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Comparison of leptin- and interleukin-6-regulated expression of the rPAP gene family: evidence for differential co-regulatory signals

Abstract

The pancreatitis-associated protein (PAP)/regenerating protein (REG) family represents a complex group of small secretory proteins, which can function as acute phase reactants, lectins, antiapoptotic factors or growth factors for pancreatic beta-cells and neural cells. Transcriptional induction of rPAP/Reg genes was studied here in PC12 cells made responsive to leptin. Northern-blots

showed quantitative differences in induction of four major family members by leptin and IL-6. Surprisingly, induction by leptin was strongly enhanced upon forskolin co-treatment whereas induction by IL-6 was counteracted. Functional studies involving progressive rPAP I promoter deletions showed, in the case of leptin, a clear correlation with predicted cis-regulatory elements. Leptin-induced stimulation was dependent on STAT3, since over-expression of dominant-negative STAT3, but not of dominant-negative STAT1, completely blocked transcriptional activation. In case of IL-6, an enhancer element outside the cloned promoter fragment is required for full stimulation. The effects of forskolin in a leptin and IL-6 context could not be explained at the promoter level, but rather events occurring upstream in the signalling cascade must be postulated to explain the differential co-regulatory effects.

Full text

INTRODUCTION

The pancreatitis-associated protein (PAP) family comprises four major members, PAP I, PAP II, PAP III and lithostathine/REG (regenerating protein). The name PAP proteins originally refers to rat acute pancreatitis, where exocrine acini express increased amounts of PAP I and Reg mRNA compared to healthy rats [1, 2], and to regenerating endocrine islets following partial pancreatectomy, where Reg mRNA is significantly induced [2, 3]. Since then, two other PAP-related mRNAs have been identified (PAP II and PAP III) which are also strongly overexpressed during the acute phase of pancreatitis [4-6]. Reg in mouse stands for three subtypes of genes (type I, II, III) based on primary structure of encoded proteins, whereby Reg III itself consists of four different genes, respectively Reg III alpha, beta, gamma and delta [7]. Although the precise function of these proteins remains elusive, their expression patterns suggest some major roles. A sharp PAP response to injury and inflammation in the pancreas is reminiscent of acute phase protein synthesis by the liver. Oxidative stress, *i.e.* enhanced release of reactive oxygen intermediates (ROIs), is believed to be part of the pathogenesis of acute pancreatitis [8]. ROIs may function as cellular mediators that trigger certain transcription factors such as AP-1 and NFkappaB [9, 10], steering cellular response towards protection and renewal. Studies with the acinar cell line AR4-2J confirm PAP I gene induction in response to oxidative stress, mediated by NFkappaB [11]. In addition, exocrine PAP proteins may function as lectins impeding bacterial infections during critical

periods for inflammation and necrosis [1]. Recently, TNF-alpha has been shown to trigger antiapoptotic mechanisms in rat pancreatic cells *in vitro*, through PAP I activation [12] and leptin in its turn is also involved in protective mechanisms against toxicity exerted by TNF [13]. The original REG protein (REG I) is thought to act on pancreatic beta-cells as an autocrine and/or paracrine growth factor [3, 14 and references therein], and a corresponding receptor cDNA has been isolated from a rat islet cDNA library [14]. Recently, rat Reg-2 (synonymous to PAP I) has been identified as an autocrine/paracrine neurotrophic factor for certain motoneurons induced by CNTF-related cytokines. It is functional during development and is an obligatory signalling intermediate in the CNTF-induced survival pathway [15, 16]. Remarkably, PAP I is also constitutively and abundantly expressed in healthy intestinal mucosa [17-20], supporting the hypothesis that PAP I contributes to control of the intestinal flora [20].

PAP/REG family members are differentially expressed in a tissue-specific manner in healthy conditions, *e.g.* PAP I and PAP III are not expressed in normal pancreas and an extended array of different tissues, while constitutively present in normal intestinal tract [5, 6, 17, 18]; PAP II is absent in normal pancreas and normal intestinal tract [4]; REG IIIalpha, beta, gamma are strongly expressed in intestine whereas REG III delta is absent [7]. Interestingly, REG and PAP I in pancreas and intestine are modulated by nutritional conditions [1, 2, 6, 18]. Concerning pancreas however, the effect of nutritional status is far below the induction level in diseased states.

