regulated CgA gene expression [11–14]. Since direct interference with CgA transcription could represent a potential mechanism underlying the inhibitory action of IFN-α on the NET-associated hypersecretion syndrome, we investigated the effect of IFN-α on the transcriptional activity of the CgA promoter in human pancreatic neuroendocrine tumor cells.

2. Materials and methods

2.1. Cell culture and transfection studies

BON neuroendocrine pancreatic carcinoma cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Karlsruhe, Germany) containing 10% fetal calf serum (Biochrom, Berlin, Germany), 100 IU/ml penicillin and 100 IU/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Transient transfections of cultured BON cells were carried out using the calcium phosphate precipitation technique (DNA transfection kit, 5′Prime 3′Prime, Boulder, CO, USA) as previously described [12]. In brief, cells were plated at a density of 4×10^5 cells/35 mm well and transfected with 1 μg of plasmid DNA per well. Following two washes with phosphate-buffered saline, medium was replaced by serum-free medium (Ultraculture, BioWhittaker, Walkersville, MA, USA). After treatment with IFN-α (Roche, Basel, Switzerland) and/or phorbol 12-myristate 13-acetate (PMA) (Sigma, Deisenhofen, Germany) for 24 h, cells were harvested and extracts were analyzed for luciferase (Luc) activity using the Luc assay system (Promega, Madison, WI, USA). Luc activity was measured using a Lumat LB 9501 luminometer (Berthold, Bad Wildbad, Germany). Light production was expressed as relative light units (RLU). Transfections were done in triplicates or quadruplicates and results were calculated as mean ± S.E.M. Values for mCgA-Luc activities were expressed as the fold increase compared to untreated controls. Co-transfection of a β-galactosidase expression construct (Promega, Madison, WI, USA) was used to correct transfections for intra-assay variance. In general, intra-assay variance between single determinations did not exceed 10–15%.

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Abbreviations: mCgA, mouse chromogranin A; IFN-α, interferon-α; CRE, cyclic AMP-responsive element; CREB, cyclic AMP-responsive element binding protein; pCREB, phospho CREB; CBP, CREB binding protein; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PKA, protein kinase A

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2.2. Plasmids

mCgA 5′-deletion constructs used in this study were based on the promoterless Luc reporter gene vector pXP1 and have been previously described [11]. Mutants of the mCgA-cyclic AMP-responsive element (CRE) were prepared by a PCR-based approach using the pXP100 construct as a template, employing different mutated 5′-oligonucleotide primers and a common 3′-primer [11]. The correctness of PCR inserts in all constructs was confirmed by dideoxy sequencing. To study the characteristics of the influence of IFN-α on the mCgA-CRE in a heterologous promoter system, we used a construct in which the region mCgA −69 to −62 bp was subcloned upstream of the enhancerless herpes simplex thymidine kinase (TK) viral promoter into the plasmid pTK-Luc [11]. Vectors encoding the viral oncoprotein E1A and the CREB co-activator protein CREB binding protein (CBP) have been previously described [15,16]. Phosphorylated CREB (pCREB) was generated by co-transfection of plasmids containing cDNAs for CREB and an activated form of protein kinase A (PKA) subunit α as previously described [12]. The characteristics of the protein kinase C (PKC)-α overexpression construct used in this study have been reported before [17].

3. Results

3.1. IFN-α dose-dependently inhibits basal and PKC-stimulated mCgA promoter activity

In BON cells transfected with the mCgA4.8kb-Luc construct, IFN-α dose-dependently inhibited basal mCgA promoter activity, showing a detectable inhibition at 10 IU/ml and maximal inhibition at 1000 IU/ml (Fig. 1A). A further increase of IFN-α concentrations did not produce stronger inhibition of mCgA promoter activity (data not shown). Activation of PKC-α-dependent signaling pathways by phorbolester (PMA) treatment potently transactivated the mCgA promoter in BON cells (Fig. 1B). Similarly, overexpression of PKC-α resulted in a 3–4-fold elevation of mCgA4.8kb-Luc activity (Fig. 1B). IFN-α treatment dose-dependently inhibited PMA-stimulated mCgA promoter activity showing a maximal effect of approximately 50% inhibition at 1000 IU/ml (Fig. 1B).

