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Identification of novel PPARγ target genes in primary human adipocytes

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Abstract

Adipogenesis is the process by which undifferentiated precursor cells differentiate into fat laden adipocytes. The nuclear proteins peroxisome proliferator-activated receptors (PPARs) play a central role in adipocyte differentiation. The goals of this study were to identify novel PPARγ responsive genes and to determine their role in regulating human adipocyte differentiation. Affymetrix profiling of gene expression in human adipocytes identified about 1000 genes that were significantly up-regulated subsequent to induction of differentiation. PPARγ expression was reduced prior to induction of differentiation using a novel, chemically modified antisense oligonucleotide. Affymetrix microarray profiling of these cells identified 278 statistically significantly down-regulated genes. Eight genes were found to contain previously documented PPARγ recognition element (PPRE) in their upstream nucleotide (promoter) sequence. Four of these genes are novel and have not previously been characterized. Chromatin immuno-precipitation experiments confirmed the binding of PPARγ to the PPRE of three of these genes. The ortholog of one of these genes, hypothetical protein FLJ 20920, has previously been reported to be involved in the control of body fat composition in *Caenorhabditis elegans*. Inhibition of expression of this protein was found to also inhibit differentiation of human adipocytes. MAST/MEME algorithm analysis was used to identify novel commonly occurring sequence motifs in the 5′ upstream region of transcripts for subset of down-regulated genes, which were grouped according to their sequence similarities. A number of clusters were identified and the largest cluster contained similar motifs from 26 genes with the literature supporting 7 of the 26 genes as being involved in fatty acid metabolism or PPARγ interaction.

Keywords: Regulatory motifs; PPARγ; Antisense oligonucleotides; DNA array

1. Introduction

Adipocyte differentiation is a complex process characterized by coordinated changes in cell morphology, hormone sensitivity, gene expression, and secretory capacity. Specific changes occur during the differentiation process including the accumulation of triglycerides as lipid droplets, the secretion of several hormones and autocrine factors (e.g. leptin and adiponectin) and characteristic changes in gene expression, for example increased expression of PPARγ, hormone sensitive lipase (HSL), glucose transporter 4 (Glut4) and adipocyte lipid binding protein 2 (aP2) (Spiegelman and Flier, 2001; Lee et al., 2003). This process can also be modelled in vitro by incubating pre-adipocytes with insulin, a PPARγ agonist, hydrocortisone and a compound that increases intracellular levels of cyclic adenosine monophosphate (cAMP), usually 3-isobutylmethylxanthine (IBMX) (Brandes et al., 1995; Gregoire, 2001). Several transcription factors such as CCAAT/enhancer binding protein-α (CEBP-α), peroxisome proliferator activated receptor gamma (PPARγ) and sterol-regulatory element-binding transcription factor 1 (SREBP1) are involved in both the in vitro and in vivo processes (Spiegelman and Flier, 1996). Of these, PPARγ is one of the key regulatory genes and plays a major role in the process of adipocyte differentiation.

Abbreviations: PPARγ, Peroxisome proliferator-activated gamma; ASO, Antisense oligonucleotides; TF, Transcription factors; FDR, False discovery rate; ChIP, Chromatin immunoprecipitation.

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The understanding of the downstream pathway genes of PPARγ is of fundamental importance in order to identify drug targets for metabolic diseases. However, there is only limited information available about the PPARγ downstream target genes particularly in primary human adipocytes. PPARs possess the same P-box sequence (the DNA binding motif) of most type II steroid hormone receptors (Schoonjans et al., 1996). To date, most PPREs are direct repeats with one intervening nucleotide (DR-1). RXR acts as the preferential binding partner and the PPAR/RXR heterodimer recognizes the PPRE. (Schoonjans et al., 1996; DiRenzo et al., 1997).

In recent years, various data analysis techniques such as cluster analysis algorithms, have provided many approaches to analyze microarray gene expression data that allow groups of genes into co-regulated clusters (Shannon et al., 2003). Similarly, promoter sequences of each gene in a cluster can be fed to *cis*-regulatory discovery algorithms to identify motifs that are common in functionally related genes (Keles et al., 2002). Motifs that are common to a set of co-expressed genes are potential candidates of binding sites for transcription factors implicated in a particular process, for example PPARγ transcriptional regulation. Previous studies have characterized several genes for the presence of PPARγ binding element (Schoonjans et al., 1996). Though the PPREs possess the same P-box (TGACCTnTGACCT) of type 11 steroid hormone receptors, the actual binding motif may differ compared to the consensus P-box element in PPARγ regulated genes (Schoonjans et al., 1996).

