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Research Article

Peroxisome proliferator-activated receptor gamma regulates expression of signal transducer and activator of transcription 5A

Hanne Olsen[1], *Lars-Arne Haldosén**

Department of Medical Nutrition, Karolinska Institutet, NOVUM, S-141 86 Huddinge, Sweden

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ABSTRACT

Signal transducer and activator of transcription 5A (STAT5A) has been shown to be important for terminal differentiation of mammary epithelial cells. In order to understand regulation of expression of STAT5A, the 5′ end of the mouse *Stat5a* gene was isolated. Putative regulatory elements was searched for and several peroxisome proliferator response elements (PPREs) were found, one with high (12/13 nucleotides) and three with less (8–10/13) similarity to the reported consensus sequence. Mouse mammary epithelial HC11 cells were treated with peroxisome proliferator-activated receptor γ (PPARγ) ligand, the thiazolidinedione (TZD) troglitazone, and an increase in STAT5A protein expression was seen. The 5′ flank of *Stat5a* gene was cloned in a luciferase reporter vector. A concentration dependent activation of the STAT5A-luciferase reporter was detected, when transiently transfected HC11 cells were treated with TZD. The activation could be inhibited by treatment with a PPARγ antagonist. It has earlier been shown that epidermal growth factor (EGF) induces MAPK phosphorylation of PPARγ resulting in a less transcriptionally active receptor. In HC11 cells, EGF inhibited TZD induced STAT5A-reporter activity suggesting that our previously reported EGF-mediated suppression of STAT5A expression is mediated in all or partly through inhibition of PPARγ activity. Furthermore, the MEK inhibitor PD98059 inhibited the EGF effect. All together, data presented suggest that PPARγ participates in regulation of STAT5A expression.

Introduction

PPARγ is a transcription factor belonging to the nuclear receptor superfamily. This receptor stimulates transcription after activation by ligand and binding to peroxisome proliferator response elements (PPREs) in promoters of target genes [1]. PPARγ is expressed in different cell types such as adipocytes, mammary epithelial cells, ovary, macrophages, B- and T-cells. Ligands for PPARγ are polyunsaturated fatty acids, eicosanoids, and the antidiabetic thiazolidinedione (TZD) drugs [2]. PPARγ has an antiproliferative effect in pre-adipocytes and mammary epithelial cells and the PPARγ pathway can induce terminal differentiation of human breast cancer cells [3–5].

Development of mammary gland occurs through growth and differentiation during fetal, pre-pubertal, and pubertal

* *Corresponding author*. Fax: +46 8 711 66 59.
E-mail address: Lars-Arne.Haldosen@mednut.ki.se (L.-A. Haldosén).
[1] Present address: Department of Medical Biochemistry and Genetics B, Panum Institute, Blegdamsvej 3b, DK-2200 Copenhagen N, Denmark.

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stage [6–8]. The mammary gland in an adult animal goes through cycles of growth, differentiation and cell death during menstrual cycle, pregnancy and lactation. All of these processes occur under the influence of a number of steroid hormones, e.g., estrogen and progesterone, polypeptide hormones, e.g., prolactin, and growth factors, e.g., epidermal growth factor (EGF), as well as interaction between cells and the extracellular matrix. Terminal differentiation of epithelial cells during late pregnancy and lactation are accompanied by production and secretion of milk proteins. Regulation of expression of the milk proteins: β-casein, whey acidic protein, and β-lactoglobulin has been extensively studied and has given important insight into intracellular signaling pathways and gene regulation in mammary epithelial cells [9]. This has resulted in the identification of a number of transcription factors being important for differentiation and milk protein expression, e.g., signal transducer and activator of transcription 5 (STAT5), glucocorticoid receptor, nuclear factor 1 (NF1), CCAAT-enhancer-binding protein (C/EBP), Ying and Yang 1, and others less characterized [10].

STAT5 was first described as a prolactin (PRL)-activated transcription factor expressed in mammary gland and important for expression of milk protein genes [11]. It is however ubiquitously expressed and activated by a number of cytokines. Two forms of STAT5 are described, STAT5A and STAT5B, which are transcribed from two separate genes. They are 95% similar at the amino acid level [12,13]. Both STAT5A and STAT5B are expressed throughout mammary gland development, with the highest protein levels seen at late pregnancy and lactation [14]. Experimental studies have shown that STAT5A and STAT5B have overlapping and non-overlapping functions with regard to the mammary gland. *Stat5a* gene deletion studies have shown the importance of STAT5A for terminal differentiation of the mammary gland. STAT5A-deficient mice were unable to lactate due to curtailed lobuloalveolar outgrowth [15]. This mammary phenotype was not seen in STAT5B-deficient mice, they were able to lactate, but could not produce enough milk to feed their pups [16]. Furthermore, both STAT5A- and STAT5B-deficient mice had somewhat reduced epididymal fat pads, while the fat pad of the double knockout was only one fifth of wild type [17]. STAT5A and STAT5B are induced during murine and human adipogenesis [18,19]. Transfection of STAT5A in preadipocytes and nonprecursor cells has been shown to increase the expression of adipogenic markers [20–22]. It has also been shown that expression of STAT5 increases during PPARγ ligand-induced adipogenesis [23].

The mouse mammary epithelial cell line HC11, has been shown to be a good model system for studying mammary cell differentiation and gene regulatory mechanisms taking place in mammary cells [24,25]. We have earlier described the expression of STAT5A and STAT5B in HC11 cells during proliferation and differentiation [26]. The level of STAT5B protein is similar to STAT5A in proliferating HC11 cells, but lower in differentiated cells. STAT5A expression increases as the cells start to differentiate due to the relief of EGF signaling through the ras/raf/MEK/MAPK pathway and PI3-kinase, which results in significantly higher levels of STAT5A as compared to STAT5B.

