The role of signal transducer and activator of transcription 5 in the inhibitory effects of GH on adipocyte differentiation

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Abstract

GH inhibits primary rat preadipocyte differentiation and expression of late genes required for terminal differentiation. Here we show that GH-mediated inhibition of fatty acid-binding protein aP2 gene expression correlates with the activation of the Janus kinase-2/signal transducer and activator of transcription (STAT)-5 signalling pathway. Within minutes of treatment, GH induced the tyrosine phosphorylation, nuclear localization and DNA binding of STAT5. Importantly, there was no evidence that STAT5 acted via an interaction with peroxisome proliferator-activated receptor γ . To further understand the mechanism of STAT5 action, we reconstituted the inhibition of aP2 in a non-adipogenic cell line. Using this system, we showed that the ability of GH to inhibit a 520 bp aP2 reporter was largely dependent upon the presence of either STAT5A or STAT5B. Mutant analysis confirmed that the tyrosine phosphorylation of STAT5 was essential for this signalling. However, STAT5's C-terminal transactivation domain was fully dispensable for this inhibition. Taken together, these data confirm a key regulatory role of STAT5 in adipose tissue and point to STAT5 as the repressing modulator of GH-mediated inhibition in primary preadipocytes.

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Introduction

Differentiation of preadipocytes into mature adipocytes is controlled by a well-established sequential cascade of transcriptional events (Christy *et al.* 1989, Freytag *et al.* 1994, Fajas *et al.* 1999, Rosen *et al.* 2000), for which the nuclear hormone receptor, peroxisome proliferator-activated receptor γ (PPAR γ) is a key regulator (Graves *et al.* 1992, Tontonoz *et al.* 1994a,b,c, Rosen *et al.* 1999, Wu *et al.* 1999). The transcriptional activity of PPAR γ is dependent both on heterodimerization with the retinoid X receptor α (RXR α) and on activation by either one of its natural ligands such as 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 or by high-affinity agonists of the anti-diabetic thiazolidinedione group of compounds (Forman *et al.* 1995,

Lehmann *et al.* 1995). Upon ligand binding, a number of transcriptional co-activators, such as the steroid receptor co-activator, PPAR γ -binding protein, thyroid hormone receptor-associated protein 220, and adenovirus E1A-associated protein p300/CREB-binding protein (CBP) are recruited to the PPAR γ /RXR α complex (Chakravarti *et al.* 1996, Kamei *et al.* 1996, Zhu *et al.* 1997, Gelman *et al.* 1999, Ge *et al.* 2002). Genes that are regulated by liganded PPAR γ include the fatty acid-binding protein aP2 (Tontonoz *et al.* 1994a,b), phosphoenolpyruvate carboxykinase (Tontonoz *et al.* 1995), and lipoprotein lipase (Schoonjans *et al.* 1996).

Growth hormone (GH) has been shown to play a critical role in regulating adipose tissue homeostasis *in vivo* (Donahue & Beamer 1993, Pomp *et al.* 1996,

Eisen *et al.* 1998, Ikeda *et al.* 1998, Lauterio *et al.* 1998, Frick *et al.* 2001). GH deficiency in humans is associated with obesity and increased adipocyte size and number, and treatment with GH restores adipose mass to normal levels (Salomon *et al.* 1989, Johannsson *et al.* 1997). Consistent with these observations, GH inhibits primary adipocyte differentiation *in vitro* (Wabitsch *et al.* 1996, Hansen *et al.* 1998). However, in other model systems, including 3T3-F442A, NIH-3T3 and 3T3-L1 cells, GH and the related hormone prolactin act as positive regulators of the adipogenic conversion (Morikawa *et al.* 1982, Clarkson *et al.* 1995, Xu *et al.* 1995, Nanbu-Wakao *et al.* 2000, 2002). Thus, considerable questions remain about the role and mechanism of action of GH in adipogenesis.

In this study we have focused our attention on establishing the role of GH-mediated inhibition of PPAR γ -activated transcription and adipocyte differentiation. We have previously demonstrated that GH does not affect expression of adipocyte determination and differentiation factor 1/sterol response element-binding protein 1 mRNA (Hansen *et al.* 1998). However, GH causes an approximately 50% reduction in the levels of PPAR γ mRNA and reduces the levels of aP2, fatty acid synthase and hormone-sensitive lipase mRNA by up to 80% (Hansen *et al.* 1998). Based on these findings, we propose that GH exerts its main inhibitory effect on genes required for terminal differentiation.

