Published in final edited form as:

Biochem Biophys Res Commun. 2002 April 26; 293(1): 274–283. doi:10.1016/S0006-291X(02)00138-9.

Genetic analysis of four novel peroxisome proliferator activated receptor- γ splice variants in monkey macrophages

Jiming Zhou^{a,b}, Katina M. Wilson^a, and Jheem D. Medh^{b,c,*}

^aDepartment of Internal Medicine, University of Iowa College of Medicine, Iowa City, IA 52242, USA

^bDepartment of Biotechnology, Sichuan University, Chengdu, Sichuan 610064, China

^cDepartment of Chemistry, Sc3112B, California State University at Northridge, 18111 Nordhoff Street, Northridge, CA 91330-8262, USA

Abstract

Peroxisome proliferator activated receptor- γ (PPAR- γ) is abundantly expressed in atherosclerotic lesions and is implicated in atherogenesis. The existence of three splice variants, PPAR- γ 1, PPAR- γ 2, and PPAR- γ 3 has been established. Using monocyte-derived macrophages from cynomolgus monkeys, we demonstrate here the identification of two new PPAR- γ exons, exon C and exon D, which splice together with already established exons A1, A2, and B in the 5' terminal region to generate four novel PPAR- γ subtypes, PPAR- γ 4, - γ 5, - γ 6, and - γ 7. PPAR- γ 4 and γ 5 were detected only in macrophages whereas γ 6 and γ 7 were expressed both in macrophages and adipose tissues. None of these novel isoforms were detected in muscle, kidney, and spleen from monkeys. We found sequences identical to exons C and D in the human genome database. These and all PPAR- γ exons known to date are encoded by a single gene, located from region 10498 K to 10384 K on human chromosome 3. We cloned and expressed PPAR- γ 1, PPAR- γ 4, and PPAR- γ 5 proteins in yeast using the expression vector pPICZB. As expected, all recombinant proteins showed a molecular weight of approximately 50 kDa. We also investigated the effect of a high-fat diet on the level of macrophage PPAR- γ expression in monkeys. RT-PCR showed a significant increase in total PPAR- γ and ABCA1 mRNA levels in macrophages of fat-fed monkeys ($n = 7$) compared to those maintained on a normal diet ($n = 2$). However, none of the novel isoforms seemed to be induced by fat-feeding. We used tetracycline-responsive expression vectors to obtain moderate expression of PPAR- γ 4 and - γ 5 in CHO cells. In these cells, expression of PPAR- γ 5 but not - γ 4 repressed the expression of ABCA1. Neither isoform modulated the expression of lipoprotein lipase. Our results suggest that individual PPAR- γ isoforms may be responsible for unique tissue-specific biological effects and that PPAR- γ 4 and - γ 5 may modulate macrophage function and atherogenesis.

Keywords

Macrophages; Atherosclerosis; PPAR- γ isoforms; Alternative splicing; Tissue-specific expression

Peroxisome proliferator activated receptors (PPAR) are members of the nuclear-receptor gene superfamily of transcription factors and are divided into three broad classes, PPAR- α , PPAR- δ , and PPAR- γ . Lipid-lowering agents such as fibrates and insulin-sensitizing thiazolidinediones are, respectively, specific ligands for PPAR- α and PPAR- γ [1,2]. Upon activation by ligand-binding, PPARs bind to a specific DNA sequence called the peroxisome

proliferator regulatory element (PPRE) and regulate the expression of genes involved in a variety of physiological functions. PPAR- γ is the most extensively studied of the three PPAR subtypes and is a key regulator of adipocyte differentiation and carbohydrate metabolism [3]. Thus it has been studied mostly in adipocytes. PPAR- γ is also implicated in dyslipidemia, inflammation, colon tumors, and atherosclerosis. In view of the crucial role of macrophages in the atherogenic process, recent studies have focused on the role of PPAR- γ in macrophages where it is expressed in a differentiation-dependent manner. Activation of PPAR- γ induces macrophage lipid accumulation and foam cell formation. It has been shown that PPAR- γ is present in macrophage foam cells within atherosclerotic lesions but not in normal vascular tissue [4]. PPAR- γ regulates the transcription of several macrophage genes including cytokines, CD36, ABCA1, gelatinase B, iNOS, and apoptosis-inducing genes [5–7]. Thus PPAR- γ is multi-functional. It also exists as multiple isoforms and it is likely that each specific isoform is responsible for a unique function.

Like all nuclear receptors, PPARs possess three major structural domains; the NH₂-terminal domain, the DNA-binding domain (DBD), and the ligand-binding domain (LBD). The DBD of all nuclear receptors is very highly conserved and consists of two zinc fingers that facilitate binding to DNA regulatory elements. Variability in the LBDs of PPAR subtypes is responsible for their unique ligand specificity, biological function, and classification into subtypes. Thus, PPARs are targets of various structurally diverse pharmacological agents.

The PPAR- γ gene consists of exons 1–6 in the open reading frame (ORF). Exons 2 and 3 encode the DBD and exons 5 and 6 encode the LBD. The 5' terminal region is the most variable and is the determinant of PPAR- γ isoform. Currently three exons have been identified in the 5'-terminal region. These are referred to as exon A1, exon A2, and exon B. Two highly conserved PPAR- γ mRNA isoforms, PPAR- γ 1 and PPAR- γ 2, have long been identified in many species including the rhesus monkey. PPAR- γ 1, the most common isoform, consists of exons A1 and A2 spliced together with exons 1–6 of the ORF. The PPAR- γ 2 mRNA consists of exon B and exons 1–6. The NH₂-terminus of PPAR- γ 2, coded by exon B, has additional amino acids (30 in mouse and monkey, 28 in human) that are not present in PPAR- γ 1 [8–11]. Recently, mRNA for a third isoform, named PPAR- γ 3, was cloned in humans [12]. The 5' UTR of this isoform consists of only exon A2. However, since the transcription initiating ATG is in exon 1, the predicted amino-acid sequences of γ 1 and γ 3 are identical. Interestingly, the tissue-specific distribution of γ 3 is more restricted compared to that of γ 1, indicating unique enhancer–promoter elements [12].