In the present work, we have continued to study the regulation of STAT5A expression. The 5′ end of the *Stat5a* gene was cloned and analyzed in luciferase reporter assays. Peroxisome proliferator-activated receptor γ (PPARγ) was found to regulate expression of STAT5A-reporter through several identified peroxisome proliferator response elements (PPRE). PPARγ ligand was also found to induce endogenous STAT5A protein expression in HC11 cells as detected by Western blot. Furthermore, the suppressive effect of EGF signaling through the MAPK pathway on STAT5 expression during proliferation was suggested to involve repression of PPARγ transcriptional activity.

Materials and methods

Cloning of 5′ end of STAT5A gene and plasmid construction

The 5′ end of *Stat5a* gene was cloned using the Genomewalker kit (Clontech) according to manufacturer's manual. 1.6 kb (–1103/+552) was cloned from the DraI library using primers for intron 1 in the mouse *Stat5a* sequence (Genbank Acc. No. NT039521), GSP1 5′GTGTGTGTACACGCGCATGTTCAGGTAA and GSP2 5′ATGTGGCTTCTATGCCTGCAGAGTCAA. 2.5 kb (–3338/–915) was cloned from the Pvu library using primers GSP1 5′ ACTTCCAGGCATTGACTTCTCCAGA and GSP2 5′ GAAGGTGGTTAGGCCTGCTTACTCAAAA. The PCR products were subcloned using TOPO-TA cloning (Invitrogen) and sequenced using BigDye terminators. The 1.6 kb and 2.5 kb PCR products were joined together by using a *Nhe*I restriction site present in the 5′ and 3′ ends of 1.6 kb and 2.5 kb PCR products, respectively, resulting in an approximately 3.9 kb fragment. The 1.6 kb (–1103/+552) and 3.9 kb (–3338/+552) fragments were cloned into pGL3Basic (Promega) for reporter analysis using the luciferase system. Deletion constructs were produced by PCR using the following primers, for (–531/+552), 5′ GCATGTTGATGTTCATCTG and 5′ GCTTATGTGGCTTCTATG; for (–246/+552), 5′ GCAGAAAAGGTTCAGGTT and 5′ GCTTATGTGGCTTCTATG. PPRE1 (–2154), PPRE3 (–390) and PPRE4 (+33) was mutated using QuikChange XL Site-Directed Mutagenesis kit (Stratagene) according to manufacturer's manual with the following primer; PPRE1 5′ GCACACGCACAGGTGGTGTCATGAGGTCAACCTCAGGG; PPRE3 5′ GGCAGAGCATCTGAAGAGCTTGTTATCTCCCATCCTCTGATCCTC and PPRE4 5′ CAGAGGAGAGGGAAGCTGTGATAAGGGGATGGCAGACTTC. The numbering of nucleotides are based on mouse est (GenBank Acc. No. BY344844) and mouse STAT5A mRNA (GenBank Acc. No U21103).

Cell culture and transfection

HC11 cells were grown in complete media: RPMI 1640 (Invitrogen), 2 mM L-glutamine, 50 μg/ml gentamycin, 10% (v/v) fetal calf serum, 10 ng/ml EGF (Sigma), and 5 μg/ml insulin (Sigma). Confluent cells were cultured in -EGF media; RPMI 1640, 2 mM L-glutamine, 50 μg/ml gentamycin, 2% (v/v) fetal calf serum, and 5 μg/ml insulin for 2 days. Cells were then induced to terminally differentiate by the addition of the lactogenic hormones: 1 μg/ml ovine PRL (oPRL; Sigma), and 10^{-7} M dexamethasone (Dex; Sigma) to the above described

-EGF media. For transient transfections, HC11 cells were split 1:7 in 12 well plates the day before. The cells were cotransfected with 100 ng reporter alone or in combination with 10 ng mouse PPARγ (a kind gift from Dr. Steven Kliewer) using Lipofectamine and PlusReagent (Invitrogen) according to manufacturer's protocol in serum free Ham F12 media (Invitrogen). Four hours later, the same volume of complete or -EGF media was added together with PPARγ ligands Ciglitazone (Sigma) or Troglitazone (Alexis). In some experiments the PPARγ inhibitor GW9662 (Alexis) and inhibitor of MAPK pathway, PD98059 (Sigma), were used. The latter was added 30 min prior to addition of EGF containing media. For concentrations used see legend to figures. The cells were grown for 48 h, after which they were washed with PBS and lysis buffer (25 mM TAE, pH 7.8, 1 mM EDTA, 10% glycerol, 1% Triton X-100 and 2 mM DTT) was added. Luciferase activity was measured with GenGlow luciferase kit (ThermoLabsystems) using AnthosLucy3. Experiments were made in triplicate and repeated three times or more.

Whole cell extract

Cells were washed with cold PBS, scraped into Eppendorf tubes, pelleted by centrifugation, and quickly frozen in N_2(l) and kept at –80°C. Cell pellet was resuspended in lysis buffer (400 mM NaCl, 10 mM HEPES pH 7.4, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 5% glycerol, 1 mM DTT, 1.5 μg/ml leupeptin, 10 μg/ml pepstatin, 2 μg/ ml aprotinin, 1 mM PMSF, and 1 mM Na_3VO_4) and kept for 10–15 min on ice. The cell lysates were thereafter clarified by centrifugation at 4°C for 10 min at 13.000 rpm in a table-top centrifuge. Protein concentration was determined with Bradford reagent (BioRad) using Spectramax 250 (Molecular Devices).

Nuclear extract

Nuclear extracts from HC11 cells were prepared using a modification of the method of Dignam et al. [27]. Cells were washed with cold PBS, scraped into Eppendorf tubes and spun down in a microcentrifuge. The cells were resuspended in RSB (10 mM Tris, pH 7.4, 10 mM NaCl, and 6 mM $MgCl_2$), kept on ice for 5 min and spun down again. The cell pellet was resuspended in RSB containing 1 mM DTT, 0.4 mM PMSF and 1 mM Na_3VO_4 and homogenized by 30 strokes in a glass homogenizer. After centrifugation, the nuclear pellet obtained was resuspended in 3 volumes of buffer C (20% glycerol, 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$ and 0.2 mM EDTA), and kept on ice for 20–30 min. The supernatant obtained after centrifugation was used as nuclear extract. Protein concentration was determined with Bradford Reagent.