GH signalling has been shown to activate several members of the signal transducer and activator of transcription (STAT) family, including STAT1, 3, 5A and 5B (Meyer *et al.* 1994, Campbell *et al.* 1995, Gronowski *et al.* 1995, Smit *et al.* 1996). In particular, STAT5A and B are believed to be major downstream targets of GH (Wood *et al.* 1995, Smit *et al.* 1996, Teglund *et al.* 1998). Binding of GH to the GH receptor (GHR) results in receptor dimerization and rapid activation of the GHR-associated Janus kinase 2 (JAK2) leading to phosphorylation of both the GHR and JAK2 (Carter-Su *et al.* 1989, de Vos *et al.* 1992, Argetsinger *et al.* 1993). Upon these phosphorylation events, STAT5 binds to the GHR and is phosphorylated on tyrosine residues enabling it to dimerize with other phosphorylated STAT5 proteins via SH2 domains. The STAT5 dimer then translocates to the nucleus where it binds specific DNA elements. Once bound to DNA, the STAT5

dimer can mediate either gene activation or gene repression depending upon the promoter and/or cellular context (Galsgaard *et al.* 1996, Stocklin *et al.* 1996, Luo & Yulee 1997, Pfitzner *et al.* 1998, Zhou & Waxman 1999a,b, Luo & Yu-Lee 2000, reviewed in Ihle 2001).

STAT5 has been implicated in the regulation of adipogenesis in a variety of different model systems (Stephens *et al.* 1996, 1999, Yarwood *et al.* 1999, Harp *et al.* 2001, Nanbu-Wakao *et al.* 2000, 2002). Given this finding, we investigated the role of STAT5 in GH signalling in primary preadipocytes. In this report, we show that GH stimulates STAT5 signalling in primary preadipocytes. Moreover, the ability of GH to inhibit transcription of an aP2 reporter is STAT5-dependent and requires tyrosine phosphorylation but not downstream signalling by STAT5. These results suggest that GH-induced STAT5 signalling in primary preadipocytes represses transcription of genes required for terminal differentiation.

Materials and methods

Cell culture and differentiation

All reagents were purchased from GIBCO-BRL Life Technologies unless otherwise is noted. Primary preadipocytes were isolated as previously described (Hansen *et al.* 1998). For preparation of nuclear, cytoplasmic and whole cell extracts cells were seeded at a density of 1.25×10^6 cells per 92 mm dish. After 2 days of culture the medium was changed to differentiation medium: DMEM with 1000 mg/l D-glucose and Glutamax/Ham's F12 (1:1 v/v) supplemented with 15 mM NaHCO₃, 15 mM Hepes, 33 μ M biotin (Sigma), 17 μ M pantothenate (Sigma), 100 units/ml penicillin, 100 μ g/ml streptomycin (Pen/Strep), 2 mM glutamine, 10 μ g/ml transferrin (Sigma), 1 μ M insulin (Novo Nordisk A/S, Bagsvaerd, Denmark), and 200 pM triiodothyronine (T₃) (Sigma). The day of induction of differentiation was referred to as day 0. HEK 293 cells were cultured in DMEM with 4500 mg/l D-glucose and Glutamax, supplemented with 10% heat-inactivated fetal calf serum (FCS) and Pen/Strep. Primary preadipocytes and HEK 293 cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂ in air. For all GH stimulations, recombinant human

GH (Novo Nordisk) was used to a final concentration of 20 nM.

Northern blot analysis

Total RNA was isolated from primary preadipocytes using TRIzol Reagent (Invitrogen) according to the manufacturer's instruction. Twenty micrograms of RNA were fractionated on a denaturing gel containing 1% agarose, 20 mM Mops, 5 mM sodium acetate, 6% formaldehyde and 1 mM EDTA, transferred to a Hybond N+ membrane (Amersham Pharmacia Biotech), hybridized using Express Hyb (Clontech Laboratories, Inc., Palo Alto, CA, USA) according to manufacturer's instructions, and the results visualized by autoradiography.

Plasmids

The 520 bp aP2-chloramphenicol acetyl transferase (CAT) reporter construct was kindly provided by Dr B M Spiegelman (Dana-Farber Cancer Institute, Boston, MA, USA). It contains the PPAR γ /RXR-responsive adipocyte regulatory element 7 (ARE7) site linked to the region -63 to +21 containing a partially deleted CCAAT box, a TATA box and a transcriptional start site in the aP2. Plasmids encoding the human RXR α and PPAR γ were kindly provided by Dr J Fleckner (Novo Nordisk). The expression vector mouse STAT5A ^{Δ 749}, sheep STAT5A^{Y694F} and sheep MGF/STAT5A were kindly given by Dr Bernd Groner (Georg Speyer Haus, Frankfurt, Germany). Plasmids encoding human p300 and mouse CBP were kindly provided by Dr R Goodman (Vollum Institute, Portland, OR, USA). The rat GHR was kindly given by Dr G Norstedt (Karolinska Institute, Stockholm, Sweden). Expression vector encoding β -galactosidase, pCH110, was obtained from Pharmacia Inc.