In this study, we have identified and sequenced two novel exons in PPAR- γ cDNA from monkey macrophages, which we have called exon C and exon D. We have shown that each of these novel exons combine with either exons A1–A2 or with exon B to form four currently undescribed PPAR- γ isoforms which we have called γ 4, γ 5, γ 6, and γ 7. Thus, the PPAR- γ gene encodes for at least seven unique isoforms resulting from alternative splicing of five exons at the 5'-terminal region. It is likely that the presence of multiple isoforms allows for the multiplicity of PPAR- γ functions.

We have cloned and expressed PPAR- γ 1, PPAR- γ 4, and PPAR- γ 5 proteins in yeast using the expression vector pPICZB. Using tetracycline-responsive expression vectors, we have expressed PPAR- γ 4 and - γ 5 in CHO cells. Our results indicate that PPAR- γ 5 may be a negative regulator of ABCA1 expression. On the other hand, we found that in monkey macrophages, a high-fat diet induced the expression of total PPAR- γ as well as ABCA1 mRNA levels. Thus, we hypothesize that each of the PPAR- γ isoforms is responsible for a unique physiological effect. Complete characterization of the structure and expression of each isoform will greatly facilitate identification of the biological function associated with each of them.

Materials and methods

Monkey macrophages

Monkey monocyte-derived macrophages were isolated from blood samples (80 ml each) obtained from cynomolgus monkeys maintained on a normal or atherosclerotic (21% w/w fat) diet for 12 months. Atherosclerotic monkeys with visible aortic lesions showed a plasma cholesterol level of 300–600 mg/dl compared to < 100 mg/dl in normal monkeys. The macrophages were separated from lymphocytes by centrifugation on a Ficoll (Sigma Chemical) density gradient. The isolated monocytes were differentiated into macrophages by culturing them for seven days in media containing 20% autologous serum. We routinely obtained $\sim 5 \times 10^6$ cells and $\sim 3 \times 10^6$ cells, respectively, from atherosclerotic and normal monkeys. After one week in culture, the cells were harvested in Tri Reagent (Sigma Chemical) for isolation of total RNA according to instructions provided with the reagent.

Isolation and sequencing of PPAR- γ isoforms

We used total monkey macrophage RNA as a template to obtain first-strand cDNA using primer set 1 (Table 1) and Superscript RNase H⁻ reverse transcriptase (Stratagene, La Jolla, CA). Subsequently, we obtained double-stranded (ds) cDNA bearing adapters by long-distance PCR (94 °C, 2 min; 94 °C, 15 s, 68 °C, 6 min, 20 cycles) using primer set 2 (Table 1). Using cDNA obtained from fat-fed monkeys as template, we were able to isolate cDNAs encoding seven distinct PPAR- γ isoforms by PCR amplification. We used different primer sets and two different cycles (1. Two step PCR: using CloneTech Advantage cDNA polymerase, 94 °C 2 min; 94 °C 30 s, 68 °C 3 min, 18–25 cycles. 2. Three step PCR: using Taq DNA polymerase and primer sets 3, 4, and 5 shown in Table 1, 95 °C 1 min; 95 °C 30 s, 45–60 °C 30 s, 72 °C 30 s to 1 min, 20–35 cycles; 72 °C 10 min). Next, we purified PCR products (designated partial length of PPAR- γ isoforms) and cloned them into pCR4-TOPO for transformation into *Escherichia coli* TOP10 competent cells (Invitrogen, Carlsbad, CA). Plasmids were isolated by minipreps (Promega, Madison, WI). For each isoform, we selected five clones at random, and subjected the rescued plasmids to automated double-strand sequencing at the University of Iowa DNA Facility using a 373S Fluorescent Automated Sequencer (Perkin–Elmer Applied Biosystems, Foster City, CA). This approach yielded four novel full-length PPAR- γ cDNAs, designated PPAR- γ 4 to - γ 7.

PPAR- γ isoform expression analysis

Using different ds-cDNAs as template and specific PPAR- γ isoform primer sets 6–11 (Table 1), we performed RT-PCR amplification to detect PPAR- γ isoform transcripts in macrophages and different tissues from normal monkeys. The house-keeping gene β -actin was used as a control. All the primers used in this research are listed in Table 1.

Sequence analyses

We analyzed the homology between various DNA sequences using the BLAST software available at the NIH website. The human PPAR- γ sequence was confirmed from the human genome resource of the National Center for Biotechnology Information. Nucleotide and deduced amino acid sequences were analyzed with the University of Wisconsin Genetics Computer Group software package (GCG, Devereux et al., 1984), and pDRAW32 software (AcaClone Software).

Construction of expression vectors and *Pichia* transformation

Using PCR amplification (primer sets 12 and 13, Table 1) we obtained cDNA for PPAR- γ 1, - γ 4, and - γ 5, each containing the 5' UTR and the full-length ORF but without the TGA stop codon. The amplified product was purified using the GENECLON II kit (Bio101, Vista, CA)

and cloned into pCR4-TOPO plasmid following supplier's instructions. After partial sequencing using T7 and T3 primers, the PPAR- γ cDNAs were excised by *Xho*I and *Xba*I digestion (restriction enzyme sites were provided by primers). They were then purified with the GENECLON II kit and cloned into the *E. coli/Pichia* shuttle vector pPICZB (Invitrogen, Carlsbad, CA) which was previously digested using the same restriction enzymes. The ligation product was transformed into competent *E. coli* Top10 cells cultured on LB plates containing Zeocin. Plasmids were isolated from 10 Zeocin-resistant transformants. Restriction enzyme digestion and partial sequencing identified the desired direction of insert and the PPAR- γ -pPICZB plasmid DNA was purified. Next, the plasmid was linearized using restriction enzyme *Pme*I to allow for integration of the vector DNA into the *Pichia* chromosome by homologous crossover. *Pichia* transformations were performed using the Easycomp kit (Invitrogen) and *Pichia* Km71H as host cells. Transformants were cultured in the dark at 30 °C on YPDS plates containing 100 μ g/ml Zeocin for 2–4 days.