Western blotting

SDS-solubilizing buffer was added to whole cell extracts and samples were boiled. Proteins were separated on a 7.5% (STAT5A) or 10% (PPARγ and RXRα) SDS-PAGE gel and transferred to a PVDF membrane by semi-dry blotting. The membrane was blocked for 1 h (or overnight) in TBS (20 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 5% milk protein. After washing, the membrane was incubated for 1 h with one of the following antibodies: rabbit anti-STAT5A (Santa Cruz Biotechnology, Inc., raised against amino acids 774–793 of mouse STAT5A, diluted 1:1000), rabbit anti-PPARγ$_{1,2}$ (Biomol, raised against amino acids 282–298 of mouse PPARγ2, diluted 1:2000), rabbit anti-RXRα (Santa Cruz, raised against amino acids 198–462 of human RXRα, diluted 1:200) or anti-beta-actin (Gene Tex, Inc., raised against human beta-actin, diluted 1:3000). The antibodies were diluted in TBS containing 0.05% Tween 20 (TTBS). The secondary antibody, goat anti-rabbit IgG coupled with horseradish peroxidase, was diluted 1:5000 in 1% milk protein/TTBS. Specific antibody signals were detected with an enhanced chemiluminescence kit (ECL; Amersham).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were incubated with 192 μg/ml polydI/dC for 10 min at room temperature in EMSA buffer (1 mM EGTA, 5 mM $MgCl_2$, 0.01% Triton X-100, 1% glycerol, 20 mM HEPES pH 7.5, 0.2 mg/ml BSA, 0.25 mM PMSF, and 5 mM DTT). Fifty thousand cpm of ^{32}P-labeled double-stranded PPARγ probe (PPRE1: GGTGGAGGCCAGAGGTCAACCT or PPRE4: GGGAAGCTGGGCAAAGGGGATGGCAGA) was added and incubated for further 20 min. For competition studies were used double-stranded PPRE and mutated PPRE (PPRE1: GGTGGAGTCATGAGGTCAACCT, PPRE4: GGGAAGCTGTGATAAGGGGATGGCAGA) as well as an estrogen response element (ACGGGTAGAGGTCACTGTGACCTCTACCCG) or a probe corresponding to the STAT5 site of the β-casein promoter (TGCTTCTTGGAATTGAAGAACCTTAATG). For supershift analysis the extract was incubated with rabbit anti-PPARγ$_{1,2}$ (Biomol, raised against amino acids 282–298 of mouse PPARγ2). Specific binding to the radioactive probe was analyzed on a 4.5% polyacrylamide gel, prerun for 1 h at 250 V in 0.25× TBE (22.5 mM Tris borate, pH 8.0, 0.5 mM EDTA). The samples were loaded and electrophoresed for 1.5 h at 250 V at 4°C, after which the gel was dried and exposed to X-ray film.

Results and discussion

Cloning and bioinformatic analysis of 5′ flank of STAT5A gene

We have earlier studied the expression of STAT5A in mouse mammary epithelial HC11 cells [26]. We found that STAT5A protein levels were influenced by the state of differentiation as well as media composition, i.e., EGF was shown to repress the expression of STAT5. To further study the regulation of STAT5A expression we isolated the promoter of the mouse *Stat5a* gene. The 5′ end of the gene was cloned by PCR in two steps, as described in Materials and methods. Two different PCR products, 1.6 kb and 2.5 kb, were obtained. The two PCR products overlapped, with the 2.5-kb PCR product extending in the 5′ direction. Ligation of these two PCR products resulted in 3.9 kb of the 5′ end of *Stat5a* gene encompassing 5′flank, exon 1 and part of intron 1 as based on genomic mouse *Stat5a* sequence (GenBank Acc. No. NT039521).

We also searched est mouse database and found a sequence (GenBank Acc. No. BY344844), which is homologous

to the published mouse STAT5A mRNA sequence (GenBank Acc. No. U21103) in the 3′ end, but extending further 293 nucleotides in the 5′ direction resulting in a larger exon 1. We have used this sequence to preliminary ascribe the transcription start site +1. Based on this our 3.9 kb PCR product extends from –3338 to +552 of mouse *Stat5a* gene.

Different bioinformatic tools were used to search for promoter elements. No TATA box was identified immediately upstream (100 bp) of the putative transcription start site (data not shown). Neither the promoter of mouse STAT3 [28], human STAT2 [29] or human STAT5A and B [30] have a TATA box suggesting that the STAT genes might lack this element. The immediate upstream region of STAT5A promoter contained two CCAAT boxes (–84 to –73 and –107 to –96; data not shown). Upstream of these elements were found several potential transcription factor consensus binding elements, e.g., for NF-1, C/EBP, AP-1 and SP1 (data not shown). We also found a close to perfect peroxisome proliferator response element (PPRE1), with 12 out of 13 nucleotides similar to the consensus sequence AGGCCAGAGGTCA [1] and three other PPREs with 8 to 10 similar nucleotides (Table 1).

PPARγ ligand induces STAT5A expression in HC11 cells

As described above, the PPARγ pathway can induce terminal differentiation of human breast cancer cells [3–5]. Furthermore, STAT5 expression is increased during PPARγ-ligand induced adipogenesis [23], but it has not been shown that this is a direct effect of PPARγ on STAT5A promoter. In view of these data we found it interesting to investigate, first if PPARγ ligands could affect expression of STAT5A in HC11 mouse mammary epithelial cells, and secondly, if the identified PPREs participate in regulation of *Stat5a* gene expression.