Transient transfection assays, β -galactosidase and CAT assays

HEK 293 cells ($3-4 \times 10^5$) were seeded in 58 mm tissue culture dishes (Nunc, Roskilde, Denmark) in DMEM with high glucose supplemented with 10% heat-inactivated FCS and Pen/Strep. Prior to transfection media were replaced with DMEM

containing carbon-stripped FCS (2 g active carbon (Merck, Darmstadt, Germany)/50 ml FCS was incubated 2 h at 4 °C, followed by centrifugation and filtration). Cells were transfected using calcium phosphate as described previously (Chen & Okayama 1987) with the following amounts for each expression vector: 0.5 μ g 520 bp aP2-CAT, 0.5 μ g SV40- β -gal and in various combinations co-transfected with 1.0 μ g RXR α , 0.5 μ g PPAR γ , 0.5 μ g MGF-STAT5A, 1.0 μ g STAT5A ^{Δ 749}, 1.0 μ g STAT5A^{Y694F} and 2.25 μ g GHR. To ensure equal amounts of DNA, empty pcDNA3.1+ was added up to a total of 10 μ g. Following transfection cells were stimulated with 0.1 μ M BRL49653 (Novo Nordisk A/S) and cultured in the absence or presence of 20 nM GH. Cells were harvested approximately 36 h after transfection. All transfections were performed as duplicate experiments and repeated two to four times. The CAT assay was performed as previously described (Galsgaard *et al.* 1996). Twenty microlitres of each cell extract were mixed with 1 ml β -gal buffer supplemented with β -mercaptoethanol and O-nitrophenyl-beta-D-galactopyranoside (ONPG). The reaction was incubated at 37 °C for approximately 2 h. OD₄₂₀ was measured and used for normalization of transfection efficiency.

Nuclear, cytoplasmic and whole cell extracts

Primary preadipocytes were grown in 92 mm plates and isolated on approximately day 5 of differentiation. Cells were stimulated with 20 nM GH for 15 min prior to isolation, which was done as previously described (Hansen *et al.* 1998). The supernatant containing the cytoplasmic proteins was recovered and used in Western blot analysis. The nuclei were resuspended in hypertonic buffer containing 20 mM HEPES (pH 7.9), 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA and 400 mM NaCl, incubated for 30 min on a rocking bench at 4 °C and subsequently centrifuged at 20 000 *g* for 30 min at 4 °C. Extracts were frozen in liquid nitrogen and stored at -80 °C. Whole cell extracts were isolated from primary preadipocytes after 2 days of culture. Prior to isolation cells were stimulated with 20 nM GH as indicated on the Figure. Cells were washed in ice-cold PBS and lysed in 0.5 ml/90 mm dish PBS containing 1% NP40 and 0.1% SDS. The lysis buffer was supplemented with 1 mM dithiothreitol, 0.5 mM

4-(2-aminoethyl)-benzene sulphonyl fluoride hydrochloride, 1 mM Na₃VO₄ and protease inhibitors (1 µg/ml leupeptin and 1 µg/ml aprotinin) just before use. Cells were lysed on ice for 30 min and the whole cell extract recovered by centrifugation at 20 000 *g* for 30 min and stored at -80 °C. Protein concentrations were measured using Bio-Rad protein assay according to manufacturer's instructions (Bio-Rad Laboratories, Munich, Germany).

Immunoprecipitation

Whole cell extract from primary preadipocytes was incubated overnight at 4 °C with 2 µg anti-STAT5 (sc-835; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) followed by a 2 h incubation with protein G-Sepharose. Precipitates were washed three times in lysis buffer (PBS containing 1% NP40 and 0.1% SDS) and proteins were eluted with 1 × SDS sample buffer.

Gel shift assay

Gel shift assays were performed as previously described (Galsgaard *et al.* 1996, Hansen *et al.* 1998). Briefly, for PPARγ/RXRα DNA binding the ARE7 (5'-gatcTGTGAACTCTGATCCAGTAAG) (Tontoz *et al.* 1994b) was used and for STAT5 DNA binding the Spi2.1-GLE1 (5'-agctATGTTCTGAGAAAATC) (Hansen *et al.* 1997) was used. As non-specific competitors unlabelled M67 (5'-agctTCATTTCCTGTAATCCCTA), αCG (5'-agctTTTTACCATGAC-GTCAATTTGATC), or E/GRE (5'-agctATTAAGTGCATTGTTCTCGA) (Life Technologies) were used. All oligonucleotides were labelled with [α -³²P]dATP (Amersham Biosciences) in a fill-in reaction using DNA polymerase (Klenow fragment) (Promega). Three micrograms of nuclear extracts from primary preadipocytes were used in each reaction. For super shift analysis 2 µg of the indicated antibodies were used: anti-STAT5 (sc-835; Santa Cruz Biotechnology), anti-PPARγ (sc-1984, sc-7273; Santa Cruz Biotechnology) or anti-PPARγ (PA3-820, PA3-821; Affinity Bio-Reagents Inc., Golden, CO, USA). Free and bound probe were separated on a 6% DNA retardation gel (#EC63652; Invitrogen) by electrophoresis, dried and visualized by autoradiography.