3.2. 5′-Deletion analysis of the IFN-α-sensitive region of the mCgA promoter

To define the IFN-α-sensitive region of the mCgA promoter, various mCgA-Luc 5′-deletion constructs were transfected into BON cells and subsequently treated with IFN-α (Fig. 2A). In untreated cells, the basal promoter activity was doubled by deletion of the mCgA fragment spanning −4.8 to −1.2 kb, suggesting the presence of (a) basal repressor element(s) within this region (Fig. 2A). Further deletion of mCgA 5′-flanking DNA down to −100 bp only modestly influenced the basal promoter activity, whereas the loss of an additional 39 bp (mCgA100-Luc versus mCgA61-Luc) resulted in a dramatic reduction of basal mCgA promoter activity (Fig. 2A). The inhibitory effect of IFN-α on the basal activity of the mCgA promoter was essentially not influenced by deletion of mCgA 5′-flanking DNA from −4.8 kb down to −100 bp (Fig. 2A). A further loss of 5′-flanking (mCgA61-Luc construct) blunted the IFN-α effect, suggesting the presence of the IFN-α-sensitive region of the mCgA promoter between −100 bp and −61 bp. Using the same set of mCgA-Luc 5′-deletion constructs under conditions of PMA stimulation, deletion down to −100 bp of mCgA 5′-flanking DNA had no effect on the stimulatory action of PMA (Fig. 2B). Further 5′-deletion down to −61 bp resulted in a complete loss of PMA-stimulated mCgA transcriptional activity as well as IFN-α-dependent mCgA inhibition (Fig. 2B). Interestingly, the basal activity of the mCgA61-Luc construct, which lacks the proximal CRE site, did not show IFN-α-sensitivity (Fig. 2B). These data indicate that basal as well as PMA- and IFN-α-responsiveness of the mCgA promoter map to the proximal mCgA core promoter region.

3.3. The mCgA-CRE mediates IFN-α-sensitivity of the mCgA promoter

Previous studies revealed that a proximal core promoter region of the mCgA gene located between −93 and −62 bp is crucial for basal and stimulated promoter activity [11,12]. To determine the role of this region for the effect of IFN-α, we used a construct in which the sequence −93 to −62 bp is joined to the heterologous, enhancerless viral TK promoter (mCgA-93/-62-TK-Luc). We found that IFN-α inhibited the activity of this construct with an identical potency and efficacy compared to the 5′-deletion constructs, suggesting that this element is conferring mCgA IFN-α-responsiveness (Fig. 3A).

