

OBESITY ASSOCIATED WITH A MUTATION IN A GENETIC REGULATOR OF ADIPOCYTE DIFFERENTIATION

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ABSTRACT

Background There is increasing evidence of genetic factors leading to obesity, but the exact genes involved have not been defined. Peroxisome-proliferator-activated receptor γ 2 (PPAR γ 2) is a transcription factor that has a key role in adipocyte differentiation, and therefore mutations of the gene for this factor might predispose people to obesity.

Methods We studied 358 unrelated German subjects, including 121 obese subjects (defined as those with a body-mass index [the weight in kilograms divided by the square of the height in meters] of more than 29). We evaluated these subjects for mutations in the gene for PPAR γ 2 at or near a site of serine phosphorylation at position 114 that negatively regulates the transcriptional activity of the protein, using a polymerase-chain-reaction-based assay coupled with specific endonuclease digestion. The activity of the mutation identified was analyzed by retroviral transfection and overexpression in murine fibroblasts.

Results Four of the 121 obese subjects had a missense mutation in the gene for PPAR γ 2 that resulted in the conversion of proline to glutamine at position 115, as compared with none of the 237 subjects of normal weight ($P=0.01$). All the subjects with the mutant allele were markedly obese, with body-mass-index values ranging from 37.9 to 47.3, as compared with a mean of 33.6 in the other obese subjects. Overexpression of the mutant gene in murine fibroblasts led to the production of a protein in which the phosphorylation of serine at position 114 was defective, as well as to accelerated differentiation of the cells into adipocytes and greater cellular accumulation of triglyceride than with the wild-type PPAR γ 2. These effects were similar to those of an *in vitro* mutation created directly at the Ser114 phosphorylation site.

Conclusions A Pro115Gln mutation in PPAR γ 2 accelerates the differentiation of adipocytes and may cause obesity. (N Engl J Med 1998;339:953-9.)

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OBESITY is reported to be the most common health problem in developed countries and is the second most common cause of preventable death in the United States.¹⁻³ It results from an imbalance between energy intake and energy expenditure. This imbalance leads to a pathologic accumulation of adipose tissue; obesity therefore reflects an interaction of development

and environment with genotype. The results of studies of twins and families suggest that up to 80 percent of the variance in body-mass index (defined as the weight in kilograms divided by the square of the height in meters) is attributable to genetic factors.^{4,5}

Several single-gene mutations have been described in rodents with obesity, but the identity of the genes associated with obesity in humans is uncertain. In the best characterized of these rodents (*ob/ob* [obese hyperglycemic] and *db/db* [diabetic] mice), there are mutations in the gene for the hormone leptin or its receptor that interrupt the feedback signal between adipose mass and hypothalamic control of food intake.⁶⁻⁸ Recently, members of families with a mutation in the coding sequence of the leptin gene⁹ or the leptin-receptor gene¹⁰ have been reported. These mutations must be extremely rare, however, since thousands of obese subjects have now been screened for variations in these genes and only a few have been found to have the mutations. Mutations of genes regulating adipocyte differentiation or triglyceride storage, on the other hand, have not been associated with obesity in either rodents or humans.

Peroxisome-proliferator-activated receptors, especially peroxisome-proliferator-activated receptor γ 2 (PPAR γ 2), are key regulators of adipocyte differentiation and energy storage.^{11,12} PPAR γ 2 is a transcription factor that directs the differentiation of pre-adipocytes to adipocytes. Overexpression of PPAR γ 2 in fibroblast cell lines through the use of retroviral vectors efficiently converts them to adipocytes.¹³ PPAR γ 2 has a reduced ability to promote the process of adipocyte differentiation when it is phosphorylated at the site of a single amino acid residue (serine at position 114 in the human PPAR γ 2 gene), suggesting a mechanism of negative regulation to limit adipocyte differentiation and lipid accumulation (Fig. 1).^{14,15} This serine phosphorylation occurs at a typical consensus site for mitogen-activated protein kinase or a related kinase and is characterized by the sequence proline-any amino acid-serine-proline. The aim of our study was to determine whether