Western blot analysis

For detection of GH-induced tyrosine phosphorylation of STAT5, proteins were separated by electrophoresis on a precast NuPAGE 4–12% Bis/Tris Gel (#NP0322; Invitrogen) with NuPAGE MOPS SDS running buffer (#NP0001; Invitrogen) using the Novex electrophoresis system and blotted to PVDF membranes (#LC2002) using a Novex blotting system (Invitrogen). For analysis of cellular localization of STAT5, proteins were separated by SDS-PAGE (4% stacking gel, 7.5% running gel) and transferred by electroblotting to ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were blocked for 1 h in 5% non-fat dry milk in PBST (PBS containing 0.1% Tween) and incubated with primary antibody overnight at 4 °C. Membranes were washed three times for 15 min in PBST and incubated with secondary horseradish peroxidase (HRP)-linked antibodies for 2 h at room temperature. Proteins were visualized using an ECL detection system (Amersham Pharmacia Biotech). Primary antibodies were used in following dilutions: anti-STAT5 (sc-835; Santa Cruz Biotechnology) 1:2000, phosphotyrosine-HRP (#61–5820; Zymed, South San Francisco, CA, USA) 1:1000. For control of equal amounts of protein the phosphotyrosine blot was re-probed with anti-STAT5 antibody.

Results

GH inhibits expression of aP2 mRNA

Our previous study has shown that GH inhibits adipogenic genes required for terminal differentiation of primary preadipocytes (Hansen *et al.* 1998). In order to demonstrate the effect of GH on aP2 mRNA expression we performed Northern blot analysis analysing total RNA from primary preadipocytes differentiated in the absence or presence of GH. Consistent with previously reports (Hansen *et al.* 1998), GH down-regulated expression of aP2 mRNA (Fig. 1). We found that primary preadipocytes kept in a serum-free medium only increased expression of aP2 modestly whereas cells that were induced to differentiate using insulin and thyroid hormone increased aP2 expression 23-fold from day 0 to day 7 (Fig. 1). On day 7 of differentiation, aP2 expression was reduced from 23-fold to 5-fold in cells that had

Figure 1 Effect of GH on aP2 mRNA expression. Northern blot analysis with total RNA (20 µg per lane) from primary rat preadipocytes cultured either in serum-free media (SF), differentiated in serum-free media supplemented with 1 µM insulin and 15 pm MT₃ (Diff) or differentiated in the presence of 20 nM GH (Diff +GH). RNA was isolated on day 0, 3 and 7 for differentiation. (A) Blots were hybridized with specific radiolabelled cDNA fragments for aP2 (top panel), and 36B4 (lower panel). (B) Amounts of mRNA were normalized to the expression levels of 36B4 mRNA and quantification of aP2 mRNA is illustrated as fold induction as a function of days of differentiation ($n=3$).

been differentiated in the presence of GH, which was even lower than the expression found under basal serum-free conditions over the same time course.

GH activates STAT5 signalling in primary preadipocytes

STAT5 is known to be an important downstream target of GH (Teglund *et al.* 1998) and has also been implicated in the regulation of adipogenesis. We therefore investigated whether GH activates STAT5 signalling in primary preadipocytes (Fig. 2). In order to do this, we analysed GH-activated tyrosine phosphorylation of STAT5 by stimulating primary preadipocytes with GH for 0, 10, 20 and 40 min (Fig. 2A). No tyrosine phosphorylation of STAT5 was observed in the absence of GH.

However, after 10 min of stimulation STAT5 was activated as identified by anti-phosphotyrosine Western blot (Fig. 2A, lane 2). Activation by GH was transient and decreased rapidly. After 40 min of stimulation only a weak band was observed (Fig. 2A).

To determine if GH activation also led to nuclear translocation of STAT5, we isolated nuclear and cytoplasmic extracts from primary preadipocytes cultured in either the presence or absence of GH for 10 min (Fig. 2B). In agreement with the phosphorylation pattern, we detected only small amounts of STAT5 in the nuclei from non-stimulated cells (Fig. 2B, lane 1) whereas the intensity of the STAT5 band in nuclear extracts was increased dramatically upon stimulation with GH (Fig. 2B, lane 2), indicating that STAT5 translocated to the nucleus in response to GH. In correlation with this, cytoplasmic extracts isolated from non-stimulated cells showed a relatively higher amount of STAT5 (Fig. 2B, lane 3) than cytoplasmic extracts from cells that had been stimulated with GH (Fig. 2B, lane 4). The appearance of STAT5 as a doublet in Fig. 2B might reflect the presence of both STAT5A and STAT5B in the extracts, although specific antibodies would be required to resolve this issue. The lack of a doublet in Fig. 2A could be caused by the longer exposure of this blot or the fact that a slightly different SDS-PAGE system with higher resolution was used for the blot shown in Fig. 2B.