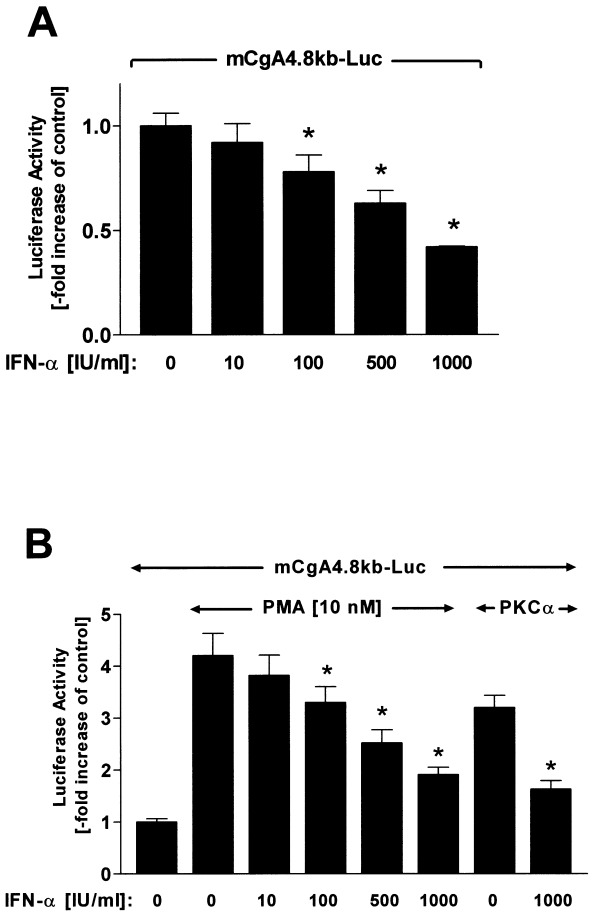

Fig. 1. IFN-α inhibits basal and PKC-stimulated mCgA promoter activity. A: BON cells were transiently transfected with 1 μg/well of the mCgA4.8kb-Luc construct and treated with increasing concentrations of IFN-α (10–1000 IU/ml) for 24 h. B: BON cells were transiently co-transfected with 1 μg/well of each mCgA4.8kb-Luc and a PKC-α expression construct. After pre-treatment with IFN-α for 24 h, stimulation with PMA was performed for an additional 12 h. Cells were harvested and lysates were analyzed for Luc activity. Luc activity is expressed as the fold increase of untreated controls and values represent the mean ± S.E.M. of four experiments. Asterisks (*) indicate statistically significant inhibition compared to untreated controls ($P < 0.001$).

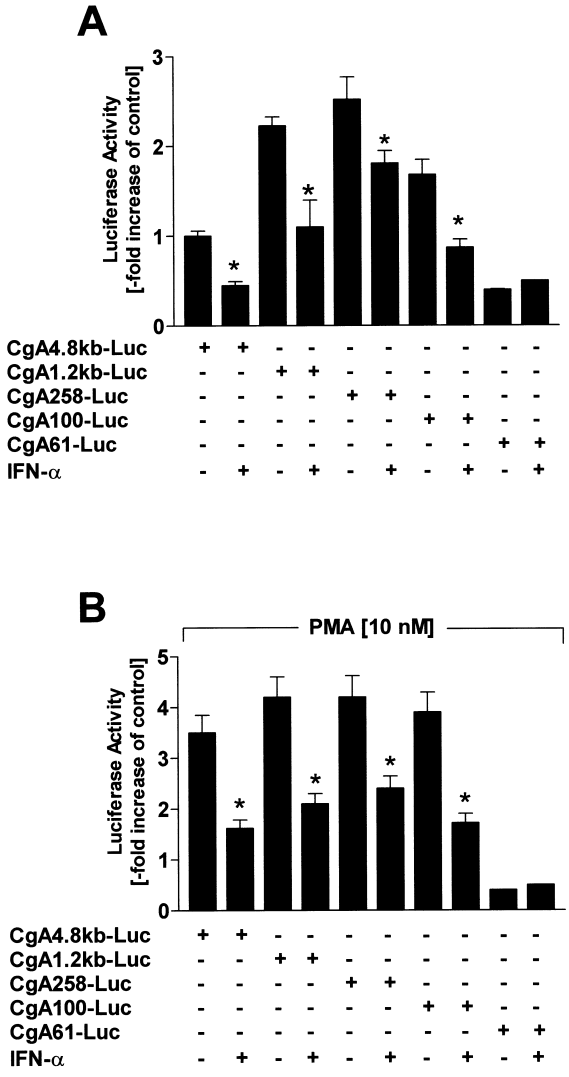

Fig. 2. 5′-Deletion analysis of the IFN-α-sensitive region of the mCgA promoter. A: BON cells were transiently transfected with mCgA 5′-deletion constructs of decreasing length and treated with IFN-α (1000 IU/ml) for 24 h. B: The same experiment as described under A was performed with subsequent stimulation with PMA for 12 h. Luc activity is expressed as the fold increase of untreated controls and values represent the mean ± S.E.M. of four experiments. Asterisks (*) indicate statistically significant inhibition compared to PMA-treated controls ($P < 0.001$).