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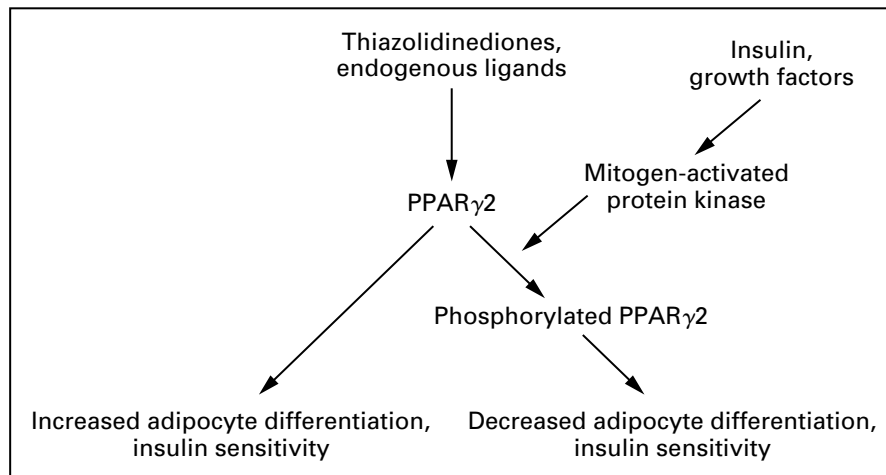


Figure 1. Role of Peroxisome-Proliferator-Activated Receptor $\gamma 2$ (PPAR $\gamma 2$) and Its Regulation by Phosphorylation.

PPAR $\gamma 2$ is activated by thiazolidinediones and unknown endogenous ligands to promote adipocyte differentiation and to increase insulin sensitivity. Mitogen-activated protein kinase, which is activated by insulin and other growth factors, catalyzes the phosphorylation of a serine site at position 114 of the PPAR $\gamma 2$ protein, leading to a decrease in adipocyte differentiation and insulin sensitivity.

mutations in or around this phosphorylation site might be associated with obesity.

METHODS

Study Subjects

We studied 358 unrelated adult subjects living in the Nordrhein–Westfalen region of Germany (in the cities of Bochum and Cologne, which together have 1.6 million inhabitants). They were recruited by random selection of subjects with type 2 diabetes mellitus and their spouses or employees of the participating institutions. Anonymous blood samples were obtained and frozen at -20°C for further analysis. Sixty-one women and 60 men were characterized as obese on the basis of a body-mass index of more than 29. This group had a mean (\pm SD) body-mass index of 33.9 ± 4.4 and a mean age of 57 ± 14 years. The group of subjects of normal weight comprised 116 women and 121 men with a mean body-mass index of 25.0 ± 2.7 and a mean age of 59 ± 16 years. These subjects have been described in detail elsewhere.¹⁶

On the basis of the criteria of the World Health Organization for oral glucose tolerance, 186 subjects had diabetes mellitus, including 79 from the obese group and 107 from the normal-weight group. Since the subjects were originally recruited for studies on the genetics of type 2 diabetes mellitus, the high prevalence of this disease in both the obese and normal-weight groups is not representative of that in the general population. The study protocol was reviewed by the appropriate institutional review committees, and all the subjects gave informed consent for the studies. Reidentification of subjects who carried a certain genotype for further analysis (e.g., family analysis) was prohibited by the committees' regulations.

Screening for PPAR $\gamma 2$ Mutations

We isolated the genomic DNA of 32 subjects from peripheral-blood leukocytes and amplified it by the polymerase chain reaction (PCR) using a sense primer (5'TGCAATCAAAGTGGAGCC3') and an antisense primer (5'CAGAAGCTTTATCTCCACAGAC3') that flank the region containing the serine-phosphorylation site of PPAR $\gamma 2$. The PCR products were cloned, and several clones from each subject were sequenced as described elsewhere.¹⁷

On the basis of the polymorphism found in a single subject during this initial screening, a PCR-based assay for the analysis of restriction-fragment-length polymorphisms was performed on the remaining DNA samples with a modified sense primer (5'TGC-AATCAAAGTGGAGCCCTGCATGTC3'). The sense primer generated an additional single-base mutation (indicated in boldface type) in the modified sense primer 3 bp upstream of the expected substitution of glutamine for proline at position 115 (Pro115Gln). This mutation led to a new *Hind*II restriction site in DNA samples from the subjects with the mutation.