Expression and purification of recombinant PPAR- γ proteins

Single Zeocin-resistant colonies were selected to inoculate 10 ml BMGY medium in 50 ml conical tubes. Cultures were grown in a shaking incubator (300 rpm) at 30 °C until the OD₆₀₀ reached 2–6 (approximately 18 h). Cells were harvested by centrifugation at 3000 rpm for 5 min and resuspended in BMMY medium at an OD₆₀₀ of 1.0. Cultures were maintained under the same conditions except for addition of methanol (0.5% final concentration) every 24 h to induce expression. After 96 h cell pellets were obtained by centrifugation for 5 min at 14,000 rpm and were stored at 80 °C for subsequent assays. Control cultures (KM71H cells transformed with empty pPICZB vector) were incubated and induced by identical methods. Cell lysates were obtained upon shearing cells with 0.5 mm glass beads. Recombinant PPAR- γ proteins with poly-histidine tags were purified by affinity chromatography using ProBond resin (Invitrogen) under native conditions. The purified proteins were concentrated using Amicon centricon-30 concentrator, resolved by SDS-PAGE, and subjected to western blot analysis using anti-myc antibody (Invitrogen).

Construction of tetracycline-responsive expression vectors

Chinese hamster ovary cells (CHO-AA8) were purchased from CLONTECH, and grown in α -MEM (GIBCO) containing 10% tetracycline-free fetal bovine serum. Full-length cDNAs of PPAR- γ 1, - γ 4, and - γ 5 were obtained by PCR, using primer set 14 (Table 1), and PPAR- γ isoform-specific cDNAs as template. After being digested by *Nhe*I and *Not*I, the fragments were cloned into the tetracycline-responsive pTRE2hyg expression vector (CLONTECH). Following the protocol described above, the constructed vectors were cloned into Top10 cells and analyzed by partial sequencing.

Transient transfection and expression analysis of pTRE2hyg-PPAR- γ s

Transient transfection was performed in CHO-AA8 Tet-off cells using Calphos Mammalian Transfection Kit (CLONTECH). Transfected cells were incubated for 48 h in the same media and treated for 24 h with 5 μ M 15d-PGJ₂, which is a PPAR- γ -specific ligand. Total RNA and ds-cDNA were prepared as described above, and used for expression analysis by virtual RT-PCR amplification.

Accession numbers

The cDNA sequences of all of the seven PPAR- γ isoforms found in monkey macrophages have been entered in the GenBank database. The accession numbers for PPAR- γ 1 to - γ 7 are, respectively, AY048694–AY048700.

Results

In recent years, it has become clear that PPAR- γ is an important player in the pathophysiology of various metabolic and inflammatory disorders including obesity, diabetes, and atherosclerosis [4,13–20]. Similar to other nuclear receptors, PPAR- γ is a transcription factor and modulates the expression of innumerable effector genes. The exact mechanism by which PPAR- γ maintains specificity for its effectors is not clear. Most likely, this is brought about by the existence of multiple isoforms, each of which may have a preference for a specific effector gene. In the present study, we have used molecular approaches to analyze the micro-heterogeneity of the monkey PPAR- γ gene. The ORF region of the PPAR- γ gene, including sequences encoding the DNA-binding domain and the LBD, is highly conserved. Table 2 illustrates that PPAR- γ 1 protein from five different animal species is highly conserved and shares 97–99% amino acid identity. We found that the 5' UTR consists of five exons that were alternatively spliced together to generate multiple isoforms.

Identification and characterization of four novel PPAR- γ isoforms in cynomolgus monkey macrophages

Total RNA isolated from monocyte-derived macrophages of cynomolgus monkeys was used to synthesize total cDNA and a PCR-based gene cloning protocol was employed to purify PPAR- γ -specific cDNA. Given the high DNA identity (over 97.9%) shared by different known species of PPAR- γ , we designed a degenerate primer set 3 (Table 1) based on conserved sequences by DNA alignment analysis. This yielded a 450-bp fragment that was cloned, double-strand sequenced, and designated partial-length monkey PPAR- γ . This cDNA fragment shares 99% amino acid identity with human PPAR- γ . Next, we used cDNA from fat-fed monkey macrophage as templates and primer sets 4 and 5 (Table 1) to perform several PCR amplifications followed by sub-cloning and sequencing. A number of cDNAs corresponding to PPAR- γ transcripts expressed in fat-fed monkey macrophages were obtained. In addition to three previously identified PPAR- γ isoforms in humans, four novel PPAR- γ cDNAs (designated as PPAR- γ 4 to PPAR- γ 7) were isolated, major features of which are summarized in Table 3. The cDNA sequences of all of the seven PPAR- γ isoforms found in monkey macrophages have been entered in the GenBank database. The accession numbers for PPAR- γ 1 to - γ 7 are, respectively, AY048694–AY048700.

In earlier studies, two PPAR- γ isoforms, namely, - γ 1 and - γ 2, showing high homology and over 90% identity, were isolated from several species including humans [8–11]. More recently, a third isoform, PPAR- γ 3 was reported in humans [12]. The present study demonstrates that the four novel PPAR- γ isoforms reported here also share high sequence identity with members of PPAR- γ subfamily except in the 5'-terminal region. The 5'-terminal region is composed of different splice variants involving five exons, two of which are identified for the first time in this report and designated as exon C (263 bp) and exon D (84 bp). The four novel isoforms result from the inclusion of these new exons in the 5' UTR. With this finding, the PPAR- γ subfamily consists of at least seven members. The structures of all seven PPAR- γ isoforms are shown schematically in Fig. 1A. They can be arranged in two groups depending on the presence of either exons A1/A2 or exon B. As shown in the figure, the ORF region encoded by exons 1–6 remains identical in all isoforms. The translation initiating ATG for each isoform is also shown. While there is an ATG in exon B, this site is inactivated by stop codons present in both exons C and D. Thus, exon B contributes 30 NH₂-terminal amino acids in monkey PPAR- γ 2 but not in PPAR- γ 4 and PPAR- γ 5. A translation initiating ATG is also present in exon C resulting in the addition of eight amino acids to the NH₂-terminus of PPAR- γ 4 and PPAR- γ 6.

In addition to these four novel isoforms, we have identified four missense point mutations of PPAR- γ transcript from fat-fed monkey macrophages, but no mutations at all in macrophages from monkeys fed a normal diet. The identified mutations include two single mutations (AAT-

AGA Asn206Ser and TAC-CAC Try192His) and two double mutations (GTG-GCG Val201Ala + ATC-ACC Iso290Thr and GAC-GGC Asp218Gly +GAC-GGC Asp748Gly), which have never been reported before.