In order to analyze if treatment with PPARγ-agonist could induce STAT5A expression, HC11 cells were grown to confluence and the medium was changed to medium without EGF. Cells were then treated for 24 h with the TZD troglitazone alone or together with the PPARγ antagonist GW9662. Whole cell extracts were made and analyzed by Western blot with an anti-STAT5A antibody. Treatment with 0.075 and 0.75 μM troglitazone increased the amount of STAT5A, but 7.5 μM troglitazone decreased the amount of STAT5A (Fig. 1). This higher concentration of troglitazone did not seem to be toxic to the cells. One explanation for this inconsistency could be that troglitazone at high concentrations down-regulates PPARγ protein levels, which has been shown to occur in 3T3-L1 adipocytes [31]. Treatment with 0.75 and 7.5 μM PPARγ antagonist GW9662 together with 0.75 μM troglitazone decreased the amount of STAT5A protein compared to treatment with 0.75 μM troglitazone alone, suggesting that the observed induction is specific for PPARγ.

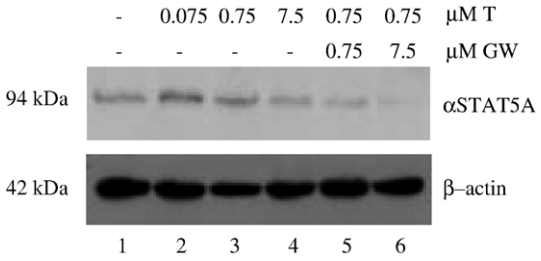


Fig. 1 – Troglitazone treatment of HC11 cells increases STAT5A expression. Confluent HC11 cells were treated with vehicle or increasing amounts of troglitazone alone or together with the PPARγ antagonist GW9662 for 24 h in medium without EGF. Whole cell extracts were analyzed by Western blot with a polyclonal antibody directed against STAT5A and beta-actin, the latter as a control for loading. The figure is representative of three independent experiments.

The presence of PPARγ in HC11 cells during proliferation, confluence, and differentiation was investigated by Western blot. HC11 cells were grown to confluence in complete media, i.e., medium containing EGF, insulin, and 10% serum. The cells were then kept for 2 days in media with 2% serum and without EGF, making the cells competent at responding to lactogenic hormones. Finally, the cells were induced to terminally differentiate by exposure to the lactogenic hormones (DIP); ovine PRL (oPRL), dexamethasone (Dex) and insulin. Cells were collected at all states and whole cell extracts were analyzed by Western blot (Fig. 2, upper part). PPARγ was present at all states, being most highly expressed during proliferation and at confluence (Fig. 2, lanes 1 and 2). This resembles the in vivo situation where PPARγ expression decreases during terminal differentiation of mammary epithelial cells [32,33]. We also investigated if retinoid × receptor α (RXRα) was expressed, an important partner for PPARγ for binding DNA [34,35]. The same extracts were used in a Western blot with an anti-

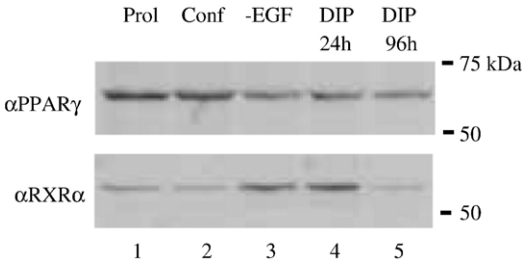


Fig. 2 – HC11 cells express PPARγ and RXRα. HC11 cells were grown to confluence in complete media containing EGF, then the cells were kept for 2 days in medium containing no EGF and reduced serum content. Finally, cells were treated with lactogenic hormones (DIP) for 24 or 96 h. Whole cell extracts were prepared at different cellular states, as indicated in figure, and analyzed by Western blot with polyclonal antibody against PPARγ or RXRα, as indicated. The figure is representative of three independent experiments.

Table 1 – The sequences of the PPREs are indicated along with nucleotide location and homology to consensus sequence according to Palmer et al. [1]

		Homology
Consensus	AGGNCA A AGGTCA	13/13
–2154	AGGCCA G AGGTCA	12/13
–548	AGGCCA C TGGCAG	8/13
–390	AAGAGC A AGGTCT	9/13
+33	TGGGCA A AGGGGA	10/13

RXRαantibody (Fig. 2, lower part). RXRα was expressed at all states, with the highest protein levels seen after withdrawal of EGF and treatment with DIP for 24 h (Fig. 3, lanes 3 and 4). After 96-h DIP treatment, the protein level was decreased to the level seen at confluence.

PPARγ DNA-binding activity in HC11 cells increases upon EGF withdrawal

HC11 cells were grown to confluence in complete medium after which they were kept for 2 days in medium without EGF and finally treated with DIP. Cells were collected at all states and nuclear extracts were made. The nuclear extracts were analyzed by EMSA with a ^{32}P-labeled probe corresponding to the PPRE1 found in the STAT5A promoter (Fig. 3A). DNA binding activity was found in nuclear extracts from HC11 cells at all states, with the highest activity after withdrawal of EGF (Fig. 3A, lane 3). The specificity of the DNA binding was tested by competition with cold probe as well as a mutated probe (Fig. 3B). The DNA binding was almost abolished with 50- and 100-fold excess cold probe (Fig. 3B, lanes 7 and 8), whereas it was only reduced with 50- and 100-fold excess mutated probe (Fig. 3B, lanes 3 and 4). Competition with another nuclear receptor response element, the estrogen response element (ERE), as well as the STAT5 element of the unrelated β-casein promoter was also performed (Fig. 3C). The DNA binding was slightly reduced when competed with the ERE probe (Fig. 3C, lanes 2 and 3), probably due to the somewhat related sequence as compared to PPRE. No reduction was obtained from competition with the unrelated STAT5 probe (β-cas, Fig. 3C, lanes 4 and 5). To further strengthen the specificity, a supershift was performed (Fig. 3D). A partial supershift was obtained with PPARγ-antibody suggesting that the DNA binding entity in nuclear extracts is PPARγ. Thus, these control experiments strongly suggest that the protein binding to the radiolabeled PPRE was PPARγ. The finding that PPARγ DNA-binding increased after EGF withdrawal is interesting. The level of PPARγ protein was decreased after EGF withdrawal (Fig. 2, upper part, lane 3). At the same time the level of RXRα protein was increased (Fig. 2, lower part, lane 3). It is possible that this increase in RXRα expression resulted in increased binding to PPRE1 probe. Also, it is interesting that the increase in PPARγ DNA-binding coincides with our earlier observed increase in STAT5A expression [26].