Functional Analyses of PPAR $\gamma 2$ Mutations

Full-length PPAR $\gamma 2$ complementary DNA (cDNA) was amplified from first-strand cDNA taken from human fat tissue (Clontech, Palo Alto, Calif.) under conditions previously described¹⁸ and cloned. This wild-type (normal) cDNA was used for site-directed mutagenesis (QuikChange, Stratagene, La Jolla, Calif.) of amino acids at positions 114 (serine to alanine) and 115 (proline to glutamine). Previous studies have shown that the mutation of serine to alanine at position 114 (Ser114Ala) ablates in vitro phosphorylation, which increases the activity of PPAR $\gamma 2$ in promoting adipocyte differentiation.^{14,15} The Pro115Gln mutation was the one found in this study.

The three samples of cDNA (wild type, Ser114Ala, and Pro115Gln) were subcloned into the retroviral expression vector pBabePuro¹⁹ and amplified in BOSC23 cells.²⁰ The supernatant containing the retrovirus was harvested and used to infect NIH 3T3 cells (American Type Culture Collection, Manassas, Va.) as previously described.²¹ The infected NIH 3T3 cells were treated with trypsin and further cultured in Dulbecco's modified Eagle's medium (4.5 g of glucose per liter) with 10 percent heat-inactivated fetal-calf serum (HyClone, Logan, Utah) and 2 μg of puromycin per milliliter (Sigma, St. Louis) to select successfully infected cells. Approximately 90 fibroblast clones of each type were isolated and pooled to obtain cell lines for further experiments.

The three pooled cell lines were grown to confluence. To promote differentiation, the medium was changed for two days to Dulbecco's modified Eagle's medium containing 10 percent fetal-calf serum, 1 μM dexamethasone (Sigma), 500 μM methylisobutylxanthine (Sigma), 0.05 μM troglitazone (Parke-Davis, Morris Plains, N.J.), and 2 μM bovine insulin (Sigma). The cells were

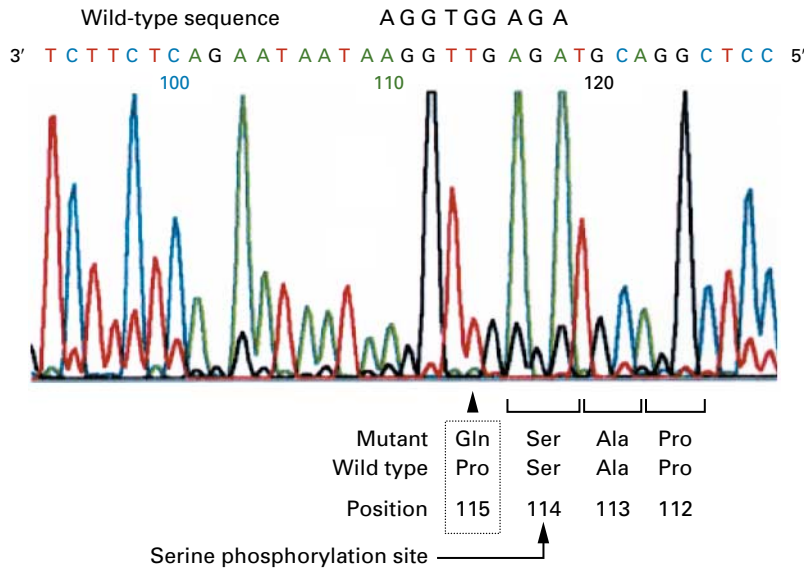


Figure 2. Sequence Analysis of a Cloned DNA Fragment from an Obese Subject with a Mutation in the Peroxisome-Proliferator-Activated Receptor γ 2 Gene.