The genomic structure analysis of PPAR- γ genes

Relatively little is known about the exon–intron organization of PPAR- γ genes. Early studies in mice and humans have proposed that PPAR- γ transcripts are products of nine exons of a single gene [1,8,12] (Fig. 1B). Transcription is regulated by different promoters (shown by black arrows) resulting in variable 5'-terminal regions.

Though monkey genomic sequences are unavailable, we took advantage of the recently released human genome database (NCBI website: <http://www.ncbi.nlm.nih.gov>) and compared our cDNA sequence data with human genomic sequences based on the relatively high conservation between monkey and human species. As expected, we were able to align cDNAs for all of the monkey PPAR- γ isoforms with regions of the human genome. All PPAR- γ sequences were encoded by a single gene, divided into 11 exons and 10 introns, and located in a reasonable serial sequence from region 10498K to 10384K on human chromosome 3 (Fig. 1C). Due to the continuous update of human genome data, the current location may shift slightly from the specifics reported here, however, we do not expect a significant change.

Based on the arrangement of aligned sequences in the human genome database, we have put together a schematic of the monkey PPAR- γ gene as shown on the bottom of Fig. 1B. Accordingly, exons are arranged in the following sequence in the gene: B, A1, A2, C, D, 1–6. The arrows above the gene indicate the currently known promoters/transcription start sites in mouse, human, and monkey PPAR- γ genes. The promoters for novel isoforms of monkey PPAR- γ reported here are shown below the gene. Isoforms PPAR- γ 4–PPAR- γ 7 have not been isolated from humans yet. Based on the high homology of human and monkey PPAR- γ 1 to - γ 3, and analysis of the human genome database, we predict that these novel isoforms also exist in humans.

Tissue-specific expression of novel PPAR- γ isoforms

Initially, we cloned all the novel PPAR- γ isoforms from fat-fed monkey macrophages. Next, we investigated if these were expressed in normal monkey macrophages or were induced as a result of fat-feeding. RT-PCR using isoform-specific primers showed that all four novel isoforms were also expressed in normal monkey macrophages. Representative data are shown in Fig. 2A.

Once we had ascertained that PPAR- γ 4 to - γ 7 are expressed in normal as well as fat-fed monkey macrophages, we investigated their expression in various tissues. We obtained cDNA from adipose tissue, skeletal muscle, kidney, and spleen from a normal monkey. Each of the PPAR- γ isoforms was amplified using isoform-specific primers and tissue cDNA as template. As shown in Fig. 2B, PPAR- γ 6 and - γ 7 were detected in adipose tissue but not in muscle, kidney or spleen. PPAR- γ 4 and - γ 5 are expressed only in macrophages (Fig. 2A) and were not detected in any of the tissues tested (data not shown). Both β -actin and total PPAR- γ (amplified using primers in the ORF region which is identical for all isoforms) were expressed in all of the tissues tested. The restricted expression pattern of four novel PPAR- γ isoforms suggests that each isoform may affect distinct functions.

Expression of recombinant PPAR- γ recombinant protein in *Pichia pastoris*

To assess whether these novel PPAR- γ isoform cDNAs are active transcripts, we constructed the full-length cDNA of PPAR- γ 1, - γ 4, and - γ 5 into eukaryotic expression vector pPICZB for translation in yeast. We chose *Pichia pastoris* for this purpose, as it is easy to use and utilizes

most of the posttranslational modification pathways typically associated with higher eukaryotes. Recombinant PPAR- γ proteins were expressed and isolated as described in 'Materials and methods'. The proteins were purified using ProBond resin (Invitrogen) in native condition, concentrated by Amicon centricon-30 concentrator, and resolved by SDS-PAGE. The identity of the recombinant protein was confirmed by Coomassie blue staining (Fig. 3A) which showed a band of 50 kDa as predicted by the cDNA sequence. The expression vector contributes a myc domain to recombinant proteins, thus, an anti-myc antibody was used to identify recombinant proteins by western blot analysis. A 50 kDa band detected using anti-myc was consistent with the 50 kDa band seen by Coomassie staining (Fig. 3B). These data confirm that recombinant proteins were expressed successfully and showed a molecular weight of approximately 50 kDa [18,21]. To our knowledge, this is the first report of the expression of full-length PPAR- γ protein in yeast which is suitable for large-scale production and protein structure analysis.

PPAR- γ expression is induced by fat-feeding

We compared the level of expression of PPAR- γ in macrophages from normal and fat-fed monkeys. We amplified total PPAR- γ using ds-cDNA prepared from monkey macrophages as a template and primer set 3 (Table 1) that was degenerate for all PPAR- γ isoforms. Representative results are shown in Fig. 2C. We found that the expression of total PPAR- γ was significantly higher in fat-fed monkey macrophages than in macrophages from normal monkeys. The data were analyzed quantitatively using Image J from NIH, results are shown alongside Fig. 2C. After correcting for β -actin levels, there is a 15–50-fold induction of PPAR- γ expression in monkey macrophages due to fat-feeding. This could be a proatherogenic effect or an adjustment to fat-feeding. Similarly, expression of ABCA1, the recently described cholesterol transporter, was also induced by fat-feeding (Fig. 2C). Quantitative analysis indicated that ABCA1 expression increased from 6–140-fold. This increase may be a direct consequence of cholesterol loading or may be an indirect effect of PPAR- γ induction. The expression of β -actin is also shown as a control for uniformity of total RNA quantity and gel-loading. We did not find any effect of fat-feeding on macrophage LPL expression (data not shown).