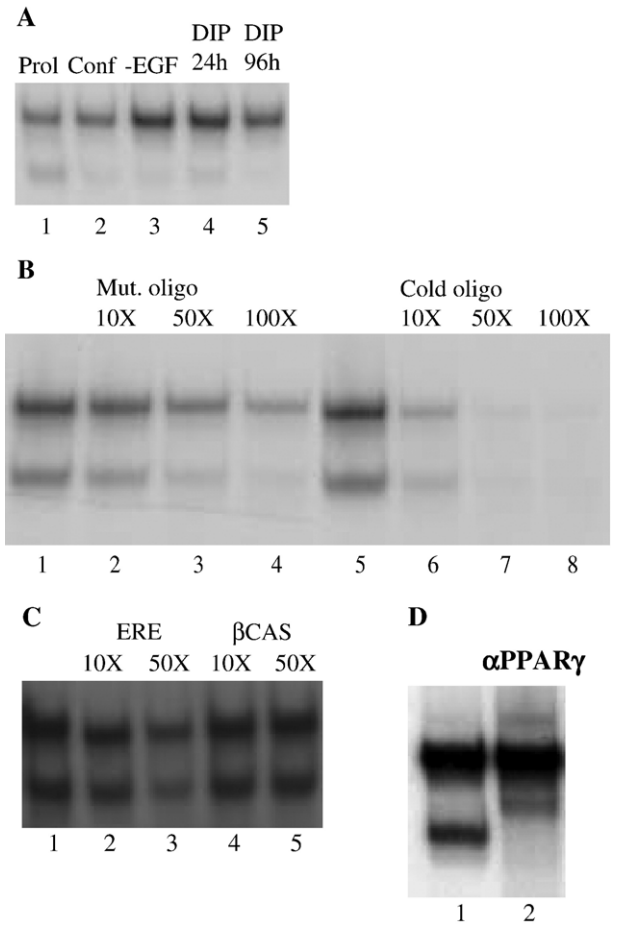
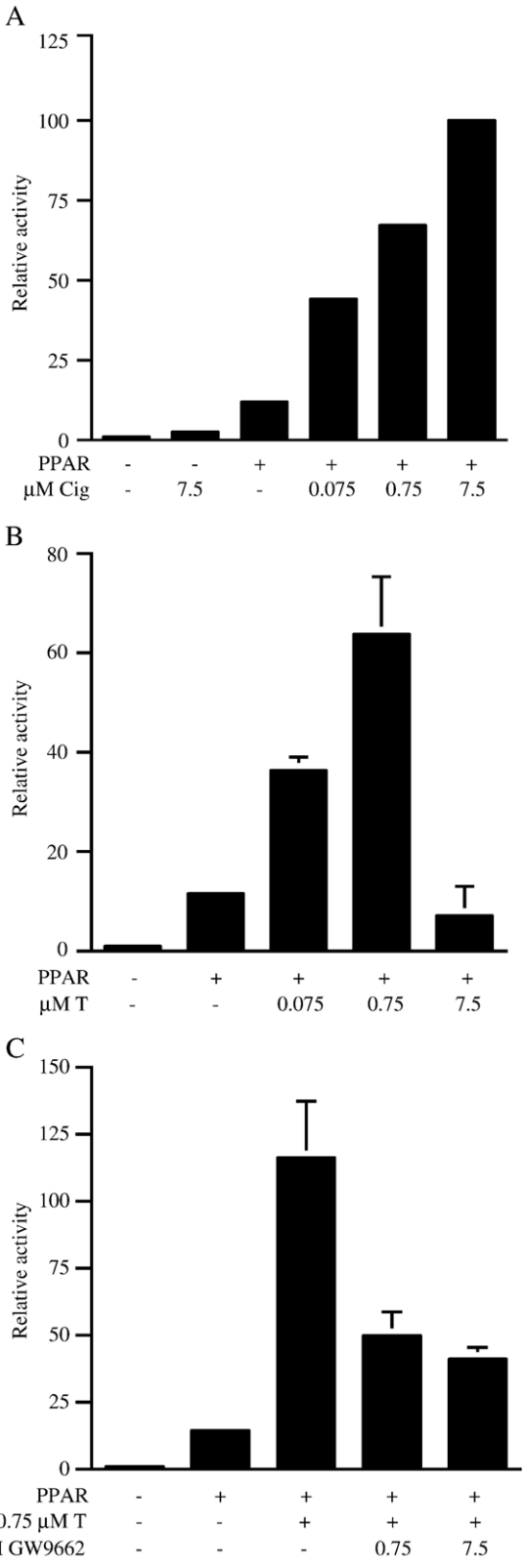


Fig. 3 – PPARγ DNA-binding activity in HC11 cells varies with developmental state. HC11 cells were grown to confluence in complete media containing EGF. The cells were then kept for 2 days in medium containing no EGF and reduced serum content. Finally, cells were treated with lactogenic hormones (DIP) for 24 or 96 h. Nuclear extracts were made at all states. (A) Samples were analyzed in a gel electrophoretic mobility shift assay with a ^{32}P-labeled probe corresponding to the PPRE1 found in the STAT5A promoter. (B) For competition analysis, cold PPRE1 as well as mutated PPRE1 was used. (C) Competition with cold probes for a STAT5 binding site (βcas) and an estrogen response element (ERE), for sequences see Materials and methods. (D) Supershift with antibody against PPARγ. The figure is representative of three independent experiments.