The complementary DNA from some of the obese subjects was subcloned and sequenced. An example of a missense mutation at bp 344 in the antisense strand (arrowhead) is shown. This mutation leads to the substitution of glutamine for proline at position 115. The serine phosphorylation site at position 114 is not directly affected.

then maintained for another seven days in Dulbecco's modified Eagle's medium with 10 percent fetal-calf serum, 0.05 μ M troglitazone, and 2 μ M bovine insulin. The triglyceride content of the cells at various stages of the differentiation process was determined by staining with oil red O (Sigma) after fixation with 10 percent formalin or chemically by using the GPO Trinder kit (Sigma). Protein content was determined with the Bradford assay (Bio-Rad, Hercules, Calif.).

To determine the phosphorylation status of PPAR γ 2, differentiated cells were processed for Western blot analysis as described elsewhere²² with use of a primary antibody against PPAR γ 2 obtained from Santa Cruz (Santa Cruz, Calif.).

Statistical Analysis

Statistical analyses were performed with SPSS software (release 7.5, SPSS, Chicago). The prevalence of mutations in the obese subjects as compared with the normal-weight subjects was analyzed with a two-tailed Fisher's exact test, and the pathophysiologic data were compared by the Mann-Whitney U test.

RESULTS

As an initial approach to identify mutations at or around the Ser114 phosphorylation site of PPAR γ 2, we amplified the genomic DNA of 32 obese subjects by PCR, as described in the Methods section, and the product was then cloned into a plasmid for sequence analysis. Since heterozygous mutations might be missed by the sequencing of single clones, several clones of each subject were analyzed. DNA from one subject contained a missense mutation at bp 344 of PPAR γ 2 in about 50 percent of the clones sequenced, a finding that is consistent with heterozygosity at this site (Fig. 2). This mutation would

change the wild-type proline at position 115 to glutamine. No mutations at Ser114 were found.

In order to study more subjects and determine the prevalence of this mutation, we used a restriction-enzyme-based assay. Since the region of interest does not contain a natural restriction site, a PCR primer was constructed by changing a nucleotide 3 bp upstream of the putative mutation to introduce a new restriction site which, when coupled with the mutation, generated a site for restriction enzyme *Hind*II when the mutant allele was present (Fig. 3). Using this assay, we studied a total of 358 subjects (121 obese subjects and 237 normal-weight subjects).

In addition to the one obese subject described above, three other obese subjects, but none of the normal-weight subjects, were found to carry a heterozygous mutation at nucleotide 344. Subsequent cloning and sequencing of this region in these obese subjects confirmed the Pro115Gln mutation. The association of the mutation with obesity (defined as a body-mass index of >29) was significant ($P=0.01$) (Table 1). Three of the four subjects carrying the mutation also had type 2 diabetes. The overall prevalence of the mutation was 1.1 percent in the 358 subjects, corresponding to an allelic frequency of approximately 0.5 percent.

The 4 subjects with mutant alleles were markedly more obese than the other 117 obese subjects (body-mass index, 37.9 to 47.3, as compared with a mean

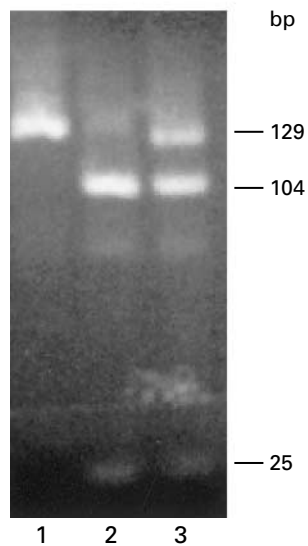


Figure 3. Results of Polymerase-Chain-Reaction (PCR) Screening for Mutations in Peroxisome-Proliferator-Activated Receptor $\gamma 2$.

PCR amplification of DNA, followed by restriction-enzyme digestion and ethidium bromide-stained agarose-gel electrophoresis, was performed as described in the Methods section. When applied to a recombinant DNA sample that was homozygous for the mutation (lane 2), the restriction endonuclease *Hind*III cut the entire DNA sample into two fragments. In DNA from a heterozygous obese subject (lane 3), fragments of three sizes were observed, owing to the digestion of the mutant allele and the absence of digestion of the wild-type allele. In DNA from a normal-weight subject with two wild-type alleles (lane 1), the fragment amplified by PCR was not cleaved by the restriction enzyme, resulting in a single band.

of 33.6 ± 4.1 ; $P=0.03$) (Table 2). The average age of the subjects with mutations was not significantly different from that of the rest of the group. The mean serum insulin concentration while fasting was significantly lower in the obese subjects with the mutation than in the rest of the obese group, suggesting a lower level of insulin resistance (Table 2).