It has long been known that diverse biological aspects of an acute phase response are coordinated by serum factors not present in healthy conditions. Transcriptional induction of rat PAP I (rPAP I) *in vitro* by specific combinations of cytokines and/or glucocorticoids has been shown [21, 22], and promoter studies delineating and mapping several putative IL-6 response elements, glucocorticoid-response elements and other transcription regulatory elements in different PAP genes have been performed [4, 6, 17, 21, 23-27]. Close similarity in promoters of different family members may explain certain coordinated constitutive and induced expression patterns [4, 6, 18, 24]. Gene localization on the same chromosome position suggests that these genes are derived from a common ancestral gene by successive gene duplications [23, 24, 28, 29].

In this study, we concentrate on the regulation of expression of rPAP I, rPAP II, rPAP III and Reg I in rat PC12 cells made responsive to leptin by stably introducing

the leptin receptor long isoform (LRlo). In continuation of previous work [30, 31], we investigated the effects of forskolin on the induction by leptin and we focussed on differential co-regulation between the leptin receptor and gp130 signaling pathways, knowing that gp130 itself is closely related to the leptin receptor.

MATERIALS AND METHODS

Cell culture and stimulations

Rat PC12-LR8 and PC12-1B8D cells were non-adherently cultured as described [30]. PC12-LR8 cells, stably expressing the leptin receptor wild-type form, are highly responsive upon leptin/forskolin co-stimulation [30]. PC12-1B8D cells express the leptin receptor long isoform at a higher level and were preferred for transfection studies. The level of leptin receptor expression was based on a leptin-SEAP binding assay [30]. PC12-LR8 cells were treated for 24 hours with medium either supplemented with 100 ng/ml mouse leptin (endotoxin level < 0.1 ng/mug of the cytokine, as determined by the Limulus amebocyte lysate (LAL) method, R&D Systems, Abingdon, Oxon, U.K.), 10 muM forskolin (Sigma), 290 U/ml human IL-6 (endotoxin level < 0.1 ng/mug of the cytokine, as determined by the LAL method, R&D Systems), plus 0.5 mug/ml soluble IL-6 Ralpha (endotoxin level much less than 0.1 ng/mug, a gift from Dr. I. Behrmann and Dr. P.C. Heinrich, Institut für Biochemie RWTH Aachen, Germany), or with combinations thereof. In some experiments, PC12-LR8 cells were induced to differentiate into neuronal cells after stimulation with beta-NGF. For this, cells were seeded in bottles coated with rat tail collagen (rat tail collagen, type 1: Becton Dickinson Labware, Bedford, MA, USA) using enriched RPMI 1640 medium with Glutamax I containing 5% inactivated foetal bovine serum, 10% inactivated horse serum and gentamycin (50 mug/ml) (all from GibcoBRL). One day after seeding, non-adherent cells were removed and medium was refreshed and supplemented with beta-NGF (10 ng/ml, R&D Systems) and forskolin (10 muM). Differentiated cells obtained within one week were finally treated as outlined above. HEK293T cells were cultured as described [32].

Representational difference analysis (RDA) and Northern-blot analysis

Two RDA procedures were performed essentially as previously described [31]. In the first RDA, we looked for leptin-upregulated genes in adherent PC12-LR8

cells, differentiated for 5 days in the presence of forskolin and beta-NGF, and ultimately treated with leptin (24 hours) in the presence of forskolin/beta-NGF, or left untreated. The second RDA experiment was performed to discriminate between gp130 and leptin receptor-mediated gene induction in non-differentiated PC12-LR8 cells treated with stimulants for 1 day. The same mRNA preparation was used for an RT-PCR procedure, optimized for amplification of rPAP II. The amplified rPAP II fragment was subcloned in pCR-Blunt vector (Invitrogen). Sequences of the primers were: forward 5'-GACAAGATGCTGCCTCGTCTG, and reverse 5'-GGTCTACTGCTTGAACTTGC. Amplification was performed for 30 cycles using Platinum Taq DNA polymerase (GibcoBRL): 1 min at 94° C, 1 min at 50° C, 1 min at 72° C, followed by a 5 min filling in reaction at 72° C. DNA sequencing revealed no difference from the published sequence [17]. For Northern-blot analysis, RNA isolation, agarose gelelectrophoresis, blotting and hybridization were all performed as previously described [30].