PPRE1, 3 and 4 are important for PPARγ ligand-induced STAT5A-reporter expression

A –3338/+552 STAT5A-promoter luciferase reporter construct was made. HC11 cells were transiently transfected with the reporter construct alone as well as cotransfected with expression vector for mouse PPARγ (mPPARγ). Cells were treated with increasing concentrations of ciglitazone for 48 h in -EGF medium. A small but significant ciglitazone-induced activation (2.5-fold) was seen with the reporter alone, but a much stronger response was seen when cells were co-transfected with mPPARγ (Fig. 4A, compare bars 2 and 6). The –3338/+552 STAT5A-luciferase reporter was found to be activated in a dose-dependent manner (Fig. 4A, bars 4–6), showing that activation of PPARγ could induce *Stat5a* gene expression. In the following, cells were cotransfected with reporter and mPPARγ. We also analyzed the effect of troglitazone on STAT5A-reporter expression (Fig. 4B). Troglitazone, at 0.075 and 0.75 μM, increased reporter expression in a dose-dependent fashion. Interestingly, treatment of HC11 cells with 7.5 μm troglitazone showed no increase in STAT5A-reporter expression. This correlates with the Western blot data (Fig. 1), which showed that when HC11 cells were treated with 7.5 μM troglitazone less STAT5A protein was seen than with lower concentrations of troglitazone. Potency of different TZD has been reported to vary with structure [36] and could explain the difference between ciglitazone and troglitazone.

Co-treatment with troglitazone and the PPARγ antagonist GW9662 reduced STAT5A-reporter expression (Fig. 4C, compare bar 3 with bars 4 and 5), which also correlates with the Western blot data on endogenous STAT5A protein levels seen in Fig. 1. In conclusion, the above data show that expression of the STAT5A-reporter was increased by ligand-activated PPARγ.



A new luciferase reporter construct was made consisting of the cloned 1.6 kb PCR product, resulting in a –1103/+552 STAT5A-promoter luciferase reporter, in which the PPRE1 was not present. HC11 cells were transiently cotransfected with mPPARγ and either of the two STAT5A-luciferase reporter constructs, and cells were treated with ciglitazone for 48 h. The ciglitazone-induced luciferase activity from the –1103/+552 construct was found to be around 50% of that of the –3338/+552 construct (Fig. 5A), suggesting that PPRE1 contributes to reporter expression. To further study the importance of the PPRE1, the site was mutated. Furthermore, two shorter forms of the promoter were generated by PCR, –531/+552 and –246/+552, which were cloned into pGL3-Basic. HC11 cells were transiently transfected with these constructs, and treated with 0.75 μM troglitazone for 48 h (Fig. 5B). Mutation of the PPRE1 site reduced the troglitazone-induced expression of the STAT5A-reporter to around 50% as compared to the –3338/+552 promoter construct, which is similar to the results obtained with the –1103/+552 construct (Fig. 5A). This further strengthens that PPRE1 is important for expression of *Stat5a* gene. The activity of the –531/+552 construct was at the same level as the mutated –3338/+552 STAT5A-reporter construct. This indicates that the –548 PPRE2 (Table 1) does not contribute to PPARγ-induced expression of STAT5A-reporter. The –531/+552 STAT5A-promoter contains two putative PPREs (PPRE3 and 4, Table 1), although with only 9 respectively 10 nucleotides similar to the PPRE consensus. The troglitazone-induced expression of –246/+552 STAT5A-reporter construct was around 20% of that of the –531/+552 construct (Fig. 5B), indicating the importance of –390 PPRE3 (Table 1) for STAT5A-reporter expression. As the expression of the –246/+552 construct was increased by troglitazone the +33 PPRE4 (Table 1) also participates in PPARγ ligand-induced expression of STAT5A-reporter. This PPRE is located in exon 1. To further analyze the importance of –390 PPRE3 and +33 PPRE4 for induction of STAT5A-reporter, the sites were mutated. The resulting constructs were transiently transfected into HC11 cells, which were treated with 0.75 μM troglitazone for 48 h (Fig. 5C). Mutation of PPRE3 reduced the PPARγ ligand induced activity of the STAT5A-reporter to approximately 30%, as compared to the full-length construct. Mutation of PPRE4 resulted in a STAT5A-reporter with almost no response to PPARγ ligand, suggesting PPRE4 is essential for the response. Comparison of the corresponding region of the rat (NW_047339) as well as the human (AC099811) *Stat5a* genes showed that this site is conserved and located in a

Fig. 4 – PPARγ ligand increases STAT5A-reporter expression. HC11 cells were transfected with –3338/+552 STAT5A-luciferase reporter alone or together with mPPARγexpression vector as indicated in the figure. The transcriptional responses to the thiazolidinediones A, ciglitazone (Cig), B, troglitazone (T) and C, troglitazone (T) together with PPARγ antagonist GW9662 were determined in -EGF medium. All experiments were done in triplicate at least three times, and data are presented as mean of relative activity ±SD. Activity of reporter plasmid alone without hormone treatment was arbitrarily set to 1.

