Cloning and Characterization of a Functional Peroxisome Proliferator Activator Receptor-γ-responsive Element in the Promoter of the CAP Gene

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c-Cbl-associating protein (CAP) is a multifunctional signaling protein that interacts with c-Cbl, facilitating the tyrosine phosphorylation of c-Cbl in response to insulin. In 3T3-L1 adipocytes and diabetic rodents, CAP gene expression is stimulated by activators of peroxisome proliferator activator receptor γ (PPARγ), such as thiazolidinediones (TZDs), resulting in increased insulin-stimulated c-Cbl phosphorylation. Sequence analysis of 2.5 kilobases of the 5′-flanking region of the CAP gene reveals a predicted peroxisome proliferator response element (PPRE) from −1085 to −1097. The isolated promotor was functional in 3T3 fibroblasts and adipocytes. Co-transfection of the CAP promotor with PPARγ and retinoic acid X receptor α caused fold stimulation of promotor activity. The TZD rosiglitazone produced an additional 2–3-fold stimulation of the promotor. Deletion of the predicted PPRE from the CAP promotor abolished its ability to respond to rosiglitazone. Gel shift analysis of the putative PPARγ site demonstrates direct binding of PPAR/Retinoid X receptor heterodimers to the PPRE in the CAP gene. These data demonstrate that TZDs directly stimulate transcription of the CAP gene through activation of PPARγ.

Although the tyrosine kinase activity of the insulin receptor is essential for the full expression of insulin action, the precise role of its different cellular substrates remains uncertain. Insulin stimulates the tyrosine phosphorylation of the c-Cbl proto-oncogene product (1). This phosphorylation requires the expression of a novel protein called CAP, which recruits c-Cbl to the insulin receptor (2). CAP is a multifunctional protein with three adjacent SH3 domains in the C terminus and a sorbin homology domain in the N terminus. CAP associates with both c-Cbl and the insulin receptor in the basal state. Insulin stimulation causes the disassociation of CAP from the insulin receptor. However, CAP remains associated with c-Cbl after insulin stimulation. Additionally, overexpression of CAP causes the formation of focal adhesions and stress fibers due to its association with p125FAK and actin stress fibers (3).

Although the role of CAP in insulin action has not been definitively proven, several lines of evidence suggest an important function. It is expressed predominantly in insulin-sensitive tissues. In the 3T3-L1 adipocyte cell line, CAP expression correlates well with insulin sensitivity (2). Moreover, stimulation of the nuclear receptor PPARγ with thiazolidinediones (TZDs) in 3T3-L1 adipocytes or in diabetic rodents leads to increased CAP expression and increased insulin-stimulated c-Cbl phosphorylation (4). The effects of TZDs on CAP expression are a direct result of increased transcription of the CAP gene. This TZD-induced increase in expression of CAP correlates well with increased insulin sensitivity both in vitro and in vivo (4).

These results provide the first line of evidence linking the anti-diabetic effects of TZDs to improvements in insulin signaling. Furthermore, it is suggested that the CAP promotor may have elements that directly bind PPARγ. We report here the cloning of the promotor of the CAP gene and the identification of a functional PPAR response element (PPRE) within the CAP promotor.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased from Life Technologies, Inc. BRL 49653 (rosiglitazone) was synthesized by the Parke-Davis Pharmaceutical Research Division of Warner-Lambert Co. (Ann Arbor, MI).

Cloning of CAP Promotor and Reporter Fusion Constructs—The bacterial artificial chromosome library at Research Genetics was screened with the 5′-end of the coding region of the CAP gene. The bacterial artificial chromosome clone was restriction digested with HindIII or SacI and subject to sequential Southern analysis with probes corresponding to the 5′-untranslated region and the putative promotor sequence of the CAP gene. The fragments were subcloned and assembled in both pBluescript (Stratagene) and pGL3 basic (Promega). All sequence analyses of the putative promotor region and putative transcription factor binding sites were done with Signal Scan software (University of Minnesota, St. Paul, MN). Plasmid constructs were generated by digestion using restriction sites within the CAP promotor. pcPH was generated by cloning a 550-base pair HindIII and SacI fragment of the CAP promotor into pGL3. pcPS was generated by insertion of a 1070-base pair SacI and Smal fragment into pGL3. pcPE was generated by insertion of the 2.6-kilobase EcoRI/Smal fragment of the CAP promotor into pGL3. pcPCEAPPRE was generated by digestion of pCPE with SacI and removal of a 435-base pair fragment that contains the PPRE of the CAP promotor and ligation of the pcPE vector.