Given that we observed phosphorylation and nuclear translocation of STAT5 in response to GH, we next examined whether GH stimulated DNA binding of STAT5 (Fig. 2C). As shown, incubation of nuclear extracts, isolated from primary preadipocytes stimulated with GH for 10 min, with an oligonucleotide containing a STAT5 response element resulted in a protein–DNA complex (Fig. 2C, lane 3). The specificity of the complex was demonstrated by competition with increasing amounts of either cold specific oligonucleotide Spi2·1-GLE or non-specific oligonucleotide (αCG) (Fig. 2C, lanes 4 and 5). The presence of STAT5 in the retarded complex was further confirmed upon pre-incubation of nuclear extracts with anti-STAT5 antibody, resulting in a super-shifted band (Fig. 2C, lane 8).

It has been previously been shown that prolactin-induced STAT5A can act as a functional repressor of glucocorticoid receptor (GR)-activated

Figure 2 GH activates STAT5 signalling in primary preadipocytes. (A) Western blot analysis of whole cell extract from primary rat preadipocytes stimulated with GH for 0, 10, 20 and 40 min. STAT5 was subsequently immunoprecipitated with anti-STAT5 antibody overnight at 4 °C. The membrane was incubated with an anti-phosphor-tyrosine antibody and subsequently re-probed with an anti-STAT5A/B antibody to verify equal STAT5 protein in each lane. (B) Western blot analysis of nuclear extracts and cytoplasmic extracts from primary rat preadipocytes with an anti-STAT5A/B antibody. Primary preadipocytes were either not stimulated (lanes 1 and 3) or stimulated for 10 min with 20 nM GH (lanes 2 and 4) prior to isolation of nuclear extracts and cytoplasmic extracts. Figures represent the results of two independent experiments. Gel shift analysis of STAT5 and PPAR γ binding to radiolabelled Spi-GLE (C) or ARE7 (D) oligonucleotides. Probes were incubated in either the absence (C and D, lane 1) or presence of 3 μ g nuclear extracts from primary rat preadipocytes not stimulated (C and D, lane 2) or stimulated 10 min with 20 nM GH (C and D, lanes 3–9). Unlabelled specific oligonucleotide was included in the incubation time at 100 $\times$ and 1000 $\times$ molar excess (C and D, lanes 4 and 5) and likewise for non-specific oligonucleotide (C and D, lanes 6 and 7). Nuclear extracts were pre-incubated for 30 min at 4 °C with antibodies as indicated above the gels. Figures represent the results of three independent experiments.

transcription by direct interaction with the GR (Stocklin *et al.* 1996). Given this finding, we examined whether PPAR γ was present in the STAT5–DNA complex. Initially, we pre-incubated the nuclear extract with an anti-PPAR γ antibody but this had no detected effect on the mobility of the protein–DNA complex (Fig. 2C, lane 9). To further test whether GH stimulated direct complex formation between STAT5 and PPAR γ , we also tested the nuclear extracts for their ability to bind to the PPAR γ -responsive element, ARE7 (Fig. 2D). As expected, we detected significant binding activity in the differentiating preadipocytes (Fig.

2D, lane 2). However, the intensity and mobility of the band was not altered in the presence of GH (Fig. 2D, lanes 3, 6 and 7). The specificity of the DNA–PPAR γ complex was demonstrated by competition with either cold specific ARE7 oligonucleotide or unspecific oligonucleotide (E/GCR) (Fig. 2D, lanes 4–7). Furthermore, pre-incubation of nuclear extracts with an antibody against PPAR γ super-shifted the specific band, indicating the presence of PPAR γ in this complex (Fig. 2D, lane 8). Most importantly, pre-incubation of nuclear extracts with anti-STAT5 antibody did not change the mobility of the PPAR γ complex,

indicating that STAT5 is not present together with PPAR γ in the protein–DNA complex (Fig. 2D, lane 9). Taken together, these data demonstrate that GH activates STAT5 signalling in primary preadipocytes and induces STAT5 DNA binding; however, no interaction between STAT5 and PPAR γ was detected.