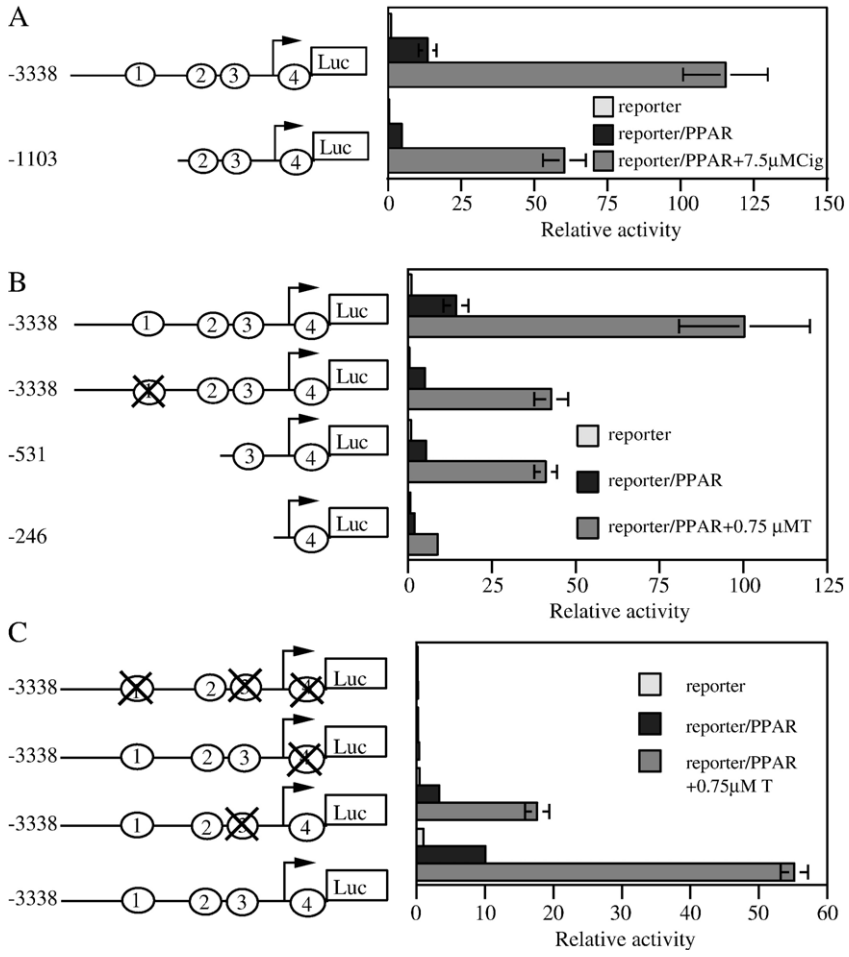


Fig. 5 – PPRE1, 3 and 4 are important for PPARγ ligand-induced STAT5A-reporter expression. (A) HC11 cells in -EGF medium were co-transfected with either –3338/+552 or –1103/+552 STAT5A-luciferase reporters and mPPARγ, and the transcriptional responses to 7.5 μM ciglitazone (Cig) were determined. (B) HC11 cells were co-transfected with mPPARγ and –3338/+552, –3338/+552 mutated in PPRE1, –531/+552 or –246/+552 STAT5A-luciferase reporters. (C) HC11 cells were cotransfected with mPPARγ and –3338/+552 and –3338/+552 mutated in PPRE3, PPRE4 or PPRE1,3,4. The transcriptional response to 0.75 μM troglitazone (T) was determined in -EGF medium. All experiments were done in triplicate at least three times, and data are presented as mean of relative activity ±SD. Activity of –3338/+552 reporter plasmid alone without hormone treatment was arbitrarily set to 1.

long stretch (more than 300 nucleotides) of high similarity. On the contrary when the bos taurus (NW_929518) sequence was examined, the site was not found, and in general the similarity between mouse and bos taurus *Stat5a* genes was found to be very low, suggesting a different type of regulation in cattle. This is however interesting and could be a result of evolution, since cattle has been extensively breeded to produce milk.

A probe corresponding to PPRE4 was made and analyzed in an EMSA with nuclear extract obtained from HC11 cells grown to confluence and kept in medium without EGF for 2 days (Fig. 6). DNA-binding activity was found and the specificity was tested with cold probe as well as a mutated probe. The DNA-binding activity was reduced with excess of cold probe, whereas no reduction was seen with excess mutated probe. In conclusion, the above data indicate that within the *Stat5a* gene sequence we have cloned, there exist three putative PPREs, –2154 PPRE1, –390 PPRE3, and +33 PPRE4 (Table 1), which are important for PPARγ ligand-induced expression of STAT5A-reporter, with the proximal +33 PPRE4 being essential.

EGF decreases PPARγ ligand-induced expression of STAT5A-reporter gene

We have earlier shown that EGF has a suppressive effect on the expression of STAT5A, which is mediated at least in part through the ras/raf/MEK/MAPK pathway [26]. PPARγ has been shown to have a MAPK consensus recognition site. When phosphorylated at this site, PPARγ is transcriptionally repressed [37,38]. We found it interesting to investigate if EGF negatively could influence PPARγ transcriptional activity on STAT5A-reporter. HC11 cells were transiently transfected with mPPARγ and the –3338/+552 STAT5A-reporter and treated with 0.75 μM troglitazone with or without EGF. As can be seen in Fig. 7A, the presence of EGF reduced the troglitazone-

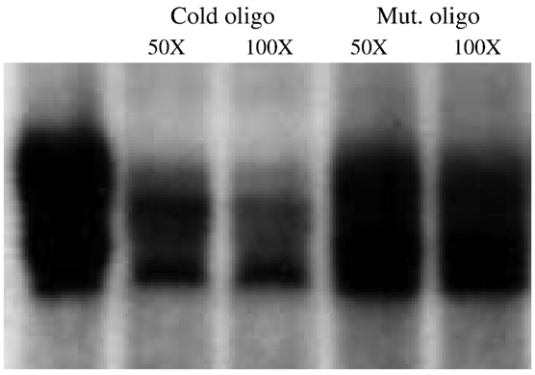


Fig. 6 – EMSA with PPRE4 and nuclear extract from HC11 cells. HC11 cells were grown to confluence in complete media containing EGF. The cells were then kept for 2 days in medium containing no EGF and reduced serum content. Nuclear extracts were made. Samples were analyzed in EMSA with a ^{32}P-labeled probe corresponding to the PPRE4 found in the STAT5A promoter. For competition analysis, cold PPRE4 as well as mutated PPRE4 was used.