Transient transfection of individual PPAR- γ isoforms in CHO cells

Our data show that fat-feeding significantly induced macrophage PPAR- γ expression and ABCA1 expression, concomitant with the development of significant atherosclerotic lesions. PPAR- γ has been implicated in the atherogenic process because of its modulation of various genes related to metabolic and inflammatory disorders. With the identification of novel PPAR- γ isoforms, we hypothesized that individual isoforms may target different genes. We focused on the two isoforms, PPAR- γ 4 and γ 5, which appear to be expressed in a macrophage-specific manner. We constructed the mammalian expression vector pTRE2hyg with inserts for the expression of full-length PPAR- γ 4 and PPAR- γ 5. PPAR- γ 4-pTRE2hyg or PPAR- γ 5-pTRE2hyg was transiently transfected into CHO-AA8 cells. The presence of PPAR- γ transcript was confirmed by RT-PCR. Quantitative analysis by the NIH Image J software indicated that transfected cells expressed 25–30-fold greater PPAR- γ , presumably the transfected isoform, compared to control cells transfected with vector alone. After activation of transfected cells with the PPAR- γ ligand 15d-PGJ₂, ds-cDNA was produced and subjected to RT-PCR analysis using primers specific for different relevant genes as shown in Table 1. RT-PCR shows a 4-fold reduction of ABCA1 transcript after PPAR- γ 5-pTRE2hyg expression compared to mock transfected cells (Fig. 4). There was no significant effect of PPAR- γ 4 expression on ABCA1 mRNA levels. Neither PPAR- γ 4 nor - γ 5 altered LPL expression levels. Also, we found that scavenger receptors, CD36 and SR-A (types I, II, and III), were not expressed in control or transfected CHO cells. We are currently evaluating isoform-specific modulation of other target genes.

Discussion

The data presented here confirm the existence of at least seven PPAR- γ isoforms in monkey macrophages. It suggests the possibility for more, as yet unidentified isoforms, resulting from alternative splicing of the five exons in the 5'-terminal region. Furthermore, based on the human genomic sequence data, we anticipate identification of similar isoforms in humans as well.

The physiological implications of multiple isoforms of PPAR- γ are not completely understood. Reports from different investigators over the past few years have ascertained that PPAR- γ activation leads to the modulation of several genes, some of which have opposing functions [4,7,11,22]. Ligand activation of PPAR- γ induces expression of CD36 while it inhibits induction of scavenger receptor A, both mediators of oxLDL uptake [4–6,15]. PPAR- γ also inhibits the expression of various inflammatory cytokines, but induces iNOS, which has proinflammatory and cytotoxic functions [15,16]. Additionally, PPAR- γ induces apoptosis and cell necrosis in macrophages. The multiplicity in PPAR- γ isoforms may contribute to the large variety of genes and biological functions affected. The mechanisms by which isoforms impart their specificity remain to be determined.

PPAR- γ isoforms may impart specificity of function at the level of both mRNA transcript and protein product. The seven isoforms of PPAR- γ transcript encode for three different forms of the protein. PPAR- γ 1, - γ 3, - γ 5, and - γ 7 each have a different 5'-UTR nucleotide sequence, but share the same protein sequence consisting of 475 amino acids. PPAR- γ 4 and - γ 6 have eight additional amino acids at the NH₂ terminus encoded by exon C. PPAR- γ 2 has 30 extra amino acids at the NH₂-terminus contributed by exon B. This variable 5' region of the protein is not part of the LBD or the DBD, which are highly conserved between all isoforms [1,2,8–12]. However, it may contribute significantly to biological function independently of the LBD and DBD [23].

Isoform-specific functions may also be a consequence of differences in mRNA sequence. It is interesting that most of the variability between isoforms is located in the 5' untranslated region. This region may bind regulatory proteins that dictate the extent of translation and mRNA splicing. The 5' sequences may also determine targeting of the protein product to regulatory elements of specific effector genes. We expect that the region upstream of the 5' UTR coding region contains promoter sequences that may determine tissue-specific expression of individual isoforms. Additionally, the 5'-UTR is thought to contain the message for post-translational protein modification. Thus, PPAR- γ 1, - γ 3, - γ 5, and - γ 7 have identical protein amino acid sequence, but each may have a different pattern of posttranslational protein modification. All of these mechanisms may lead to unique protein folding, ligand specificity, and effector genes. Interestingly, exon B is in the coding region of PPAR- γ 2, but it is a part of the untranslated region in PPAR- γ 4 and - γ 5. This kind of functional switching in a single gene has not been previously reported. This apparent overlap between the 5'UTR and ORF suggests new, non-traditional functions for the 5'-region of mRNA, which needs to be further investigated.

Recent reports have provided compelling evidence for PPAR γ involvement in arterogenesis and obesity. However, the contributions of specific isoforms have not been extensively studied. PPAR- γ 2 is known to be the predominant isoform in adipocytes [11,24,25], and shows significant positive correlations with obesity [13,22,25,26]. However, additional reports suggest that other PPAR- γ isoforms besides - γ 2 may also contribute towards obesity [4,7,15, 27,28]. Recently, macrophage PPAR- γ has been implicated as an active participant in the atherogenic process [4,7,15,19,29–31]. Thus, our finding that the two novel isoforms containing exon B, PPAR- γ 4, and - γ 5 were only expressed in macrophages, while PPAR- γ 6 and - γ 7 were detected in adipose tissue as well, may suggest that PPAR- γ 4 and - γ 5 play a role

in arterogenesis, while PPAR- γ 6 and - γ 7 may be involved in obesity. All of the novel isoforms were detected in macrophages but none in muscle, kidney, or spleen indicating a role in macrophage-specific function such as regulation of inflammation, immune response, and atherogenesis [32,33].

The tight linkage of obesity, insulin resistance, and arterogenesis is well established. In recent years, thiazolidinediones including troglitazone, rosiglitazone, and pioglitazone were approved for use as insulin-sensitizing anti-diabetic pharmaceutical agents. It is well known that PPAR- γ is the molecular target of these compounds [29,34]. In view of the fatal side effects which led to the withdrawal of troglitazone, the varied biological functions of PPAR- γ , and the multiple isoforms of PPAR- γ reported here, it is extremely important to analyze the transcriptive effect of troglitazone, rosiglitazone, and pioglitazone on individual PPAR- γ isoforms.

While there is no evidence as yet that the various PPAR- γ isoforms have different ligand specificities, we have preliminary evidence that different isoforms have varying effects on individual genes. Using transient transfection in CHO cells, we have shown that PPAR- γ 5 downregulates the expression of ABCA1 but PPAR- γ 4 does not. On the other hand, one recent report suggests that activation of PPAR- γ leads to the induction of macrophage ABCA1 expression [7]. The specific isoform responsible in this instance has not been determined. The overall direction of regulation of each gene is probably dependent on the relative abundance of opposing isoforms.