GH inhibits PPAR γ -activation of the aP2 promoter in a STAT5-dependent manner

Our results with primary preadipocytes showed that STAT5 is regulated in primary preadipocytes in response to GH. In order to determine whether STAT5 is required for GH-mediated inhibition of the aP2 promoter, we used HEK 293 cells transiently transfected with the 520 bp aP2-CAT reporter construct containing the PPAR γ /RXR α -binding site ARE7 (Graves *et al.* 1991). To validate the HEK 293 cells as a model system for aP2 promoter regulation, we transiently transfected the HEK 293 cells with the 520 bp aP2-CAT reporter either alone or together with PPAR γ 2 and RXR α in the presence of BRL49653. Co-transfection of PPAR γ and RXR α and simultaneous stimulation with BRL49653 led to a 15-fold increase in transcriptional activity (Fig. 3A). To reconstitute the STAT5 signalling pathway we transiently transfected HEK 293 cells with expression vectors encoding the GHR and STAT5A, together with PPAR γ 2, RXR α and the 520 bp aP2-CAT reporter (Fig. 3A). Expression of GHR together with PPAR γ 2/RXR α and BRL49653 led to an additional increase in CAT activity from 15-fold to 20-fold (Fig. 3A, bar 3), which was reduced to 6-fold in the presence of GH (Fig. 3A, bar 4). The transcriptional activity measured in HEK 293 cells transfected with the 520 bp aP2-CAT reporter, PPAR γ 2, RXR α , GHR and STAT5A (Fig. 3A, bar 3) was arbitrarily set to 100% (Fig. 3B, bar 1). In the absence of STAT5A or STAT5B stimulation by GH had no effect on the 520 bp aP2 promoter. Upon co-transfection with STAT5A or STAT5B and simultaneous stimulation with 20 nM GH, the relative CAT activity was decreased from 100% to ~ 40% for STAT5A and to 30% for STAT5B (Fig. 3B, bars 4 and 6). We next tested if STAT1 or/and STAT3 could mediate inhibition by GH. However, neither expression of STAT1 or STAT3, nor of both STAT1 and STAT3 combined, led to a reduction in the transcriptional activity (data not

shown), suggesting that GH inhibits aP2 transcription through a mechanism that requires specifically the presence of STAT5A or STAT5B.

Having established that GH-mediated inhibition of the 520 bp aP2 promoter is STAT5-dependent, we were able to use this model system to investigate the potential mechanisms of repression (Fig. 3C). First, the importance of tyrosine residue Y694 in STAT5A was analysed. This residue is phosphorylated in response to GH and required for dimerization of STAT5 proteins and hence translocation to the nucleus (Gouilleux *et al.* 1994, Smit *et al.* 1997). To that end, HEK 293 cells were transfected with the 520 bp aP2-CAT reporter construct together with PPAR γ 2, RXR α , GHR and either wild-type STAT5A (STAT5A^{wt}) (Fig. 3C, bars 1 and 2) or STAT5A^{Y694F} (Fig. 3C, bars 3 and 4) in the absence or presence of 20 nM GH (Fig. 3C, bars 2 and 4). STAT5A^{Y694F} was unable to mediate the inhibitory effect of GH (Fig. 3C, bar 4), indicating that tyrosine phosphorylation is essential for GH-mediated inhibition via STAT5A.

We also wished to establish whether inhibition of aP2 transcription required STAT5's transcriptional activity. To address this issue, we utilized a dominant negative C-terminal truncated mutant, STAT5A ^{Δ 749}, in which the transactivating domain had been deleted (Fig. 3C, bars 5 and 6). This STAT5 mutant is able to dimerize with STAT5A^{wt}, translocate to the nucleus and bind DNA but it cannot activate transcription. Surprisingly, addition of GH in context with the truncated STAT5A ^{Δ 749} resulted in 62% inhibition of CAT activity (Fig. 3C, bar 6) relative to non-stimulated cells transfected with STAT5A ^{Δ 749} (Fig. 3C, bar 5). Together, these data suggest that GH down-regulates the aP2 promoter activity through a mechanism that requires the presence of STAT5. Moreover, activation of STAT5 by tyrosine phosphorylation is needed but downstream activation of transcription by STAT5 is not required for the inhibitory effect of GH.

Increasing concentrations of p300 partly reverse the inhibitory effect of GH

STAT5 has been shown to repress transcription through a variety of different mechanisms depending upon both the promoter and cellular context. In the case of STAT5-mediated inhibition of

nuclear factor- κ B (NF- κ B) signalling this repression can be overridden by increasing concentrations of the co-activator, p300 (Luo & Yu-Lee 2000). Given this finding, we tested whether expression of p300 could rescue PPAR γ -activated transcription of the 520 bp aP2-CAT reporter (Fig. 4). Cells were co-transfected with PPAR γ , RXR α , GHR and STAT5A, stimulated with BRL49653 and treated with GH together with increasing concentrations of p300. Increasing the concentration of p300 from 0 to 1.0 μ g reduced the inhibitory effect of GH from 60 to 30% (Fig. 4, bar 5). Increasing the concentration of p300 from 1.0 to 5.0 μ g did not reduce the effect of GH any further (data not shown). This result shows that p300 can partially suppress the inhibitory effect of GH in a dose-dependent manner.