S1 Nuclease Assay—Total RNA (40 μg) isolated from 3T3-L1 adipocytes or mouse fat tissue was hybridized with a γ-32P-end-labeled 70-mer probe overnight at 42 °C. The antisense 70-mer probe contains a sequence complimentary to the first 40 bases of the 5′-end of the longest CAP cDNA clone, a sequence complimentary to the 20-base genomic sequences immediately upstream of the 5′-end of the longest clone, and a 10-base nonspecific sequence (see Fig. 3B). The samples were then digested with 250 units of S1 nuclease at 37 °C for 60 min, followed by ethanol precipitation. The reaction products were subjected to polyacrylamide (8%)/urea (7 M) gel electrophoresis and visualized by autoradiography. Undigested probe was loaded on lane 1 (see Fig. 2). The size of the protected fragment was estimated by comparison with a DNA sequencing ladder run on the same gel.

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The abbreviations used are: CAP, c-Cbl-associating protein; PPAR, peroxisome proliferator activator receptor; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor; TZD, thiazolidinedione; CMV, cytomegalovirus; C/EBP, CAAT/enhancer-binding protein.
Characterization of a PPRE in the CAP Gene

Cell Transfections and Reporter Assays—NIH3T3 and 3T3-L1 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium and 10% calf serum. Transfections of NIH3T3 fibroblasts were done by using LipofectAMINE (Life Technologies, Inc.) reagent according to the manufacturer’s instructions. 250 ng of pGL3 basic firefly luciferase constructs (Promega) were co-transfected with 50 ng of PCMV-β-galactosidase and/or CMV-PPARγ and CMV-RXRα. PPARγ and RXRα were cloned into the pSG5 expression plasmid as described previously (5). Translation products were examined by SDS-polyacrylamide gel electrophoresis. Double-stranded cDNAs for murinePPARα and murineRXRα were in vitro translated using the TNT Quick Coupled Reticulocyte Lysate kit (Promega) according to the manufacturer’s instructions. Translation products were examined by SDS-polyacrylamide gel electrophoresis.

Gel Electromobility Shift Assay—cDNAs for murinePPARα and murineRXRα were in vitro translated using the TNT Quick Coupled Reticulocyte Lysate kit (Promega) according to the manufacturer’s instructions. Translation products were examined by SDS-polyacrylamide gel electrophoresis. 5 μl of each TNT reaction were used for the mobility shift assay.

In vitro translated murinePPARα and murineRXRα were co-incubated on ice for 30 min before the addition of radiolabeled (50,000 cpm/sample) double-stranded probe. 10 μg of 3T3-L1 pre-adipocyte and adipocyte nuclear extracts were prepared as described previously (8), except that the extracts were not dialyzed but were added directly to the binding reaction, containing radiolabeled (50,000 cpm/sample) double-stranded probe, for 15 min at room temperature. Complexes were resolved by non-denaturating acrylamide gel electrophoresis.

Fig. 1. Structure of the 5′-flanking region of the mouse CAP gene. A, schematic of the genomic organization and restriction map of the CAP promoter and 5′-coding exons. The black box represents the promoter. The hatched box is the location of the PPRE. The gray boxes represent exons, and the open boxes represent intronic sequences. B, sequence and putative transcription factor binding sites in the CAP promoter. Binding sites for Sp-1, activator protein-2, C/EBP, and PPAR are highlighted. The start of transcription, as determined by S1 nuclease protection, is indicated by the arrow. The cDNA encoding exon 1 of the CAP gene (GenBankTM accession number U58883) is underlined. The single-stranded probe used for the S1 nuclease protection assays is italicized.

RESULTS

Cloning of the CAP Promoter and Determination of Transcription Start Site—Southern analysis and sequencing of frag-
ments from a bacterial artificial chromosome clone containing the 5′-flanking region of the CAP gene revealed a structure depicted in Fig. 1, A and B. To determine the start of transcription of the CAP gene, a 70-nucleotide single-stranded DNA probe was generated that overlapped a region containing the longest known CAP cDNA sequence. The probe was hybridized with 3T3-L1 adipocyte or mouse fat tissue mRNA and digested with S1 nuclease to identify the start of transcription (Figs. 1B and 2). The presence of one predominant transcript was confirmed by reverse transcription-polymerase chain reaction. The start of translation of the CAP gene was found to lie within exon 4 of the CAP gene (data not shown). A putative promoter sequence was identified by analysis of 2.6 kilobases of the 5′-flanking region of the CAP gene using Signal Scan software (University of Minnesota). Although no TATA box was found within the promoter, it does possess the characteristics of a TATA-less promoter (9, 10). The basal promoter region is GC rich and contains multiple Sp-1 binding sites. Additionally, a CAAT box was identified as well as several activator protein-2 and C/EBP sites. The putative PPRE was found at −1085 to −1097 in the CAP promoter (Fig. 1B). The CAP promoter PPRE has a sequence of direct repeats of a hexamer (DR1) type similar to the PPREs from other genes, as shown in Table I (11, 12).