Since the mCgA-CRE located at −71 to −64 bp has been shown to be essential for basal and stimulated mCgA promoter activity in neuroendocrine cells, we evaluated the role of this element using mCgA100-Luc-based constructs in which different mutations of the CRE site were introduced. In addition, we investigated the ability of a single copy of the CRE to confer IFN-α-sensitivity to the per se IFN-α-insensitive TK promoter. Deletion of the CRE in the context of 100 bp of 5′-flanking mCgA DNA (mutants M13, M15) resulted in a dramatic reduction of basal and PMA-stimulated activity (Fig. 3B). While the mutant CRE sequence M13 (A*catcacc*A) showed a reproducible but not statistically significant IFN-α-sensitivity, the CRE mutation M15 (G*acca*T) was completely IFN-α-insensitive (Fig. 3B). In contrast, conversion of the mCgA-CRE into an 'ideal' CRE (mutant M14 = CGT*c*ATT) resulted in enhanced basal and PMA-

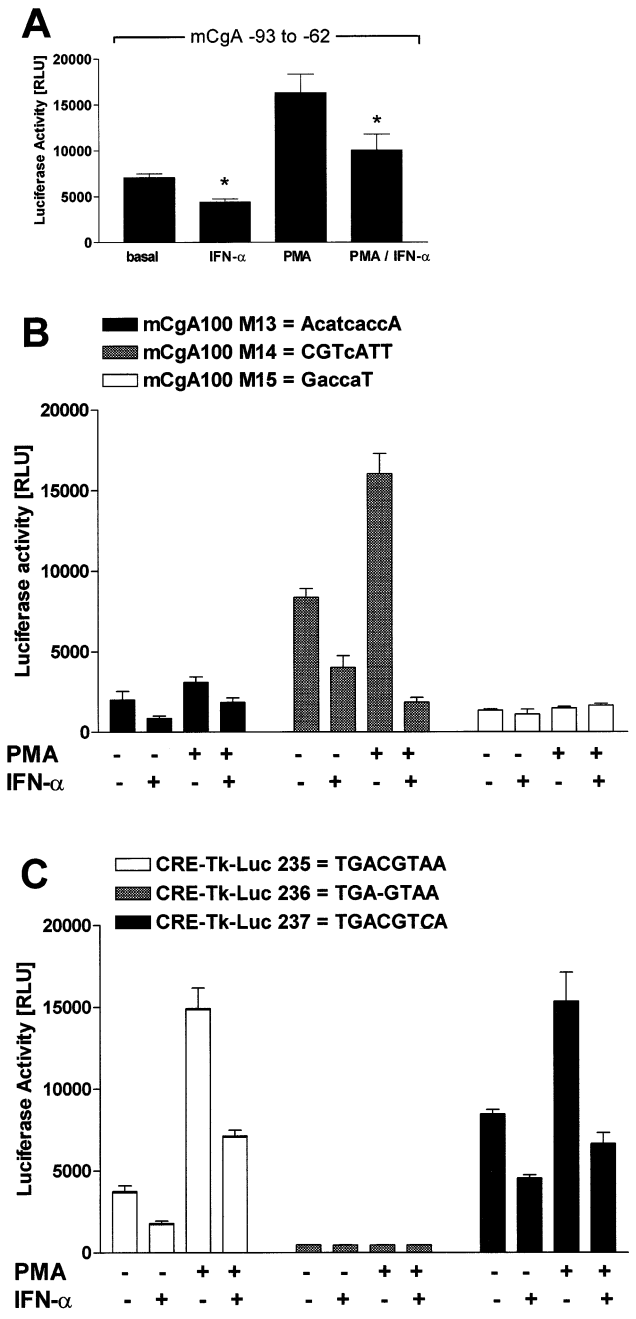

Fig. 3. The mCgA-CRE mediates IFN-α-sensitivity of the mCgA promoter. A: BON cells were transiently transfected with the mCgA-93/-62-Tk-Luc construct, which comprises the mCgA core promoter region. B: BON cells were transfected with mCgA100-Luc-based constructs in which three different mutations were introduced into the sequence of the mCgA-CRE at −68 bp. In the mutant CgA M14, the mCgA-CRE was converted to the 'ideal' CRE sequence of the human somatostatin gene. Basepairs that