In the present study we have firstly identified, through transcriptional profiling, a group of genes that are increased in expression during human primary adipocyte differentiation. Furthermore, we identified an overlapping group of genes that are down-regulated when PPARγ is inhibited by an antisense oligonucleotide (ASO) before the differentiation process is begun. As expected, a sub-set of this group of genes is regulated by PPARγ, and eight genes in this subset contain a well-characterized PPRE element in their 5′ upstream transcripts. One of these PPRE-containing genes is FLJ20920, a novel gene of unknown function. CHIP analysis demonstrated that the PPRE in this gene directly binds PPARγ in human adipocytes. Furthermore, inhibition of expression of this gene with an ASO resulted in attenuation of the differentiation response. In addition, a second sub-set of genes down-regulated in PPARγ ASO treated cells were found to possess novel 5′-upstream domains, suggesting a possible role for these sequences as regulators of PPARγ function.

2. Materials and methods

2.1. Human adipocyte differentiation

Human pre-adipocytes (Zen-Bio, Inc., Research Triangle Park, NC) obtained from abdominal fat reduction surgeries were cultured up to 80% confluency in T175 flasks in Preadipocyte Growth Medium (Zen-Bio, Inc.). Differentiation was induced by treating the cells with DM, or differentiation medium which contained insulin, dexamethasone, IBMX and a PPARγ agonist (Zen-Bio, Inc.). Subsequently the cells were maintained in AM, or adipocyte media which is the same as DM, but without IBMX or the PPARγ agonist (Zen-Bio, Inc.) for the indicated time period before being harvested.

2.2. Triglyceride assay

Triglyceride accumulation was measured using the Infinity™ Triglyceride Reagent Kit (Sigma-Aldrich, St. Louis, MO) which uses a lipoprotein lipase to liberate glycerol from triglycerides. The glycerol is then enzymatically metabolized in a manner that generates H_2O_2. The H_2O_2 is then used to produce a red-colored dye which can be quantitated spectrophotometrically. At the indicated time, cells were washed, lysed in a mild detergent, IGEPAL CA-630 (Sigma-Aldrich), at room temperature and the triglyceride assay reagent added. After a 1 h incubation, the optical density of the solution was measured at 515 nm using an UV spectrophotometer. Glycerol concentration was calculated from a standard curve for each assay, and data is normalized to total cellular protein as determined by a Bradford assay (Bio-Rad Laboratories, Helena, CA). Results are expressed as a percent±standard deviation relative to the transfectant-only controls.

2.3. RT-PCR

To monitor mRNA expression, cells were lysed in a guanadinium containing buffer and RNA was extracted using RNeasy (Qiagen, Valencia, CA). The amount of RNA obtained was determined using Ribogreen (Molecular Probes, Eugene, OR). Real-time RT-PCR was performed on GLUT4, HSL, aP2 and PPARγ. The expression levels were normalized to total cellular RNA.

2.4. Antisense oligonucleotides and transfection conditions

All antisense oligonucleotides used in this study were 20 bases in length, and contained 2′-*O*-(2-methoxy) ethyl (2′-MOE) modifications on the 5 sugar residues at both the 5′- and 3′-ends of the molecule. The compounds contained phosphorothioate linkages throughout with 10 contiguous deoxynucleotide residues in the central portion of the molecule. This class of compound has been shown previously to be highly potent and selective for target gene down-regulation (McKay et al., 1999). For each gene targeted, a total of 78 ASOs were designed, synthesized and evaluated in a cell culture RT-PCR assay for their ability to reduce the target mRNA expression (data not shown), a procedure that facilitated the identification of the most active ASO. The most active oligonucleotides from these screens were chosen for all further studies. The sequence of ISIS 155990 targeted to PPARγ is: 5′-AGCAAAAGATCAATCCGTTA 3′. The sequence of ISIS 331539 targeted to FLJ20920 is: 5′ TGTGGTCAGATCTGGGAGCC 3′. The control oligonucleotide mixture used in these studies, ISIS 29848, is a mixture of 20 base oligonucleotides with completely random sequence (i.e. NNNNNNNNNNNNNNNNNNNN), which is anticipated to contain a mixture of 4^{20} different