Identification of a Functional PPRE in the CAP Gene—To determine the functionality of the CAP promoter as well as its putative PPRE, transient reporter assays using various CAP promoter constructs (Fig. 3A) were performed in NIH3T3 fibroblasts. Luciferase fusion constructs were prepared by subcloning restriction fragments from the cloned CAP promoter into the pGL3 basic luciferase reporter vector as described under “Experimental Procedures.” Results obtained from reporter assays in NIH3T3 fibroblasts showed that the CAP promoter was functional (Fig. 3B). Fusion of the various CAP promoter constructs to the luciferase reporter produced a greater than 20-fold increase in luciferase activity, compared with the pGL3 basic vector alone, for all CAP promoter constructs tested (Fig. 3B). Co-transfection of PPARγ and RXRα with the fusion construct pCPE containing the putative CAP PPRE led to an additional fold stimulation of luciferase activity. Addition of rosiglitazone led to an additional 2–3-fold stimulation of luciferase activity from the pCPE fusion construct. In contrast, the addition of PPARγ and RXRα, with or without rosiglitazone, was ineffective in increasing the luciferase activity of the shorter pCPE and pCPS fusion constructs. Additionally, deletion of the region containing the PPRE of the CAP promoter (fusion construct pCPEΔPPRE) abolished the response to rosiglitazone.

To further characterize the activity of the CAP promoter and its PPRE, fusion constructs were electroporated into 3T3-L1 adipocytes. 3T3-L1 adipocytes contain high levels of endogenous PPARγ. Moreover, CAP expression in 3T3-L1 adipocytes is responsive to TZDs (4). Electroporated CAP reporter constructs pCPE and pCPS exhibited a 10-fold higher level of luciferase activity compared with pGL3 basic vector (Fig. 4). The pCPE construct containing the CAP PPRE produced an additional 2-fold stimulation of luciferase activity compared with pCPS. The activity of the pCPE fusion construct was 2-fold greater in the presence of 20 μM rosiglitazone. Deletion of the region of the CAP promoter containing the PPRE (pCPEΔPPRE) abolished both the increase in basal luciferase activity and the response to rosiglitazone.

**Gel Shift Analysis of PPAR/RXR Heterodimer Binding to the CAP PPRE—**To demonstrate direct binding of PPARγ/RXRα heterodimers to the CAP PPRE, gel shift analysis with the CAP PPRE was performed (Fig. 5A). A double-stranded oligonucleotide probe for wtCAP PPRE was end-labeled with 32P and incubated with in vitro translated proteins as well as 3T3-L1 nuclear extracts. As shown in Fig. 5B, neither PPARγ nor RXRα alone bound to the CAP PPRE oligonucleotide. However, PPARγ/RXRα heterodimers bound to the wtCAP PPRE oligonucleotide. This binding is specific because it could be competed with unlabeled wtCAP PPRE oligonucleotide. Incubation with either mutCAP PPRE (an oligonucleotide with a single-base deletion in the DR1 motif) or a nonspecific double-stranded oligonucleotide did not displace the labeled wtCAP PPRE oligonucleotide. Furthermore, nuclear proteins from 3T3-L1 adipocytes were able to form protein-DNA complexes with wtCAP PPRE with a specificity similar to that seen in the experiments done with the in vitro translated PPARγ/RXRα heterodimers (Fig. 5C). As described above, unlabeled wtCAP PPRE competed for DNA-protein complexes that were formed with nuclear proteins. mutCAP PPRE and nonspecific double-stranded oligonucleotide probes did not compete with the radiolabeled DNA-protein interaction.