match with the original mCgA-CRE sequence are shown in capital letters, mismatches in small letters. C: BON cells were transfected with various heterologous Tk-Luc promoter constructs comprising the mCgA-CRE in the unaltered (CRE-Tk-Luc 235) or mutated form (CRE-Tk-Luc 236 and CRE-Tk-Luc 237). Where indicated, treatment with IFN-α (1000 IU/ml) and/or PMA (10 nM) was done as described in Fig. 1. Luc activity is expressed as arbitrary RLU and values represent the mean ± S.E.M. Asterisks (*) indicate statistically significant inhibition compared to untreated controls.

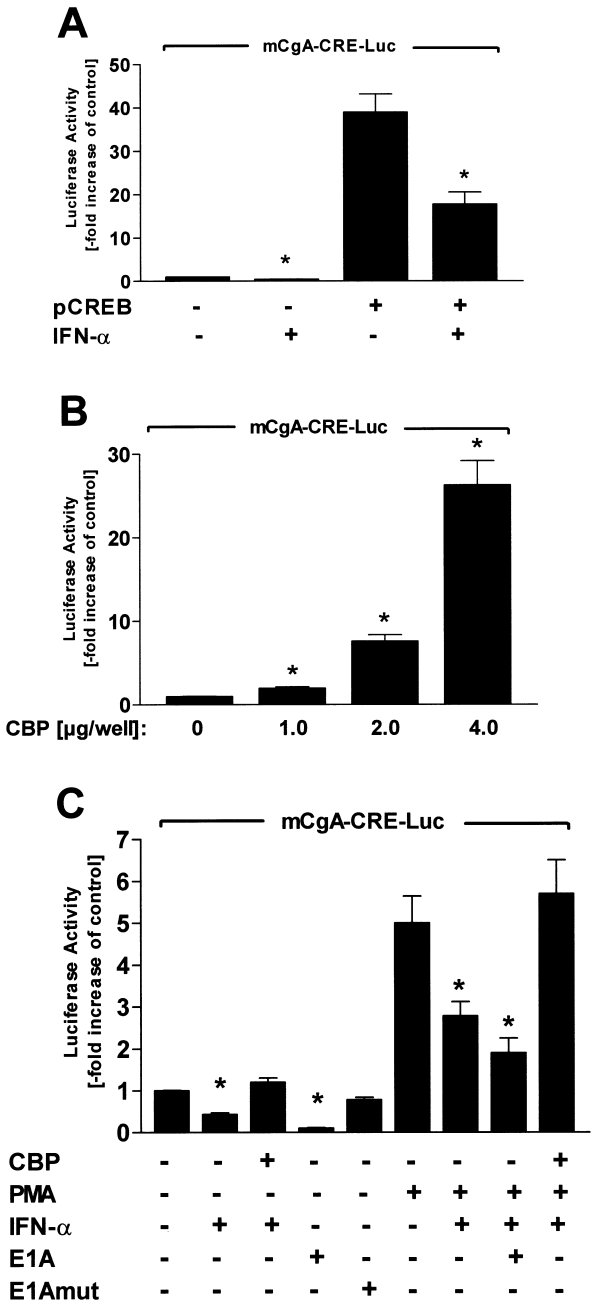

Fig. 4. Effect of IFN-α on the mCgA-CRE is mediated through interaction with CREB and CBP. BON cells were transiently transfected with the CRE-Tk-Luc 235 construct, which contains a single copy of the mCgA-CRE linked to the heterologous TK promoter. (A) Co-transfections were performed with expression vectors for CREB and the constitutively active α-subunit of PKA, (B) increasing amounts of an expression vector for CBP, (C) constructs encoding CBP, the viral oncoprotein E1A or a functional incompetent E1A mutant (E1Amut). Where indicated, treatment with IFN-α (1000 IU/ml) and/or PMA (10 nM) was done as described in Fig. 1. Luc activity is expressed as the fold increase of untreated controls and values represent the mean ± S.E.M. Asterisks (*) indicate statistically significant inhibition compared to untreated controls.