Generation of rPAP I promoter mutants and pXP2d2-rPAP I reporter constructs

Via PCR using pGL3-rPAPluc [30] as template, three deletion fragments were obtained using suitable oligonucleotides. Forward primers were designed containing about 20 nucleotides at the 3' side in perfect match with target sequences and preceded at the 5' side by a KpnI restriction site. One reverse primer was designed for the three deletions, hybridizing to a sequence in the luciferase gene beyond the EcoRI site. The oligonucleotide sequences were as follows:

forward: deletion 1: 5' GGGGTACCGTATGTAAGGTAAGTTGGTTG

deletion 2: 5' GGGGTACCTTGTGTTTCCCAGAACAG

deletion 3: 5' GGGGTACCTCACACCTGAGGTAAAGCAG

reverse (deletion 1, 2, 3): 5' CAGTACCGGAATGCCAAGC.

PCR was performed using Pfu polymerase, including 2.5% DMSO, 10 ng template and 500 ng primer DNA in the reaction mixture. Amplification was performed for 40 cycles after hot start (1 min at 94° C, 1 min at 59° C, 1 min at 72° C), followed

by a 5 min filling in reaction at 72° C. Amplified promoter pieces were subcloned in pCR-Blunt vector (Invitrogen) in the unique StuI site, excised afterwards by KpnI/EcoRI digestion and ligated into the pGL3 vector (*i.e.* "full size" promoter excised with KpnI and EcoRI from pGL3-rPAPluc reporter [30]).

The cloning strategy was further adapted since it appeared from initial transfection experiments that the pGL3 vector suffers from high background expression when leptin/forskolin co-stimulation was performed, suggesting interference of enhancer functions in that vector. To avoid this, "full size" and deleted promoters were transferred into the pXP2d2 luciferase reporter vector (a gift from Pr. Dr. Nordeen, University of Colorado, Health Sciences Center), a pXP2 derived vector from which two cryptic AP-1 sites are deleted [33]. Transfer of deletion mutants was done by KpnI/BglII based fragment exchange, excision of "full size" promoter was based on XhoI digestion/KpnI partial digestion and transfer in KpnI/XhoI opened pXP2d2 vector. All final constructs were sequenced after PCR amplification using primers annealing on bordering pXP2d2 sequences.

Two additional deletion mutants were also subcloned in pCR-Blunt vector, after fragment amplification by PCR using pXP2d2-rPAP I "full size" promoter as template and specifically designed primers: forward:

deletion 4:5' GGGGTACCGGCTGCTAACAGGAAAGAGG

deletion 5: 5' GGGGTACCAGCCAATGCCAAGTGCTTCCC

reverse (deletion 4 and 5): 5' CCGTGATGGAATGGAACAACAC.

The forward primers included again a KpnI site, the reverse primer hybridized to a luciferase sequence beyond the SphI site, to produce fragments of significant length. Promoter fragments were excised from pCR-Blunt vector by KpnI and BstEII digestion and transferred into linearized pXP2d2 vector. All final constructs were confirmed by sequence analysis.

Construction of dominant-negative (dn) variants of STAT1 and STAT3 (signal transducer and activator of transcription 1 and 3)

The pCAGGS-STAT3 and pCAGGS-STAT3dn (Y705 to F mutation) plasmids were kindly provided by Dr. T. Hirano (Biomedical Research Center, Osaka University Medical School, Osaka, Japan). The human STAT1 cDNA was obtained through RT-PCR using mRNA from the human 2fTGH fibrosarcoma cell line, using standard procedures with platinum Pfx polymerase (Life Technologies). Primers for amplification were as follows:

forward: 5'GGGTCGACATGTCTCAGTGGTACGAAC and reverse: 5'GGGGTTACCGGAGATCTCTATACTGTGTTCATCATAC. Restriction-based cloning *via* SalI-BstEII in the pMET7mcs construct resulted in the pMET7-STAT1 plasmid. The Y701 to F mutation was introduced by site-directed mutagenesis using Pfu polymerase (QuikChange, Stratagene) with primers 5'GATGGCCCTAAAGGTACCGGATTTATCAAGACTGAGTTG and 5'CAACTCAGTCTTGATAAATCCGGTACCTTTAGGGCCATC. All constructs were verified by sequence analysis. Functionality of STAT1dn was verified in IFN-stimulated 2ftGH cells (E. Pattyn, unpublished observations).