Figure 3 Effects of GH on the aP2 520 bp promoter. HEK 293 cells were transiently transfected with 0.5 μ g of the internal standard (SV40- β -gal) and 0.5 μ g of the 520 bp aP2-CAT reporter gene. Co-expression of cDNA encoding PPAR γ (0.5 μ g), RXR α (1.0 μ g), GHR (2.25 μ g), various STAT5 expression vectors and stimulation of cells with BRL49653 (0.1 μ M) and/or GH (20 nM) are specified below graphs. Cells were cultured approximately 36 h before harvest. Transfections were done in duplicates and CAT activity was normalized to β -galactosidase activities. Error bars indicate s.d. (A) Cells were either transfected with the 520 bp aP2-CAT reporter alone (bar 1) or in the presence of PPAR γ , RXR α or BRL49653 in the absence (bar 2) or presence of GHR, STAT5A (0.5 μ g) (bars 3 and 4) and treated with GH (bar 4). CAT activities are presented as fold induction of transcriptional activity relative to 1 for cells transfected with the 520 bp aP2-CAT reporter alone ($n=8$). (B) Cells transfected with 520 bp aP2-CAT, PPAR γ , RXR α or GHR and stimulated with BRL49653 were either not treated (bar 1) or treated with GH (bar 2). Cells were co-transfected with either STAT5A (0.5 μ g) (bars 3 and 4) or with STAT5B (0.5 μ g) (bars 5 and 6) in the absence or presence of GH (bars 4 and 6). CAT activities are presented as percent transcriptional activity relative to 100% for cells transfected with the 520 bp aP2-CAT reporter, PPAR γ , RXR α , GHR, indicated STAT5 cDNA and stimulated with BRL49653 ($n=3$). (C) Cells were co-transfected with STAT5A^{wt} in the absence or presence of 20 nM GH (bars 1 and 2) (representing bars 5 and 6 of (B)) or co-transfected with the mutants STAT5A^{Y694F} (1.0 μ g) (bars 3 and 4) or STAT5A ^{Δ 749} (1.0 μ g) (bars 5 and 6) and cells were either not treated or treated with GH (bars 4 and 6). CAT activities are presented as percent transcriptional activity relative to 100% for cells transfected with the 520 bp aP2-CAT reporter, PPAR γ , RXR α , GHR or STAT5A and stimulated with BRL49653 ($n=4$).

Figure 4 Increasing concentrations of p300 partly reverse the inhibitory effect of GH. HEK 293 cells were transiently transfected with 0.5 µg of the internal standard (SV40-β-gal) and the 520 bp aP2-CAT reporter (0.5 µg), PPARγ (0.5 µg), RXRα (1.0 µg), GHR (2.25 µg) and STAT5A (0.5 µg), stimulated with 0.1 µM BRL49653 and either not treated or treated with 20 nM GH. Cells were co-transfected with increasing concentrations of p300 as indicated. Transfections were done in duplicates and cells were cultured approximately 36 h before harvest. CAT activities are presented as percent transcriptional activity relative to 100% for cells transfected with the 520 bp aP2-CAT reporter, PPARγ, RXRα, GHR, STAT5A and stimulated with BRL49653 ($n=3$). Error bars indicate s.d.

Discussion

GH has been shown to play a critical role in regulating adipose tissue homeostasis *in vivo* (Donahue & Beamer 1993, Pomp *et al.* 1996, Eisen *et al.* 1998, Ikeda *et al.* 1998, Lauterio *et al.* 1998, Frick *et al.* 2001). We have previously shown that GH inhibits the differentiation of primary adipocytes and that this effect is primarily exerted through the repression of genes required for terminal differentiation (Hansen *et al.* 1998). Although other growth factors such as epidermal growth factor and platelet-derived growth factor have been shown to antagonize PPARγ-activated adipogenesis via a MAP kinase-dependent mechanism (Adams *et al.* 1997, Font *et al.* 1997), the inhibition of primary adipocytes by GH does not require the MAP kinase pathway (Hansen *et al.* 1998). In this study, we provide evidence to suggest that the inhibitory effect of GH is mediated via STAT5. First, we show that GH induces tyrosine phosphorylation, nuclear translocation and DNA binding of STAT5 in primary preadipocytes. Secondly, we show that GH-activated STAT5

serves as a repressor of the PPARγ-stimulated 520 bp aP2 promoter activity. In fact, the inhibitory effect of GH was found to be dependent on the presence of STAT5.