Since Pro115 flanks Ser114 at the phosphorylation site of PPAR $\gamma 2$, it appeared likely that this mutation might lead to decreased serine phosphorylation and therefore increased differentiation of fibroblasts into adipocytes, as has been observed with an artificial mutation of serine at position 114.^{14,15} To test this hypothesis, the nonmutant (wild-type) PPAR $\gamma 2$, the subject-derived Pro115Gln PPAR $\gamma 2$, and an artificially mutated Ser114Ala PPAR $\gamma 2$ were each evaluated with regard to phosphorylation and support of adipocyte differentiation. The Ser114Ala mutant had more activity than the wild-type PPAR $\gamma 2$, because the potential negative effect of serine phosphorylation on the activity of the protein is lost owing to the substitution of alanine.¹⁴ Each construct was used for retrovirus-mediated, stable transfection of NIH 3T3 fibroblasts.

TABLE 1. FREQUENCY OF THE PRO115GLN MUTATION IN THE PPAR $\gamma 2$ GENE IN OBESE AND NORMAL-WEIGHT SUBJECTS.

VARIABLE	OBESE SUBJECTS	NORMAL-WEIGHT SUBJECTS	TOTAL*
Type 2 diabetes	3/79	0/107	3/186
No type 2 diabetes	1/42	0/130	1/172
Total†	4/121	0/237	4/358

* $P=0.62$ for the comparison between the subjects with type 2 diabetes and those without it.

† $P=0.01$ for the comparison between the obese subjects and those of normal weight.

These cells do not normally accumulate triglyceride but do differentiate into adipocytes when transfected with PPAR $\gamma 2$ and stimulated appropriately.¹⁴

PPAR $\gamma 2$ protein was demonstrated in extracts of all three transfected cell lines by Western blot analysis (Fig. 4A), and the expression was significantly increased as compared with the endogenous expression in nontransfected NIH 3T3 cells (data not shown). After stimulation by insulin, the mobility of wild-type PPAR $\gamma 2$ on sodium dodecyl sulfate-polyacrylamide gels was retarded, a finding consistent with the effect of phosphorylation of serine at position 114. This gel shift was absent in cells expressing either of the mutant proteins, indicating that neither the Pro115Gln mutant nor the Ser114Ala mutant was phosphorylated at position 114.

To assess adipocyte differentiation and lipid accumulation, the transfected NIH 3T3 cells were treated with a differentiation medium that supported and maintained the cells for an additional nine days in culture. Staining with oil red O, a fat-specific dye, revealed more lipid accumulation in the cells containing either the Ser114Ala or the Pro115Gln mutants than in those containing wild-type PPAR $\gamma 2$ after six and nine days of differentiation (Fig. 4B). This finding was confirmed by chemical analysis of both mutant cell lines, which revealed an accumulation of triglyceride in the mutant cells that was at least 2.5 times that found in the wild-type cell line, after normalization for cell protein content (Fig. 4C). Thus, both the natural mutation of proline to glutamine at position 115 and the artificial mutation of serine to alanine at position 114 increased the activity of PPAR $\gamma 2$ and accelerated adipocyte differentiation and triglyceride accumulation.

DISCUSSION

Obesity in humans results from a combination of environmental influences and genetic factors that af-

TABLE 2. CLINICAL CHARACTERISTICS OF THE SUBJECTS WITH THE *PPAR*γ2 MUTATION AND THE OBESE SUBJECTS WITHOUT THE MUTATION.*

SUBJECT No.	AGE	SEX	BODY-MASS INDEX	FASTING SERUM INSULIN CONCENTRATION	TYPE 2 DIABETES
	yr			μU/ml	
1	65	M	47.3	11	Yes
2	32	F	38.5	ND	No
3	54	M	43.8	9	Yes
4	74	M	37.9	8	Yes
Mean	56±18	—	41.9±4.5	9.3±1.5	—
Obese subjects without the mutation	57±13†	—	33.6±4.1‡	16.7±4.6§	—

*Plus–minus values are means ±SD. ND denotes not determined. To convert values for insulin to picomoles per liter, multiply by 6.