Transfection, leptin binding assay and reporter analysis

Transfections were performed by electroporation in PC12-1B8D cells. Co-transfections of pXP2d2- rPAP I reporter constructs were performed with pcDNA6/V5HISA-SEAP to judge transfection efficiency enabling normalization of data [30]. The SEAP gene was excised from pMET7-SEAP [30] by EcoRI/XhoI digestion and ligated into the constitutive expression vector pcDNA6/V5HISA similarly digested. Optimal ratio of reporter and SEAP vector was found to be 9/1. Since it also appeared from initial experiments that pools of stably transfected cells revealed a comparable pattern of promoter induction as transiently transfected cells, all subsequent experiments were performed under transient conditions. 48 hours after transfection, stimulations were performed for 24 hours with the effectors listed above or with combinations thereof. Unstimulated cells were included as a control. HEK293T cells were typically co-transfected with pMET7 vector carrying the leptin receptor long form, and the reporter and SEAP vector or beta-galactosidase expression vector (pUT651, Eurogentec, Seraing, Belgium) using a calcium phosphate precipitation procedure [32]. Luciferase and beta-galactosidase activity assays were performed as described [30, 32].

RESULTS

During previous investigations, we identified by using a subtractive cloning strategy (Representational Difference Analysis, RDA) rPAP I as a late target gene upon leptin stimulation of PC12-LR8 cells, which stably express the long isoform of the leptin receptor. Leptin combined with forskolin, an adenylate cyclase activator providing a net increase in cAMP concentration [34], led to a significant increase of the rPAP I induction compared to leptin alone. Forskolin alone did not affect expression of rPAP I which was also not detected in untreated cells [31].

To extend the search for genes regulated by leptin, we performed an RDA on differentiated PC12-LR8 cells treated with leptin for 24 hours or left untreated. Among leptin-upregulated genes, we identified rPAP III (data not shown). An additional RDA was performed to compare gene induction *via* the leptin receptor and the related gp130-subunit of the IL-6 receptor complex. Since PC12 cells do not express the IL-6Ralpha chain, we used the IL-6/sIL-6Ralpha complex to activate gp130. In non-differentiated PC12-LR8 cells, this RDA revealed rPAP I and Reg I as genes strongly upregulated through gp130 (data not shown). Since RDA is a random subtractive strategy, it is striking that several distinct members of the PAP/Reg family were identified. Combined with rPAP II which was amplified by RT-PCR, a panel encompassing four major members of the rPAP/Reg family became available for expression studies.

Northern-blot analysis on the induction characteristics of these PAP/Reg family members after treatment with leptin or IL-6 is shown in Figure 1. Induction of rPAP I by a 24 hours treatment of PC12-LR8 cells with IL-6/sIL-6Ralpha was far more pronounced than the moderate induction by leptin. Induction of rPAP II, rPAP III and Reg I was not detected after a 24 hours treatment with leptin. Compared to rPAP I, induction of rPAP II, rPAP III and Reg I by IL-6 was also significantly less. In all cases, untreated PC12-LR8 cells did not express any of the genes under investigation.

As can be seen in Figure 1, forskolin co-treatment led to a significant increase of leptin-dependent rPAP I induction as previously described [31] and also to a similar increase of rPAP III, whereas the rPAP II and Reg I induction is very limited. Remarkably, and in contrast to the co-stimulation with leptin treatment, we observed an inhibitory effect of forskolin on rPAP I, rPAP II, rPAP III and Reg I gene induction by IL-6.

To further examine the regulatory mechanisms underlying rPAP I gene induction by leptin and IL-6, we used the leptin responsive pXP2d2 luciferase reporter vector containing a 1254 nucleotide rPAP I promoter fragment [30]. Leptin-driven signaling to the rPAP I promoter was STAT3- but not STAT1-dependent, since overexpression of dn STAT3 significantly blocked reporter activity, compared to overexpression of WT STAT3 or to conditions of mock transfection. In contrast, overexpression of dn STAT1 did not down-modulate reporter-based luciferase activity (Figure 2). Similar data for STAT3dn and STAT1dn were also obtained for LIF-dependent rPAP I induction (data not shown).