stimulated promoter activity, as well as a more pronounced sensitivity to IFN-α. In addition, a single copy of the mCgA-CRE ligated upstream of the enhancerless viral TK promoter was able to confer IFN-α-sensitivity to the same extent as observed with the longest 5′-deletion construct mCgA4.8kb-Luc (Fig. 3C). Point mutation of the CRE at −68 (CRE-Tk-Luc 236) resulted in a dramatic reduction of basal and PKC-stimulated mCgA activity and a complete loss of IFN-α-sensitivity. In contrast, conversion of the mCgA-CRE into an 'ideal' CRE motif corresponding to the CRE of the somatostatin gene resulted in full IFN-α-responsiveness of the TK-Luc construct (CRE-Tk-Luc 237).

3.4. IFN-α affects the mCgA-CRE through interaction with CREB and CBP

Since the CREB adapter molecule CBP/p300 has been shown to play a crucial role for CREB-dependent transactivation of target genes, we investigated the functional role of CREB and CBP for the effect of IFN-α on the mCgA-CRE. We found that overexpression of pCREB potently enhanced mCgA-CRE-dependent transactivation (35–45-fold induction), while IFN-α treatment inhibited this effect of CREB by approximately 50% (Fig. 4A). In addition, CBP overexpression dose-dependently stimulated the activity of the mCgA-CRE in the TK-Luc system, demonstrating the functional importance of CBP for mCgA-CRE-dependent transactivation in BON cells (Fig. 4B). Overexpression of CBP also reverted the inhibitory effect of IFN-α on basal and PMA-dependent activity of the mCgA-CRE. In contrast, sequestration of CBP by overexpression of the viral oncoprotein E1A, which also binds CBP directly, resulted in diminished mCgA-CRE-dependent transactivation (Fig. 4C).

4. Discussion

Analysis of neuroendocrine tumor samples from IFN-α-treated patients revealed diminished expression of CgA mRNA, suggesting that the CgA gene represents a molecular target for IFN-α [8]. Since the vesicular matrix protein CgA influences crucial steps of neuroendocrine secretory processes, inhibition of CgA expression could represent a potential mechanism by which IFN-α affects the secretory capacity of neuroendocrine tumor cells [9,10]. To investigate a potential interference of IFN-α with the molecular machinery controlling mCgA gene transcription, we analyzed the effects of IFN-α on the mCgA promoter in vitro. Since previous studies revealed a crucial role of PKC-related signaling cascades in regulated CgA gene expression in neuroendocrine cells, we also investigated the influence of IFN-α on PKC-stimulated regulation of the mCgA promoter [11–14].

In a recent study, we found that IFN-α treatment of BON cells exerts enhanced phosphorylation of STAT1/-2 and stimulation of IFN-stimulated response element (ISRE)-dependent transactivation [18]. In addition, our current study demonstrates that IFN-α treatment of BON cells inhibits the basal and PKC-stimulated transcriptional activity of the mCgA promoter. 5′-Deletion analysis combined with mutational analysis of the proximal mCgA core promoter (−93 to −62 bp) revealed that the CRE element located at −71 to −64 bp is indispensable for basal and PKC-stimulated mCgA activity in BON cells. These data are in accordance with previous studies, in which the proximal CRE site was uncovered as a crucial determinant of basal and neuroendocrine-specific transcriptional activity of the human and mCgA genes, which are highly homologous in their promoter sequences [11–13]. Furthermore, our studies revealed a critical role of the proximal mCgA-CRE for transmission of IFN-α-sensitivity to the