Results reported here also indicate an induction of total PPAR- γ by fat-feeding in monkeys. These monkeys displayed significant atherosclerotic lesions, though it is not clear if the lesions are the cause or the effect of PPAR- γ induction. We were not able to determine which specific isoform was induced. There was a concomitant increase in ABCA1 expression, which is thought to be atheroprotective because it facilitates cholesterol removal from macrophages. However, cholesterol loading of macrophages is also known to upregulate ABCA1 expression, which is possibly independent of PPAR- γ . Thus, results presented here and reported by other investigators implicate PPAR- γ in regulation of ABCA1 expression [7]. Based on all of our data, we hypothesize that the macrophage-specific isoforms PPAR- γ 4 and - γ 5 are important in atherosclerosis whereas PPAR- γ 6 and - γ 7 may play a significant role in obesity. However, we found that macrophage LPL expression was not altered by fat-feeding or by PPAR- γ 4 and γ 5 overexpression in CHO cells.

PPAR- γ is a highly conserved gene in which polymorphisms are rare. So far only three mutations have been identified in the PPAR- γ gene [35–38]. Many studies have investigated these mutations extensively, with the hypothesis that they may be related to metabolic disorders such as obesity and diabetes. However, the results are contradictory and it has not been possible to establish a clear relationship between the presence of a mutation and a pathophysiological condition [35–38]. In our studies, we have consistently found polymorphisms in fat-fed monkeys at an extremely high frequency in spite of the small sample size. Additionally, double mutations have never been reported before, but we have found two. This suggests that fat-feeding may introduce errors in transcription and offers a new area for investigation. Further studies using genetically engineered systems are required to fully understand PPAR- γ and its biological functions.

Acknowledgments

We thank Drs. Francis J. Miller Jr. and Donald D. Heistad for providing blood samples from normal and fat-fed monkeys. The work was supported by program project grant HL49264 from the National Institutes of Health and a grant-in-aid award (to JDM) from the American Heart Association. We thank Dr. Ronald E. Law (UCLA) for a critical review of the manuscript and helpful suggestions.

References

- Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocrine Rev* 1999;20:649–688. [PubMed: 10529898]
- Weatherman RW, Fletterick RJ, Scanlan S. Nuclear-receptor ligands and ligand-binding domains. *Annu. Rev. Biochem* 1999;68:559–581. [PubMed: 10872460]
- Willson TM, Brown PJ, Sternbach DD, Henke BR. The PPARs: from orphan receptors to drug discovery. *J. Med. Chem* 2000;43:527–550. [PubMed: 10691680]
- Tontonoz P, Nagy L, Alvarez J, Thomazy VA, Evans RM. PPAR γ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* 1998;93:241–252. [PubMed: 9568716]
- Moore KJ, Rosen ED, Fiizgerald ML, Randow F, Andersson LP, Altshuler D, Milstone DS, Mortensen RM, Spiegelman BM, Freeman MW. The role of PPAR- γ in macrophage differentiation and cholesterol uptake. *Nature Med* 2001;7:41–47. [PubMed: 11135614]
- Chawla A, Barak Y, Nagy L, Liao D, Tontonoz P, Evans RM. PPAR- γ dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. *Nature Med* 2001;7:48–52. [PubMed: 11135615]
- Chinetti G, Lestavel S, Bocher V, Remaley AT, Neve B, Torra IP, Teissier E, Minnich A, Jaye M, Duverger N, Brewer HB, Fruchart J, Clavey V, Staels B. PPAR- α and PPAR- γ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nature Med* 2001;7:53–58. [PubMed: 11135616]
- Zhu Y, Qi C, Korenberg JR, Chen X, Noya D, Rao MS, Reddy JK. Structural organization of mouse peroxisome proliferator-activated receptor γ (mPPAR γ) gene: alternative promoter use and different splicing yield two mPPAR γ isoforms. *Proc. Natl. Acad. Sci. USA* 1995;92:7921–7925. [PubMed: 7644514]
- Elbrecht A, Chen Y, Cullinan CA, Hayes N, Leibowitz MD, Moller DE, Berger J. Molecular cloning, expression and characterization of human peroxisome proliferator-activated receptors γ 1 and γ 2. *Biochem. Biophys. Res. Commun* 1996;224:431–437. [PubMed: 8702406]
- Fajas L, Auboeuf D, Raspe E, Schoonjans K, Lefebvre A, Saladin R, Najib J, Laville M, Fruchart J, Deeb S, Vidal-Puig A, Flier J, Briggs MR, Staels B, Vidal H, Auwerx J. The organization, promoter analysis, and expression of the human PPAR γ gene. *J. Biol. Chem* 1997;272:18779–18789. [PubMed: 9228052]
- Hotta K, Gustafson TA, Yoshioka Y, Ortmeyer HK, Bodkin NL, Hansen BC. Relationships of PPAR γ and PPAR γ 2 mRNA levels to obesity diabetes and hyperinsulinaemia in rhesus monkeys. *Internal. J. Obesity* 1998;22:1000–1010.
- Fajas L, Fruchart J, Auwerx J. PPAR γ 3 mRNA: a distinct PPAR γ mRNA subtype transcribed from an independent promoter. *FEBS* 1998;438:55–60.
- Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR γ 2, a lipid-activated transcription factor. *Cell* 1994;79:1147–1156. [PubMed: 8001151]
- Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-Deoxy- $\Delta^{12;14}$ -prostaglandin J₂ is a ligand for the adipocyte determination factor PPAR γ . *Cell* 1995;83:803–812. [PubMed: 8521497]
- Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptors- γ is a negative regulator of macrophage activation. *Nature* 1998;391:79–82. [PubMed: 9422508]
- Jiang C, Ting AT, Seed B. PPAR- γ agonists inhibit production of monocyte inflammatory cytokines. *Nature* 1998;391:82–86. [PubMed: 9422509]
- Nagy L, Tontonoz P, Alvarez J, Chen H, Evans RM. Oxidized LDL regulates macrophage gene expression through ligand activation of PPAR γ . *Cell* 1998;93:229–240. [PubMed: 9568715]
- Ricote M, Huang J, Fajas L, Li A, Welch J, Najib J, Witztum JL, Auwerx J, Pallinski W, Glass CK. Expression of the peroxisome proliferator-activated receptors γ (PPAR γ) in human atherosclerosis and regulation in macrophages by colony stimulation factors and oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. USA* 1998;95:7614–7619. [PubMed: 9636198]
- Li AC, Brown KK, Silvestre MJ, Willson TM, Palinski W, Class CK. Peroxisome proliferator-activated receptors γ ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. *J. Clin. Invest* 2000;106:523–532. [PubMed: 10953027]