A series of deletion mutants was prepared to map the cis-regulatory elements involved in rPAP I promoter stimulation (Figure 3). *Via* the MatInspector program, five potential binding sites for relevant cis-regulatory factors were predicted. The first deletion removed about half of the promoter sequence, but does not include such predicted regulatory sequences. By the second deletion, two potential CREB binding sites were removed. The third deletion eliminated two potential STAT binding sites also predicted before [17, 21]. The fourth and fifth deletion progressively eliminated the last putative CRE element and STAT interaction site respectively. Data obtained in a representative experiment using PC12 1B8D cells are shown in Figure 3. Similar data were gathered on other PC12 clones stably expressing lower numbers of the leptin receptor than PC12 1B8D cells, ruling out clonal variation in general and different receptor numbers in particular as relevant to the results obtained (data not shown).

Leptin as well as IL-6 activate the 1254 bp rPAP I promoter segment (Figure 3B, C). However, IL-6 appeared to be a much weaker inducer than leptin. The leptin-responsive promoter activity significantly decreased by the first deletion and was not further affected by the second deletion. Induction partially recovered after the third deletion, suggesting the loss of positively regulatory elements by the first deletion. Contrary to data obtained in PC12 cells, our co-transfection study in HEK293T cells revealed a doubling of the signaling activity after the first deletion (data not shown) indicating the critical role of (a) cell-specific factor(s) as shown before [25]. Leptin responsiveness ultimately decreased by deletion 4 and 5 leaving a 118 nt promoter fragment devoid of critical elements of promoter activity. The activity of the "full" and deleted rPAP I promoters showed an identical pattern when stimulation was performed with leptin (Figure 3B) compared to IL-6 (Figure 3C).

To elucidate the differentially co-regulatory effect of forskolin, we included forskolin stimulation in our progressive rPAP I promoter study (Figure 3). Forskolin alone is a weak inducer but showed strong co-stimulatory effects on induction by leptin in both PC12-1B8D and HEK293T cells (not shown). This synergistic effect persisted with every deletion mutant analyzed. Also, but in complete contrast to Northern-blot data (Figure 1), forskolin in combination with IL-6 provided significant co-stimulation. Similar data were obtained in forskolin/LIF co-stimulated HEK293T cells (data not shown).

DISCUSSION

The involvement of PAP/REG proteins in feeding and digestion physiology was apparent from earlier investigations [1, 2, 6, 18]. However, transcriptional regulation of this gene family by leptin was not studied before. Leptin is a main factor controlling feeding behaviour by its activity on hypothalamic centers, but can also directly act on pancreas and intestine. These effects depend on the expression of the long isoform of the leptin receptor and on rapid STAT activation [35-38]. PC12 cells expressing the leptin receptor were found to be a highly relevant system for studying leptin-induced genes [31].

In the past, it has repeatedly been stressed that the full-length leptin receptor has signalling capabilities similar to the IL-6 receptor and stimulates transcription through IL-6 responsive gene elements and hematopoietin receptor responsive gene elements [*e.g.* 39, 40]. Differences in intensity of transcriptional activation of rPAP/Reg genes by leptin compared to IL-6 as we noticed, may originate from differential STAT3 recruitment, since the leptin receptor contains a single STAT3 recruitment site compared to four sites in gp130. Furthermore, efficacy of recruitment might also differ between distinct motifs, as has been reported for the individual STAT3 binding motifs in gp130 [41, 42].

The regulatory mechanisms underlying rPAP I gene induction by leptin and IL-6 were further studied using a pXP2d2 luciferase reporter construct containing a 1,254 nucleotide rPAP I promoter fragment. This vector was preferred since it lacks two cryptic AP-1 sites in the plasmid backbone [33]. Using over-expression of dn STAT1 and dn STAT3, it was obvious that STAT3 and not STAT1 is involved in transcriptional activation of the rPAP I promoter.

From our functional studies involving progressive rPAP I promoter deletions, leptin appeared to be a much stronger inducer than IL-6, in contrast to Northern-blot data (Figure 1). This indicates that the cloned promoter fragment (nt - 1,253, + 1) lacks additional upstream regulatory sequences that primarily concern induction by IL-6. The leptin- and IL-6- responsive promoter activity is subject to similar changes throughout our promoter study, suggesting that similar cis-regulatory elements within the promoter segment analyzed are involved in induction by leptin and IL-6. At least one of these elements is not predicted by the MatInspector program, but has been noticed before [17]. It concerns a putative "IL-6RE" for high level induction of many acute phase genes at position -860 [17], *i.e.* within our first deletion, explaining the observed loss of promoter activity by the first deletion.