induced activation of the STAT5A-reporter by 75%. In another experiment, HC11 cells were transiently transfected with mPPARγ and the –3338/+552 STAT5A-reporter and treated with increasing concentrations of the MEK inhibitor PD98059 in EGF-containing media (Fig. 7B). Treatment with PD98059 resulted in a dose-dependent increase in STAT5A-reporter expression. The above data suggest that the suppressive effect of EGF on STAT5A expression is mediated through MAPK phosphorylation of PPARγ, resulting in a less transcriptionally active PPARγ. Interestingly, Yang et al. [39] have shown that PPARγ expression was elevated and PPARγ transcriptional activity was decreased in epidermal growth factor receptor 2 (HER2)-overexpressing MCF7 breast cancer cells. Down-regulation of HER2 by anti-HER2 antibody was shown to result in decreased levels of activated MAPK and PPARγ protein and increased PPARγ transcriptional activity. Also, Prusty et al. [40] have shown that MEK/ERK activation during adipogenesis results in enhanced activity of factors that increase PPARγ expression. These results correlate well with our results. High levels of PPARγ protein (Fig. 2, upper part lane 1 and 2), and low PPARγ transcriptional activity were found when EGF was present (Fig. 7A), while low levels of PPARγ protein (Fig. 2, upper part lane 3, 4 and 5), and high PPARγ transcriptional activity was found, when EGF was not present (Fig. 7A). Thus, also in HC11 cells, EGF and the following activation of MAPK, promoted expression but inhibited transcriptional activity of PPARγ.

Based on our results, PPARγ seems to participate in regulation of STAT5A expression in mammary epithelial cells. The physiological importance of this is at present unknown. PPARγ-null mice die in utero but PPARγ has recently been deleted in mammary epithelium using MMTV- and WAP-Cre transgene mice [33]. Analysis of these mice showed that PPARγ is not required for functional mammary gland development. No quantification of STAT5A expression was done in these PPARγ-deleted mice. Deletion of *Stat5a* gene results in impaired terminal differentiation of mammary gland [15]. It is possible that the loss of PPARγ in mammary epithelial cells in MMTV- and WAP-Cre transgene mice did not significantly influence STAT5A expression. Our sequence analysis of the cloned 5´flank of mouse *Stat5a* gene identified binding elements for several other transcription factors, e.g., C/EBP and NF-1, which have been ascribed a role in regulation of differentiation and gene regulation in mammary epithelial cells [41–43]. Thus, it is possible that these transcription factors also participate in regulation of STAT5A expression

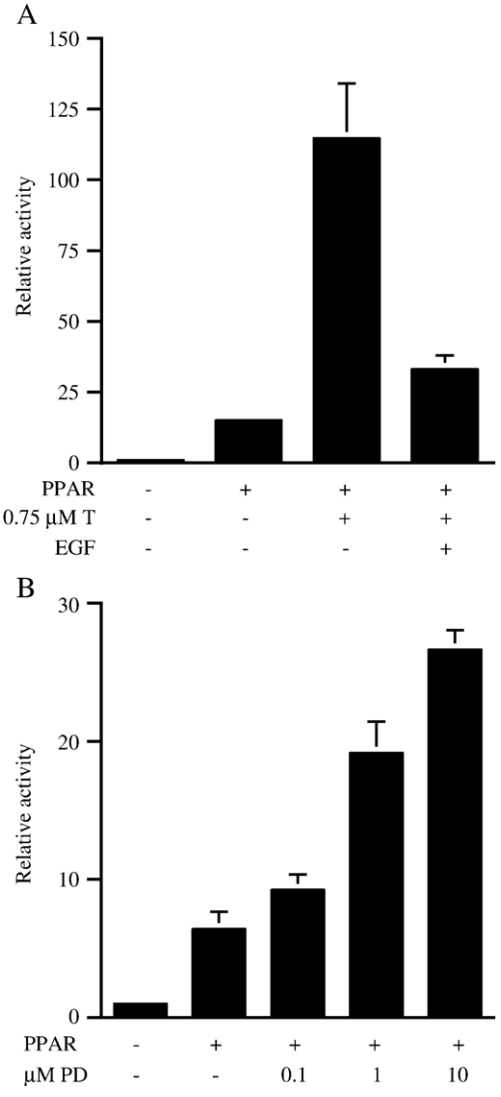


Fig. 7 – EGF down-regulates PPARγ induced STAT5A reporter expression. HC11 cells were co-transfected with –3338/+552 STAT5A luciferase reporter and mPPARγexpression vector. (A) The transcriptional responses to 0.75 μM troglitazone (T) alone or together with 10 ng/ml EGF were determined. (B) The transcriptional responses in complete media, i.e., media containing EGF, were determined in the presence of increasing concentrations of PD98059 (PD). All experiments were done in triplicate at least three times. Data are presented as mean of relative activity ±SD. Activity of reporter plasmid alone without hormone treatment was arbitrarily set to 1.

and that they compensated for the loss of PPARγ in the above-described study.

PPARγ-ligands have been shown to inhibit growth and induce differentiation of cancer cells in vitro, including breast cancer cells [5,44,45]. Also, PPARγ-ligands have been shown to decrease experimentally induced mammary cancer [46,47]. Based on these studies PPARγ has been suggested to be a new molecular target for breast cancer prevention and therapy [4,48]. Future studies are needed to determine if PPARγ-ligands increase STAT5A expression in breast cancer cells and if STAT5A then is important for the induced differentiation.

PPARγ-ligands have also been shown to increase expression of STAT5 in adipocyte precursors [23]. Furthermore, it has been shown that ectopic expression of STAT5A in adipocyte precursors and nonprecursor cells results in expression of adipogenic markers and accumulation of triglycerides [20,22]. Thus, it seems that STAT5A could be a down-stream mediator of PPARγ in adipocytes. Milk produced in mammary gland contains fat, which is either produced in mammary epithelial cells or mobilized from fat depots [49]. Interestingly, a recent study has shown that STAT5A contributes to lactational stimulation of promoter III in the gene expressing the rate limiting enzyme for de novo synthesis of fatty acids, acetyl-CoA carboxylase α [50].

In conclusion, we have shown that PPARγ-ligands induced STAT5A protein expression in HC11 cells. The 5´flank of the mouse *Stat5a* gene was shown to contain several PPREs, which were important for PPARγ-ligand-induced STAT5A-reporter expression. Furthermore, EGF was shown to decrease PPARγ-ligand-induced expression of STAT5A-reporter possibly through MAPK-effectuated repression of PPARγ transcriptional activity.

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