20. Takata Y, Kitami Y, Okura T, Hiwada K. Peroxisome proliferator-activated receptors- γ activation inhibits interleukin-1 β -mediated platelet-derived growth factors- α receptor gene expression via CCAAT/Enhancer-binding protein- δ in vascular smooth muscle cells. *J. Biol. Chem* 2001;276:12893–12897. [PubMed: 11278956]
21. Mukherjee R, Jow L, Croston GE, Paterniti JR. Identification, characterization, and tissue distribution of human Peroxisome proliferator-activated receptor (PPARs) isoforms PPAR γ 2 versus PPAR γ 1 and activation with retinoid X receptor agonists and antagonists. *J. Biol. Chem* 1997;272:8071–8076. [PubMed: 9065481]
22. Vidal-Puig A, Jimenez-Linan M, Lowell BB, Hamann A, Hu E, Spiegelman B, Flier JS. Regulation of PPAR γ gene expression by nutrition and obesity in rodents. *J. Clin. Invest* 1996;97:2553–2561. [PubMed: 8647948]
23. Werman A, Hollenberg A, Solanes G, Bjorbaek C, Vidal-Puig AJ, Flier JS. Ligand-independent activation domain in the N-terminus of peroxisome proliferator-activated receptors γ (PPAR γ). *J. Biol. Chem* 1997;272:20230–20235. [PubMed: 9242701]
24. Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM. mPPAR γ 2: tissue-specific regulator of an adipocyte enhancer. *Gene Dev* 1994;8:1224–1234. [PubMed: 7926726]
25. Vidal-Puig A, Considine RV, Jimenez-Linan M, Werman A, Pories WJ, Caro JF, Flier JS. Peroxisome proliferator-activated receptor gene expression in human tissues. *J. Clin. Invest* 1997;99:2416–2422. [PubMed: 9153284]
26. Globerman H, Zauberman Y, Makarov T, Beamer BA, Yen C, Shuldiner AR, Harel C, Karnieli E. Analysis of the peroxisome proliferator activated receptor gamma (PPARgamma) gene in HAIRAN syndrome with obesity. *Clin. Endocrinol* 2000;52:479–485.
27. Ribot J, Rantala M, Kesaniemi YA, Palou A, Savolainen MJ. Weight loss reduces expression of SREBP1c/ADD1 and PPAR γ 2 in adipose tissue of obese women. *Eur. J. Physiol* 2000;11:1–14.
28. Park KS, Ciaraldi TP, Abrams-Carter L, Mudaliar S, Nikoulina SE, Henry RR. PPAR γ gene expression is elevated in skeletal muscle of obese and type2 diabetic subjects. *Diabetes* 1997;46:1230–1234. [PubMed: 9200661]
29. Rosen ED, Spiegelman BM. Peroxisome proliferator-activated receptor γ ligands and atherosclerosis: ending the heartache. *J. Clin. Invest* 2000;106:629–631. [PubMed: 10974014]
30. Marx N, Sukhova G, Murphy C, Libby P, Plutzky J. Macrophages in human atheroma contain PPARgamma: Differentiation-dependent peroxisomal proliferator-activated receptor gamma (PPARgamma) expression and reduction of MMP-9 activity through PPAR-gamma activation in mononuclear phagocytes in vitro. *Am. J. Pathol* 1998;153:17–23. [PubMed: 9665460]
31. Marx N, Bourcier TD, Sukhova G, Murphy C, Libby P, Plutzky J. PPARgamma activation in human endothelial cells increases plasminogen activator inhibitor type-1 expression: PPARgamma as a potential mediator in vascular disease. *Arteriosclero. Thrombo. Vasc. Biol* 1999;19:546–551.
32. Spiegelman BM. PPAR γ in monocytes: less pain any gain? *Cell* 1998;93:153–155. [PubMed: 9568708]
33. Rocchi S, Auwerx J. Peroxisome proliferator-activated receptor- γ : a versatile metabolic regulator. *Ann. Med* 1999;31:342–351. [PubMed: 10574507]
34. Reaven GM. Insulin resistance and human disease: a short history. *J. Basic Clin. Physiol. Pharmacol* 1998;9:387–406. [PubMed: 10212844]
35. Yen C, Beamer BA, Negri C, Silver K, Brown KA, Yarnall DP, Burns DK, Roth J, Shuldiner AR. Molecular scanning of the human peroxisome proliferator activated receptor γ (hPPAR γ) gene in diabetic Caucasians: identification of a pro12ala PPAR γ 2 missense mutation. *Biochem. Biophys. Res. Commun* 1997;241:270–274. [PubMed: 9425261]
36. Evans D, Mann WA, Heer JD, Michel U, Wendt D, Kortner B, Wolf A, Beisiegel U. Variation in the gene for human peroxisome proliferator activated receptor γ (PPAR γ) does not play a major role in the development of morbid obesity. *Int. J. Obesity* 2000;24:647–651.
37. Valve R, Sivenius K, Miettinen R, Pihlajamaki J, Rissanen A, Deeb SS, Auwerx J, Uusitupa M, Laakso M. Two polymorphisms in the peroxisome proliferator-activated receptor- γ gene are associated with severe overweight among obese women. *J. Clin. Endocrinol. Metab* 1999;84:3708–3712. [PubMed: 10523018]

38. Meirhaeghe A, Fajas L, Helbecque N, Cottel D, Auwerx J, Deeb SS, Amouyel P. Impact of the peroxisome proliferator activated receptor γ 2 Pro12Ala polymorphism on adiposity, lipid and non-insulin-dependent diabetes mellitus. *Int. J. Obesity* 1999;24:195–199.

Fig. 1. Structure of PPAR- γ cDNA isoforms and gene. (A) cDNA structures of seven PPAR- γ isoforms identified in monkey macrophages are shown. The ATG codon indicates the translation initiation site. (B) The genomic structures of mouse (m), human (h), and monkey (M) PPAR- γ gene are compared. Arrows indicate currently known transcription start sites, (#) from [10]. (C) Partial structure of human chromosome 3 as reported in the NCBI human genome bank with a blast analysis showing all of the monkey PPAR- γ exons aligning with 93–100% sequence identity.