To elucidate further the differentially co-regulatory effect of forskolin in a leptin and IL-6 context, we also applied forskolin stimulation in our functional study involving progressive deletions of the rPAP I promoter. In contrast to Northern-blot data, forskolin in combination with IL-6 significantly provided co-stimulation, again underscoring the lack of a critical element(s) in the promoter fragment that are used solely by IL-6. This effect on a minimal promoter suggests that similar mechanisms function at an upstream level in the signaling cascade for both leptin and IL-6.

Leptin has been shown to suppress cAMP levels in pancreatic beta cells by activation of phosphodiesterase 3B and it has been suggested that PDE3B is also a mediator of leptin action in other tissues, *e.g.* the liver and adipose tissue [43]. Nevertheless, the use here - at high concentration - of forskolin, a direct stimulator of adenylate cyclase leading to constitutive activity, may overcome the cAMP lowering effect induced by leptin. The application of forskolin was optimized before in signaling experiments to the rPAP I promoter in PC12 cells [31]. The molecular mechanism(s) by which enhanced cAMP concentrations differentially co-regulate expression of this gene family through activated leptin- and IL-6 receptor complexes is (are) as yet unclear, but may rely on the balance of the same crucial factors that ultimately assist in differential expression *in vivo* in healthy and diseased conditions. In the case of IL-6 (but also of leptin), interference by forskolin might occur in several ways: (1) cAMP-dependent PKA activation may lead to phosphorylation of CREB (cAMP response element binding protein) and the precise arrangement of CRE (cAMP response elements) and (tandem) STAT binding sites in the promoter could in the end

determine the induction pattern. The effect could also be indirect, since activated CREB could induce c-fos [44] and therefore influence expression of genes with c-fos/c-jun (AP-1) consensus binding sites in their promoter [2]. Forskolin is critical for STAT3 expression itself, in view of a combined low affinity STAT3 binding and CRE site in the STAT3 promoter, which binds a complex of STAT3 and an unknown CRE-binding protein leading to full activation. Provided STAT3 availability in the cytosol is limiting, forskolin treatment could raise the level of cytosolic STAT3 [45]. (3) Forskolin suppresses in PC12 cells the early induction of SOCS-3 (suppressor of cytokine signalling 3) [31], a negative regulator of leptin- [46] and of IL-6-receptor activity [47, 48], enabling co-stimulatory effects of forskolin at later time points. This could explain the differential forskolin effect if SOCS-3 induction/recruitment would differ between activated leptin- and IL-6-receptor complexes [4]. A hitherto unknown mechanism may be involved in the interference by forskolin.

CONCLUSION

In conclusion, knowledge of the mechanisms by which leptin and IL-6 control expression of the multigene rPAP/Reg family may help to understand how these different family members exert their specific functions. We define a hitherto unknown regulatory element which is responsive to IL-6 only, and intriguingly, induction of the rPAP/Reg gene family by leptin or IL-6 appears to be subject to opposite effects by forskolin. This latter observation cannot be explained by the complex interplay of known regulatory proteins and cellular effectors at the core promoter level but implies an upstream regulatory event in the signaling cascade.

