A Novel Peroxisome Proliferator-activated Receptor γ Isoform with Dominant Negative Activity Generated by Alternative Splicing*

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We examined the peroxisome proliferator-activated receptor γ (PPARγ) locus in an attempt to identify expressed sequence tags and/or conserved non-coding sequences in the intron sequences containing open reading frames and potentially able to encode new proteins. We identified a new PPARγ transcript, defined γORF4, which harbors a readthrough in intron 4. The expected translated protein lacks the ligand-binding domain encoded by exons 5 and 6. We identified the transcript in human tumor cell lines and tissues, synthesized the cDNA, and cloned it in expression vectors. Using transient transfections, we found that γORF4 cDNA is translated into a predominantly nuclear protein that does not transactivate a reporter gene. Moreover, the isoform is dominant negative versus PPARγ. Interestingly, γORF4 was expressed in vivo in a series of sporadic colorectal cancers. In some cases, it was expressed, albeit at lower levels, also in the mucosa adjacent to the tumors, suggesting that it may be related to tumorigenesis. A tumorigenic effect of γORF4 is in line with our finding that γORF4 has not only lost the capacity to restrain cell growth but has acquired the potential to stimulate it. In conclusion, this study demonstrates that γORF4 is expressed in vivo, that it has lost some PPARγ properties, and that it affects PPARγ functioning. The ability to counteract PPARγ suggests that γORF4 plays a role in the pathogenesis of colorectal cancers.

PPARG is located on chromosome 3p25.2. It spans about 100 kb and comprises six coding exons that are translated into the protein domains common to all PPAR isoforms and nuclear receptors (1–3). Analysis of the PPARG 5′-flanking region has identified at least four promoters that are alternatively used to produce two PPARγ protein species, γ1 and γ2 (3, 4). These species differ at the N terminus in that the γ2 isoform carries 30 additional amino acids encoded by exon B (3). The sequences transcribed from the other promoters are present as untranslated regions at the 5′-end of the mRNAs; thus, γ3 and γ4 transcripts have the same coding potential as γ1 (4, 5). Similar differential promoter usage, with no changes in the protein species produced, has been reported for the α (6) and β/δ isoforms (7). PPARs also undergo diverse genomic rearrangements that result in distinct transcripts. PPARG undergoes a gene translocation event that produces a PAX8-PPARγ chimeric, the product of which has been detected thus far only in thyroid follicular tumors (8); PPARA undergoes an exon-skipping mechanism that generates alternatively spliced products in cell lines and tissues (9). Somatic loss-of-function PPARG mutations have also been reported in sporadic colorectal cancers (10), suggesting that any event that hampers PPARγ activity causes loss of the differentiated phenotype. In fact, PPARγ plays a pivotal role in adipocyte and epithelial cell differentiation as well as in the regulation of energy metabolism (11). Its pre differentiated and antiproliferative effect occurs in several cell types. Specifically, exposure to PPARγ ligands inhibits the growth of various human cancer cell lines in vitro, of tumors in vivo, and of tumors transplanted into nude mice (12–17). These data support the notion that PPARG exerts a protective function in cancer progression (18). Differently, whether or not PPARγ is a tumor-promoting factor in vivo remains to be established (19–22).

Alternative splicing is a post-transcriptional mechanism whereby proteins that exert distinct functions are generated from a single transcript (23–25). From 30 to 60% of human genes are thought to undergo this regulatory mechanism that results in protein diversity (25). It appears to function during embryogenesis as in the case of sex determination in Drosophila melanogaster (26) and in the activation of various Hox genes by which different proteins are generated in different tissues at different developmental stages (27). More recently, alternative splicing has also been implicated in apoptosis (23, 28), in the origin and development of a large number of human genetic diseases (29), and in tumorigenesis (Refs. 30–32 and references therein).