†P=0.9 for the comparison with the subjects with the mutation.

‡P=0.03 for the comparison with the subjects with the mutation.

§P=0.007 for the comparison with the subjects with the mutation.

fect energy storage and caloric intake. In subjects with severe obesity, this interaction is associated with an increased number (hyperplasia) and size (hypertrophy) of fat cells.¹ The hyperplastic adipocytes are derived from a poorly defined precursor pool of preadipocytes, which appear to be fibroblasts on morphologic analysis. Activation of the transcription factor *PPAR*γ2 appears to be a key element in the regulation of the process of adipocyte differentiation.^{11,13} The activity of *PPAR*γ2 is decreased by phosphorylation of a single serine site at position 114.^{14,15}

In this study, we identified a mutation that affects the serine phosphorylation of *PPAR*γ2 and appears to contribute to the development of severe obesity. This mutation was found in approximately 3 percent of obese subjects in a German population, or about 1 percent of all subjects screened. Since neither a founder effect nor linkage disequilibrium can be fully ruled out by the methods used in this study, further studies will be needed to determine the prevalence of this mutation in other populations and in families with obesity.

As predicted by its structure and according to our functional analysis, the mutation of proline to glutamine at position 115 blocks the phosphorylation of *PPAR*γ2 at Ser114, just adjacent to the mutation. This mutation reduces the inactivation of *PPAR*γ2, leading to a constitutively more active protein. Since our screening technique focused on a specific mutation alone, a search for other *PPAR*γ2 mutations should be considered in future studies of the genetics of obesity. Yen et al. recently identified a polymorphism converting serine to alanine at position 12 at the amino-terminal end of *PPAR*γ2.²³ However, the function of this site remains uncertain, and

there is no evidence that this mutation is associated with obesity or that it increases *PPAR*γ2 activity.²³ By contrast, the subjects with the mutation in our study were massively obese, even as compared with the other obese subjects in the study. Also, the Pro115Gln mutation, as compared with the wild-type sequence, clearly results in increased rates of differentiation of adipocytes when transfected into NIH 3T3 cells.

Although the endogenous ligand for *PPAR*γ2 is unknown, a new class of antidiabetic drugs called thiazolidinediones, which includes troglitazone and pioglitazone, has been found to act as a synthetic ligand for this transcription factor.²⁴ These drugs act as insulin sensitizers, decreasing insulin resistance in patients with type 2 diabetes.²⁵ There is no evidence that the administration of troglitazone results in greater weight gain than the administration of other antidiabetic drugs²⁶; however, studies in rodents suggest that the long-term administration of this drug may increase the total number of adipocytes.²⁷

Although obesity is usually associated with insulin resistance in proportion to the excess in body weight,^{28,29} one might predict that activating mutations of *PPAR*γ2 might cause obesity that would be associated with less insulin resistance than expected. Although we did not measure the degree of insulin resistance in our subjects, the obese subjects with the *PPAR*γ2 mutation had lower serum insulin concentrations while fasting than the other obese subjects, suggesting a lower degree of insulin resistance.²⁸

In summary, the missense mutation Pro115Gln in the gene for the transcription factor *PPAR*γ2 is associated with marked obesity. In vitro analyses showed a permanent activation of *PPAR*γ2 that led to an accelerated rate of adipocyte differentiation