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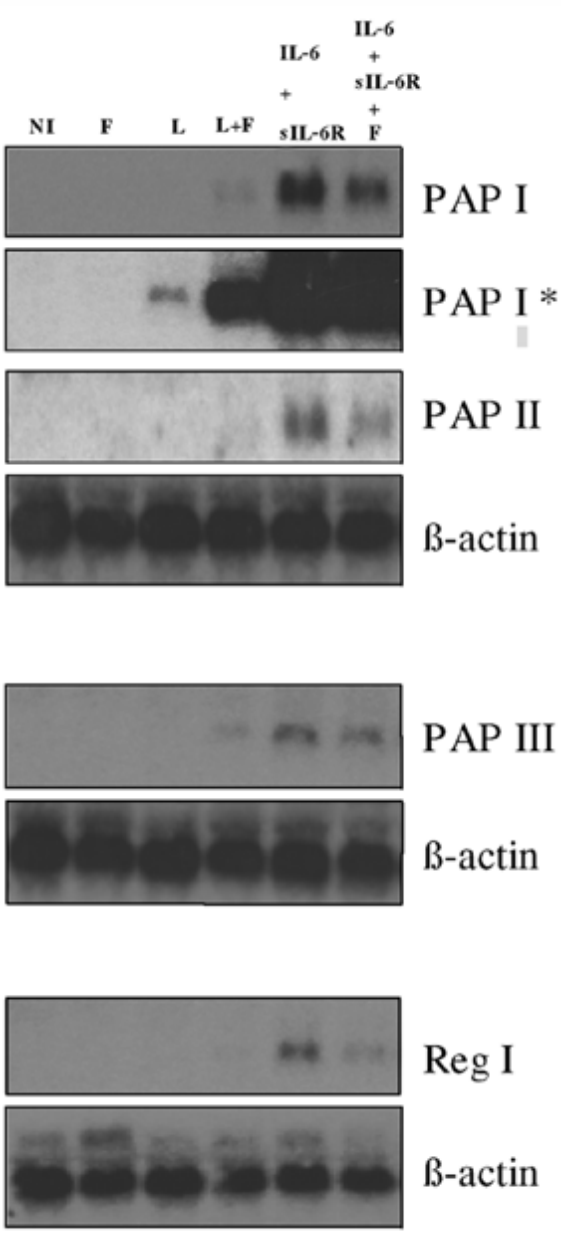


Figure 1

rPAP I, rPAP II, rPAP III and Reg I gene expression in leptin and IL-6 treated PC12-LR8 cells, and effect of forskolin co-treatment. PC12-LR8 cells were left untreated (non-induced: NI) or were treated for 24 hours with either forskolin (F: 10 muM), leptin (L: 100 ng/ml), IL-6 plus sIL-6Ralpha (290 U/ml and 0.5 mug/ml respectively), or with a combination of one of these cytokines and forskolin. Northern-blot analysis is shown and hybridization with a mouse beta-actin probe was performed as a control and is included below the respective blots. Exposure to BioMax MS film was 2 hours, 2 days, 16 hours and 16 hours for PAP I, PAP II, PAP III and

Reg I respectively. A longer exposure (2 days) of the PAP I lanes is also included: PAP I*. Exposure time for beta-actin was 1 hour in each case.

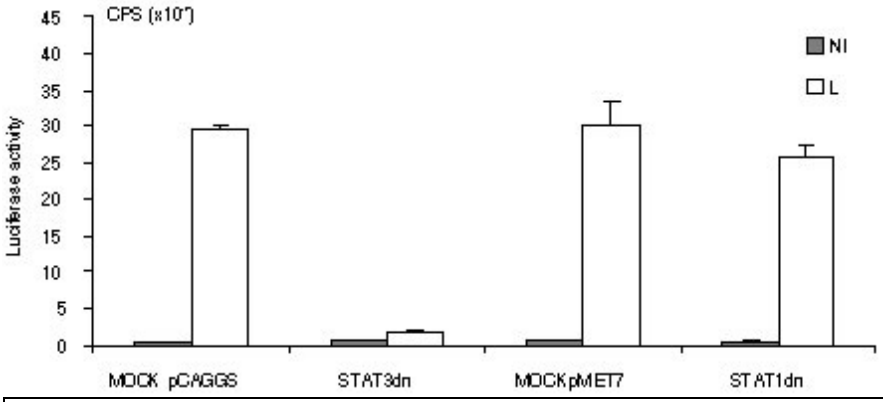


Figure 2

Effect of STAT3dn and STAT1dn overexpression on leptin stimulation of the rPAP I promoter. Typically, 2.5 x 10^5 HEK293T cells were transiently co-transfected with pMET7-LRlo (600 ng), the pXP2d2-rPAP I- luciferase reporter vector (150 ng), either the pCAGGS-STAT3dn vector (40 ng), the pMET7-STAT1dn vector (80 ng) or the corresponding empty vectors (mock transfection). Cells were treated after one day with leptin for 24 hours, or were left untreated (non-induced: NI). Luciferase activity data shown are the mean value and standard deviation of assays performed in triplicate. Co-transfection was performed with pUT651 (20 ng) enabling dosage of beta-galactosidase and normalization of data.