| Gene                     | Species     | Element | Sequence            | Protein function                              |
|--------------------------|-------------|---------|---------------------|-----------------------------------------------|
| Acyl CoA oxidase         | Rat         | ACOA    | AGGACAAAGGTCA       | Peroxisomal β-oxidation                       |
| Acyl-CoA synthase        | Rat         | ACS(CI) | AGGTCACAGCTCA       | Peroxisomal β-oxidation                       |
| ALBP (aP2)               | Mouse       | ARE6    | GGGTGAATGGTC        | Fatty acid-binding protein                    |
| Apolipoprotein CIII      | Human       | APOCIII | TGGCAGAAGGTCA       | Triglyceride clearance                        |
| Bifunctional enzyme      | Rat         | BIF     | AGGTCCAGTTCT        | Peroxisomal β-oxidation                       |
| Cytochrome P450 A1       | Rat         | CYP4A1  | AGGGTAAAGTTCA       | ω-Oxidation                                   |
| Cytochrome P450 A6       | Rabbit      | CYP4A6  | AGGGGAAAAGTGA       | ω-Oxidation                                   |
| L-FABP                   | Rat         | FABP    | AGGCCATAGGTCA       | Fatty acid binding                           |
| Fatty acid transport protein | Mouse     | FATP    | GGCGGCAAGGGCA       | Fatty acid transport                          |
| HMG-CoA synthase         | Rat         | HMG     | GGGCGAAGGTCT        | Liver ketogenesis/sterol synthesis            |
| Lipoprotein lipase       | Rat         | LPL     | GGGGGAAGGGA         | Triglyceride clearance                        |
| Malic enzyme             | Rat         | MEP     | GGGTCAAGTTGA        | Fatty acid synthesis                          |
| Muscle-type carnitine    | Human       | MCPT1   | AGGGGAAAAGGTCA      | Fatty acid transport                          |
| Palmitoyltransferase     | Rat         | PKC1    | CGGCCCAAGGTCA       | Glycerogenesis and gluconeogenesis            |
| Uncoupling protein I     | Mouse       | URE1    | GGCTCAAGGGTCA       | Thermogenesis                                 |
| c-Cbl-associated protein | Mouse       | CAPRE   | AGGCTAAAGGTCA       | Insulin signal transduction                   |

* ALBP, adipocyte lipid-binding protein; L-FABP, L-fatty acid-binding protein; HMG, hydroxymethylglutaryl; PEPCK, phosphoenolpyruvate carboxykinase.
Numerous studies in animal models and patients with type 2 diabetes have established a direct link between PPAR activation by TZDs and insulin sensitivity (13). However, the precise mechanism by which TZDs improve insulin sensitivity remains uncertain. Most of the genes discovered to date that are PPAR-responsive primarily participate in lipid synthesis and/or clearance. Moreover, despite the profound improvement in insulin sensitivity observed after TZD treatment, few genes that play a role in insulin signaling are PPAR-responsive. We recently demonstrated that the transcription of CAP is directly increased in response to TZDs in both 3T3-L1 adipocytes and in Zucker Fatty Rats (4). We have extended and confirmed this finding with the cloning and characterization of the promoter of the CAP gene and its PPRE.

Cloning of the 5'-flanking region of the CAP gene led to the characterization of its promoter. The CAP gene has one start site for transcription, as identified by S1 nuclease protection and reverse transcription-polymerase chain reaction. This start of transcription lies 362 nucleotides upstream of the identified start of translation of the CAP protein. Whereas the identified CAP promoter lacks a TATA box, it possesses characteristics of a TATA-less promoter, including a GC-rich proximal sequence, a CAAT box, and several binding sites for the transcription factor Sp-1, which has been shown to activate TATA-less promoters (9, 10). Sp-1 has the ability to recruit cofactors, including TATA binding factors, which interact with transcription factor IID and initiate transcription. The CAP promoter also contains binding sites for the transcription factor activator protein-2 and several potential binding sites for the transcription factor Sp-1.
C/EBP family of transcription factors. C/EBP isoforms are expressed in a differentiation-dependent manner during 3T3-L1 adipocyte differentiation, with C/EBPδ and C/EPBβ expressed in preadipocytes, and C/EBPα expressed in adipocytes (14). These data complement the known differentiation-dependent expression of the CAP gene in 3T3-L1 adipocytes (2).

Sequence analysis of the CAP gene identified a PPRE of the DR1 type in the CAP gene. In reporter assays, the region of the CAP promoter containing the putative PPRE was necessary for the stimulation of luciferase activity by PPARγ activation. Additionally, gel shift analysis demonstrated specific and direct binding of the CAP PPRE to PPARγ/RXRα heterodimers. The protein-DNA complexes formed were sequence-specific for the CAP PPRE, as shown by competition analysis. Deletion of the spacing base of the DR1 motif in the CAP PPRE prevented binding competition with the CAP PPRE-PPARγ/RXRα complex. Taken together, these data indicate that TZD-stimulated CAP expression is mediated through direct binding of PPARγ to the CAP PPRE.

We have previously reported that TZDs increase the expression of CAP both in 3T3-L1 adipocytes and Zucker (fa/fa) diabetic rats. The increase in CAP expression leads to an increase in insulin-stimulated c-Cbl phosphorylation in 3T3-L1 adipocytes. This observation established the first direct link between TZD-mediated increases in insulin sensitivity and insulin signal transduction. We establish here that one mechanism by which TZD induces CAP expression is through direct binding of activated PPARγ/RXRα heterodimers to a PPRE in the CAP promoter. These observations further support the role of the CAP/c-Cbl interaction in insulin action and the link between PPARγ activation and insulin sensitivity. Future studies on the precise role of the CAP/c-Cbl pathway will lead to a greater understanding of the mechanism by which PPARγ activators improve insulin sensitivity.

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