There is now considerable evidence that STAT5 can function as a transcriptional repressor. This includes inhibition of NF-κB (Luo & Yu-Lee 2000), GR (Stocklin *et al.* 1996) and PPARα, PPARδ and PPARγ (Zhou & Waxman 1999a,b). Although tyrosine phosphorylation of STAT5 appears to be essential for its activity, there does not appear to be a unifying mechanism of downstream signalling. For example, depending upon the target, repression may or may not require either the STAT5 transactivation domain or a direct interaction between STAT5 and the transcription factor it is repressing (Stocklin *et al.* 1996, Luo & Yu-Lee 1997). Given this heterogeneity, we also investigated the mechanism by which STAT5 mediates GH-dependent repression of aP2. First, we showed that mutation of tyrosine 694 was sufficient to abolish STAT5 function. Thus, consistent with the previous studies (Luo & Yulee 1997, Pfitzner *et al.* 1998, Zhou & Waxman 1999a), tyrosine phosphorylation and presumably nuclear localization of STAT5 is essential for its repressive activity. Secondly, we tested whether STAT5 might form a direct complex with PPARγ, the presumed target of this repressive effect. Although we used two different approaches, we were unable to detect any evidence for an interaction between GH-induced STAT5 and PPARγ. Clearly, we cannot rule out the possibility that this complex exists *in vivo* but is destroyed during the preparation of nuclear extracts. However, this finding is entirely consistent with the previous observations that STAT5 represses the ability of PPARα to activate transcription in transient transfection assays without any evidence of direct interaction (Zhou & Waxman 1999a,b). Thus, in contrast to the direct binding and repression of GR, STAT5 appears to inhibit PPARγ through an indirect mechanism, more analogous to that reported for NF-κB (Luo & Yu-Lee 2000).

To further establish the mechanism of inhibition, we determined how this related to STAT5's transcriptional properties. Consistent with the transfection studies of Zhou & Waxman (1999a), we found that a C-terminal truncated STAT5A^{Δ749} lacking the transactivation domain was able to mediate the GH-dependent inhibition

of our aP2 reporter. Hence, transactivation by STAT5A and thereby downstream gene expression induced by STAT5A is fully dispensable for the inhibitory effect of GH. Given this finding, we were surprised to find that overexpression of p300 can partially suppress, although not completely block, STAT5's inhibitory effect. It is entirely possible that p300 is acting in an indirect manner to increase global transcription and therefore to increase the activity of PPAR γ . Alternatively, since both the N-terminal and C-terminal domains of STAT1 have been shown to interact with CBP/p300 (Zhang *et al.* 1996), it is possible that STAT5 is acting to repress transcription by competing with the limiting pools of CBP/p300 or other transcriptional co-activators. This would explain how the STAT5A Δ^{749} could affect PPAR γ -mediated transcription by sequestering transcriptional co-activators. If indeed the N-terminal domain of STAT5 is able to interact with CBP/p300, as is the case for STAT1, this could cause the inhibition of aP2 transcription in response to GH. Importantly, since we were unable to completely override the effect of STAT5 by p300 overexpression, STAT5's repressive properties must be at least partially independent of p300 binding. Clearly, this could be through the sequestration of some other, limiting co-activator or through an unrelated mechanism. The inhibitory effect of GH on the differentiation of primary rat preadipocytes is in striking contrast to the finding that GH acts a positive regulator in the adipogenic conversion of the 3T3-F442A and NIH-3T3 mouse cell lines (Morikawa *et al.* 1982, Clarkson *et al.* 1995). Interestingly, there is considerable evidence suggesting that STAT5 acts in a positive manner in these adipogenic cell line systems. First, the JAK2/STAT5 signalling pathway is required for GH-dependent differentiation of 3T3-F442A preadipocytes (Yarwood *et al.* 1999). Secondly, STAT5 mediates adipocyte differentiation of NIH-3T3 and 3T3-L1 cells upon stimulation by the GH-related cytokine prolactin (Nanbu-Wakao *et al.* 2000, 2002). Finally, STAT5 expression is induced in a PPAR γ ligand-dependent manner and seems to be tightly linked to the adipogenic conversion of 3T3-L1 cells (Stephens *et al.* 1996, 1999). Combined with our findings in primary preadipocytes, these observations suggest that STAT5 plays an important role in mediating the effects of GH on adipocyte differentiation.

Moreover it raises the question as to whether cytokine-induced STAT5 affects adipogenesis differently depending on species and the stage of adipocyte differentiation. Importantly, it was recently shown that antagonism of PPAR γ activity ameliorated high fat diet-induced obesity and insulin resistance (Yamauchi *et al.* 2001). A better understanding of the mechanisms behind functional antagonism of PPAR γ consequently has important therapeutic potential for treatment of obesity and related metabolic syndromes.

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