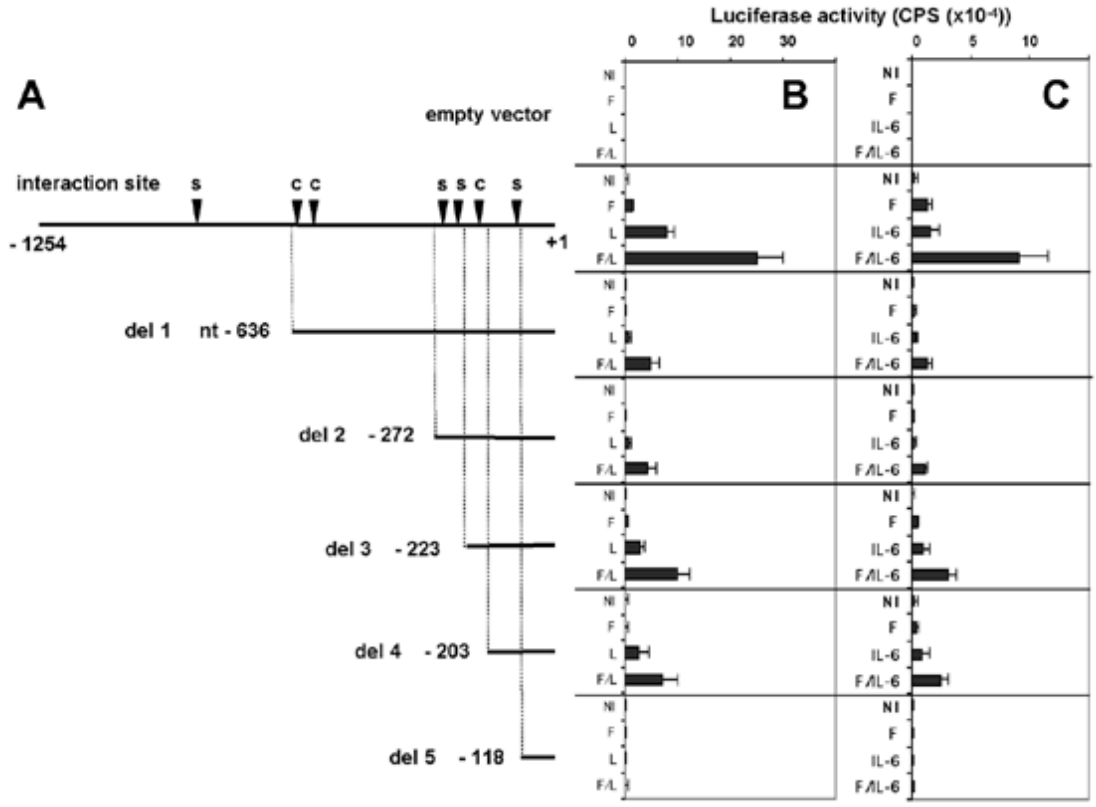


Figure 3

rPAP I promoter study. Panel **A:** diagrammatic presentation of the rPAP I promoter. Potentially regulatory sites and positions of the progressive deletions are indicated. Numbers refer to relative positions to the transcription initiation site. c: CREB interaction site, s: STAT interaction site. The respective positions are: s: nt - 860 (square), c: - 625 and - 576 (round), s: - 266 (round, square) and - 249 (square), c: - 210 and s: - 126 (round). (round): identified by the MatInspector program and (square): described by Dusetti *et al.* [17, 21]. Panels **B** and **C:** activity profiles of the rPAP I promoter and deletion mutants cloned in pXP2d2 and transfected in PC12-1B8D cells, upon differential stimulation with leptin and IL-6/sIL-6-Ralpha. PC12-1B8D cells were transiently transfected with cloned rPAP I promoter and deletion mutants in the pXP2d2 luciferase reporter vector and treated after two days with different effectors or combinations of effectors for one day, or were left untreated (non-induced: NI). The following treatments were tested: leptin (L), forskolin (F),

leptin + forskolin (F/L), IL-6 + sIL-6Ralpha (IL-6), IL-6 + sIL-6Ralpha + forskolin (F/IL-6). Twenty-four hours after treatment, cells were lysed and luciferase activity was measured. Data shown are the mean value and standard deviation of 8 assays. Co-transfection was performed with pcDNA6/V5HISA-SEAP to judge transfection efficiency and normalization of data was based on the SEAP activity. Panel **B** represents the effects of leptin and forskolin, panel **C** concerns the effects of IL-6 and forskolin.

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CSF-1, tumour progression, metastasis, de-differentiation.

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IL-1beta, TNF-alpha, GM-CSF, neuron, mRNA, protein.

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