mCgA promoter. Mutation of this element largely diminished basal and PKC-dependent mCgA promoter activity and also resulted in a loss of IFN-α-sensitivity (Fig. 3B,C). In contrast, a single copy of the mCgA-CRE was able to confer IFN-α-sensitivity to the per se IFN-α-insensitive heterologous viral TK promoter. Mutation of the mCgA-CRE at −71 to −64 bp resulted in a loss of most of the intrinsic activity, PMA-responsiveness as well as IFN-α-sensitivity, further confirming the importance of this element for regulation of the mCgA promoter. The fact that IFN-α also inhibited the transcriptional activity of the 'ideal' CRE of the somatostatin gene (5′-TGACGTCA-3′) (mutant M14 in Fig. 3 and CRE-Tk-Luc in Fig. 3C) supports the view that CRE-like sequences in general can function as IFN-α-responsive elements. Interestingly, the somatostatin gene CRE (5′-TGACGTCA-3′) displayed a more pronounced transcriptional inhibition by IFN-α, demonstrating that subtle differences in the sequence of this element can also enhance the effectiveness of IFN-α on CRE-regulated transcription.

Analysis of the transcription factor(s) binding to the proximal CgA-CRE of the mCgA gene in neuroendocrine cell lines identified the transcription factor CREB as the principal nuclear protein responsible for basal and stimulated control of this element [11–16]. CREB belongs to the family of leucine-zipper transcription factors and represents the predominant nuclear protein binding to the CRE consensus sequence 5′-TGACGTCA-3′ present in the 5′-flanking region of a number of genes, such as somatostatin, tyrosine hydroxylase, pro-enkephalin and VIP [9]. CREB-dependent transactivation is generally controlled through phosphorylation of the serine residue at position 133 of the transcription factor [9,11–14]. In addition, recent studies suggested a model in which CREB-dependent transactivation of CREs is critically determined by the availability of the co-activator protein CBP/p300, which physically interacts with CREB at its transactivation domain and links the transcription factor to the basic transcriptional complex [20,21]. Furthermore, it has been demonstrated that IFN-regulated JAK/STAT signaling cascades can interfere with CBP-dependent gene regulation [20,21]. IFNs exert their anti-viral and anti-proliferative effects through activation of a specific set of genes termed IFN-stimulated genes (ISGs) [5]. Transactivation of these genes in response to IFN is initiated by Janus kinase-dependent phosphorylation of the latent cytoplasmic transcription factor ISG factor 3 (ISGF3), which binds to the ISRE [5,21]. Recent studies demonstrated that CBP/p300 can be found in the ISGF3 complex and that it can physically interact with STATs and that this interaction is required for IFN-stimulated transactivation of target genes [19–21]. In contrast, acquisition of cellular CBP by STATs has been shown to interfere with expression of genes, whose transactivation depends on the availability of CBP/p300 [19].

To determine the role of the CREB and CBP for mCgA gene transcription in BON cells, we overexpressed both proteins in the presence of the mCgA-CRE-Tk-Luc construct, which contains a single copy of the mCgA-CRE. Expression of either protein resulted in a potent transactivation of the mCgA-CRE, which could be inhibited by IFN-α treatment. In addition, expression of the viral oncoprotein E1A, which has been shown to act as a CBP 'scavenger' [20], reproduced the effect of IFN-α on the mCgA promoter. Finally, the inhibitory effect of IFN-α on the mCgA-CRE was fully reverted by overexpression of either CBP or CREB, suggesting the functional involvement of both proteins in this context. These data are in accordance with a recent investigation analyzing the inhibitory effect of IFN-γ on the macrophage scavenger receptor (MSR) gene, whose transactivation is critically determined by a nuclear complex comprising CBP and AP-1 [19]. The molecular analysis of IFN-γ-dependent inhibition of the MSR transactivation revealed that STAT1 competes with AP-1 for acquisition of cellular CBP protein and that elevation of cellular STAT1 levels resulted in diminished CBP levels and inhibition of MSR gene transcription [19].

Taken together, our data strongly support a concept in which IFN-α inhibits CgA gene transcription through inhibition of the proximal CRE element, which is crucial for basal and PKC-dependent expression of the gene. Furthermore, our study indicates that the inhibitory effect of IFN-α involves interference with CREB/CBP-dependent transactivation of this element. Inhibition of CgA gene transcription in neuroendocrine tumor cells by IFN-α treatment therefore represents one potential molecular aspect underlying the beneficial effect of IFN-α on the hypersecretion syndrome associated with neuroendocrine tumor disease.

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