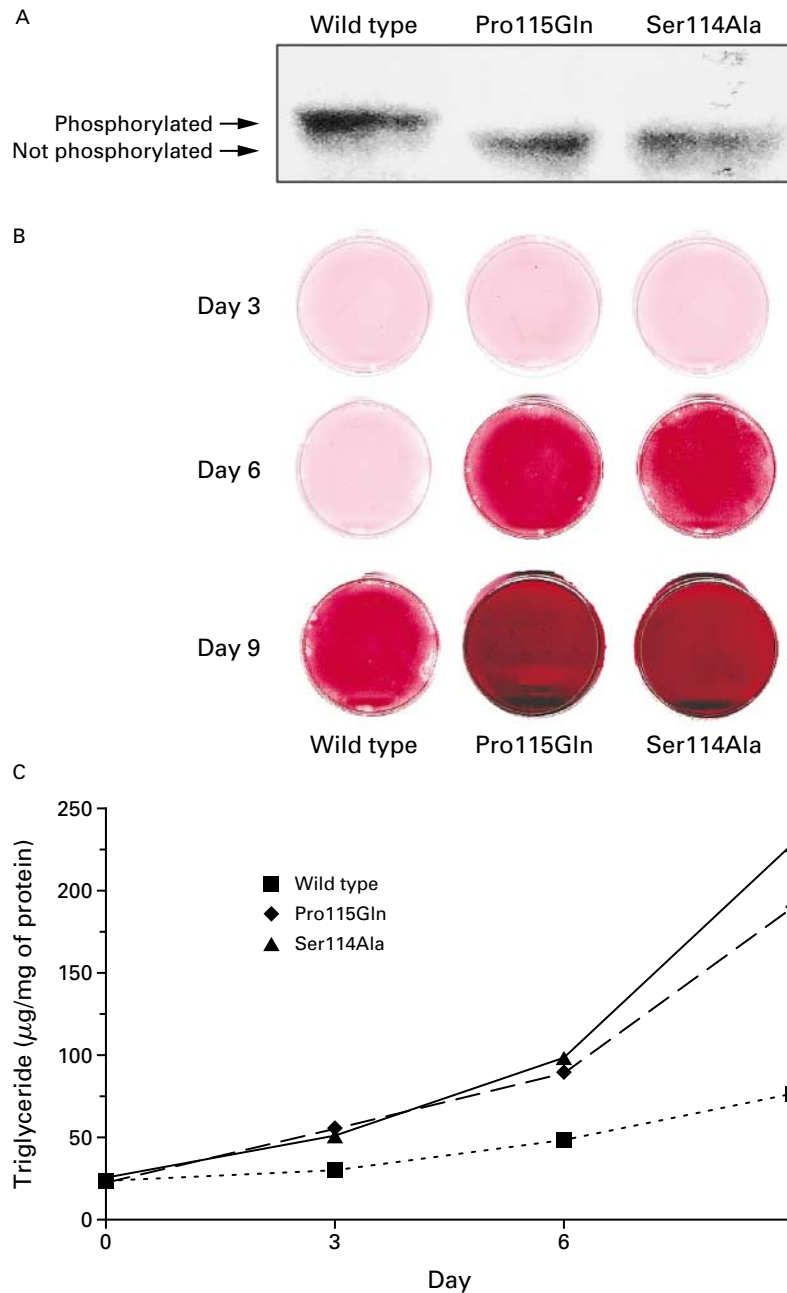


Figure 4. Functional Analyses of Constitutively Active Mutant Peroxisome-Proliferator-Activated Receptor γ 2 (PPAR γ 2).

NIH 3T3 cells transfected with the wild-type unmutated *PPAR γ 2* and two mutant *PPAR γ 2* genes were differentiated with 0.05 μ M troglitazone as described in the Methods section. Panel A shows the results of a Western blot analysis in which an antibody against PPAR γ 2 was used. As compared with the wild-type protein, both mutant proteins migrated more rapidly. This effect was consistent with the phosphorylation of the wild-type protein at position 114, which does not occur in either of the mutants. Panel B shows the cells stained with oil red O to identify lipid accumulation. As compared with the wild-type PPAR γ 2, both the natural mutation of proline to glutamine at position 115, found in the obese subjects, and the artificial mutation of the Ser114 phosphorylation site accelerated the accumulation of triglyceride during differentiation. In Panel C, the same effect is evident in the results of a quantitative triglyceride assay to assess lipid accumulation. The standard deviations of the triglyceride values were less than 10 percent of the mean values and are therefore not shown.

and increased fat accumulation in a tissue-culture model of adipogenesis. Although the degree of obesity was pronounced, there was no association with type 2 diabetes or hyperinsulinemia, thus possibly defining a specific subclass of obesity.

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