Fig. 2.

Expression of PPAR- γ isoforms in normal and fat-fed monkey macrophages and normal monkey tissues. (A) The novel PPAR- γ isoforms- γ 4 to - γ 7 are all expressed in both normal and fat-fed monkey macrophages. (B) PPAR- γ 6 and - γ 7 are expressed in adipose tissue but not in muscle, kidney, or spleen of normal monkeys. Expression of β -actin and total PPAR- γ are shown as internal controls. (C) PPAR- γ as well as ABCA1 expression in macrophages is induced by fat-feeding in monkeys. Results are shown for two normal and four fat-fed monkeys. Quantitative analysis of the data using the Image J software from NIH is shown alongside. P/ β (%) and A/ β (%) represent, respectively, the levels of PPAR- γ and ABCA1 as a percentage of the level of β -actin.

Fig. 3.

Expression of recombinant PPAR- γ 1, - γ 4, and γ 5 in yeast. Recombinant PPAR- γ isoforms were expressed and purified as described in 'Materials and methods'. The recombinant proteins were resolved by 10% SDS-PAGE. (A) The gel was stained with Coomassie blue and reveals protein bands at ~50 kDa for recombinant PPAR- γ 1, - γ 4, and - γ 5. The lower band is unidentified. (B) Resolved proteins were transferred to a PVDF membrane and subjected to western blot analysis using anti-myc as the primary antibody revealing a 50 kDa protein for both PPAR- γ 1 and - γ 4.

Fig. 4.

Expression of ABCA1 and LPL in CHO cells transiently expressing PPAR-γ4 and -γ5.

Tetracycline-responsive CHO cells were transiently transfected with expression vectors specific for PPAR-γ4 and -γ5 or were mock-transfected with vector alone (CT). The levels of total PPAR-γ, ABCA1, and LPL transcripts were compared after activation with 5 μM 15-d-PGJ₂. The level of actin is shown as a control for the protocol. Results of densitometric analysis using the Image J software are shown alongside with a calculation of the level of PPAR-γ, ABCA1, and LPL expression (P/β, A/β, and L/β, respectively) as a percentage of β-actin levels.

Table 1

PCR primer sets used for this research

Product	Sense primer	Anti-sense primer
1. First strand cDNA	AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG	ATTCTACAGGCCGAGGCGGCCGACATGT30VN
2. ds-cDNA	AAGCAGTGGTATCAACGCAGAGT	ATTCTACAGGCCGAGGCGGCCGACATGT30VN
3. First PPAR γ fragment	CCACTATGGAGTTCATGCTTGTGAAGG	TGCAGGGGGGTGATATGTTTGAAGTGG
4. PPAR γ 5' fragment	AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG	TGCAGGGGGGTGATATGTTTGAAGTGG
5. PPAR γ 3' fragment	CCACTATGGAGTTCATGCTTGTGAAGG	ATTCTACAGGCCGAGGCGGCCGACATGT30VN
6. β -Actin	ATCTGGCACCACACCTTCTACAATGAGCTGCG	CGTCATACTCCTGCTTGCTGATCCACATCTGC
7. PPAR γ detection	CCACTATGGAGTTCATGCTTGTGAAGG	TGCAGGGGGGTGATATGTTTGAAGTGG
8. PPAR γ 4 detection	AGCAAACCCCTATTCCATGCTG	CGTCAGGATGATTCTTGTCTCTGG
9. PPAR γ 5 detection	AGCAAACCCCTATTCCATGCTG	CGTCAGGATGATTCTTGTCTCTGG
10. PPAR γ 6 detection	CCTTTACCTCTGCTGGTGAC	TAATCCCAGCACTTTGGGAGGCCG
11. PPAR γ 7 detection	CCTTTACCTCTGCTGGTGAC	TAATCCCAGCACTTTGGGAGGCCG
12. Yeast PPAR γ 1 expression	CTCGAGCCTTTACCTCTGCTGGTGAC	TCTAGAGTACAAGTCCTTGTAGATCTCCTG
13. Yeast PPAR γ 4/5 expression	CTCGAGAGCAAACCCCTATTCCATGC	TCTAGAGTACAAGTCCTTGTAGATCTCCTG
14. Mammalian PPAR γ 4/5 expression	ATTCGCCCTTGCTAGCAAACCCCTATTCCATGCTG	GTCGACGGGCGAATTGAATTTAGC
15. ABCA1 detection	CCCGAATGTCATCAACAATGCC	GAGGACACATAGGACTTCTGC
16. LPL detection	GGTTTATCAACTGGATGGAGGAGG	GGAAACTTCAGGCAGAGTGAATGGG
17. CD36 detection	CTGTGATCGGAACCTGTGGGCTC	TTGCTGATGTCTAGCACACC
18. SR-A detection	GATTGGGAACATTCTCAGACCTT	CTTGTCCAAAGTGAGCTGCCTT

V=A, G, or C; N=A, G, C, or T.

Table 2
Similarity and identity (%) of PPAR γ 1 deduced polypeptide sequences of different species

Accession number	Source	R. monkey	Human	H. mouse	Pig	O. Cuniculus
AY048694	Cynomolgus monkey	99.78/99.78	99.37/99.16	98.74/98.10	98.50/98.11	98.10/97.90
AF033342	Rhesus monkey		99.58/99.37	98.95/98.32	98.74/98.32	98.32/98.11
X90563	Human			98.53/98.11	98.74/98.11	97.90/97.47
U01841	House mouse				98.53/97.47	98.11/97.26
AJ006756	Pig					98.11/97.47

Table 3

Major features of cynomolgus monkey PPAR- γ isoforms

Isoform	PPAR- γ 1	PPAR- γ 2	PPAR- γ 3	PPAR- γ 4	PPAR- γ 5	PPAR- γ 6	PPAR- γ 7
Accession number	AY048694	AY048695	AY048696	AY048697	AY048698	AY048699	AY048700
cDNA length (bp)	1800	1765	1713	2028	1849	2063	1884
ORF (bp)	1425	1515	1425	1449	1425	1449	1425
Polypeptide aas	475	505	475	483	475	483	475
MW (KD)	54.5	57.6	57.6	55.3	54.5	55.3	54.5
Isoelectric point	6.62	5.81	6.62	6.62	6.62	6.62	6.62