The aim of our study was to determine whether or not alter-
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MATERIALS AND METHODS

Tissue Samples—We collected 25 tissue samples from patients affected by colorectal cancer who underwent surgery at the "Fatebenefratelli" Hospital, Benevento, Italy. Informed consent was obtained from all patients. The sporadic origin of the tumors was determined from clinical, imaging, and laboratory data as well as from the personal and familial history, which ruled out any disorder of the large intestine in the relatives of the proband up to the second degree. All patients were at their first surgery after the initial diagnosis. No patient was diabetic or had taken non-steroidal anti-inflammatory drugs on a regular basis for inflammatory diseases of the large bowel. Tumor samples were collected immediately after surgery, immersed in acrylamide gel, and stored until time of analysis. Samples of intestinal mucosa were obtained from the adjacent tissue and processed in parallel as internal controls of the assays. The specimens were analyzed by histology and immunohistochemistry to verify correct pathological staging. All tumors were stages 2–4 according to Broder’s classification, and all metastases were at their first surgery after the initial diagnosis. No patient was infected by colorectal cancer who underwent surgery at the “Fatebenefratelli” Hospital, Benevento, Italy. Informed consent was obtained from all patients.

RNA Extraction and RT-PCR Assay—RNA was extracted from tissues and cell lines by Trizol™ (Invitrogen). Random primed double-strand cDNA was synthesized using Superscript III (Invitrogen). The specific primers for ORF4 included forward, 5'-GCC CCA GAG TCA TTG CAT AC-3', PPARγ (exons 1–6) forward, 5'-ATG ACC ATG TTG CAT GGA-3'; PPARγ (exons 1–6) reverse, 5'-CTA GAA TTC CTT GAT GAA-3'; and reverse, 5'-CTA GAA TTC CTT GAT GAA-3'; yORF4 reverse, 5'-GCC AAC ACT AAA CCA CA-3'; and reverse, 5'-GCC AAC ACT AAA CCA CA-3'. The fixed cells were then stained with anti-V5, anti-HA, or anti-FLAG epitopes (Roche Applied Science). Images were generated with an Axiophot fluorescent microscope (Carl Zeiss, Jena, Germany).

Protein extracts were run on a 10–12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes for dot blot analysis. Immunodetection was performed with the primary antibodies followed by incubation with horseradish peroxidase-coupled secondary antibodies, and the signals were revealed with enhanced chemiluminescence detection reagent (Amersham Biosciences). For displacement experiments, a blocking peptide directed against yORF4-specific amino acids was added to the reaction mixtures containing antibody-proteins at increasing concentrations as indicated. Protein loading was normalized by incubating the same filters with anti-β-actin antibody, and the band intensity was quantified by densitometry (Bio-Rad). The antibodies used in Western blot and dot blot analysis were: PPARγ (C terminus) sc-7273 and HA probe sc-805 (Santa Cruz Biotechnology); β-catenin C19220-050 (BD Transduction Laboratories); FLAG M2 monoclonal antibody A5441 and β-actin A1978 (Sigma), V5 antibody (Invitrogen).

Expression Vectors—yORF1 and PPARγ2 and yORF4 cDNAs were cloned in pcDNA3 expression vectors (Invitrogen) in-frame with 5'-end HA or FLAG epitopes or in the pcDNA3.1/V5-His-TOPO in-frame with the V5 and His epitopes at the 3'-end. The various cDNAs were obtained by RT-PCR starting from total RNA extracted from tumor tissues. The right orientation was determined by restriction map, and the correct sequence was determined by automated DNA sequencing of the plasmids.

Cell Lines, Transient Transfections, and Transactivation Assays—Cos7, NIH-3T3, and Caco2 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine and 10% fetal calf serum in a 5% CO2 humidified atmosphere at 37 °C. Cos7 and Caco2 cells were transfected by Lipofectamine 2000 (Invitrogen), and NIH-3T3 were transfected by FuGENE 6 (Roche Applied Science). Where indicated, 16 h after transfection, troglitazone (Cayman Chemical) dissolved in dimethyl sulfoxide or the vehicle alone was added at the indicated concentrations and incubated for a further 24 h. All transfections were carried out with a luciferase reporter gene under the transcriptional control of the herpes simplex thymidine-kinase (TK) promoter fused to three copies of the peroxisome proliferator-respons e element (PPRE) derived from the acyl-CoA oxidase gene (PPRE-TK-Luc). Expression vectors containing 5'- or 3'-tagged PPARγ-cDNA were transfected alone or in combination. In all cases, 0.2 µg of the CMV-β-galactosidase-containing plasmid were cotransfected to normalize for transfection efficiency. 48 h later, luciferase and β-galactosidase activity were determined on cell extracts, and luciferase was normalized to β-galactosidase. All experiments were performed in duplicate and repeated at least five times. Data are given as mean ± S.E. Differences between controls and the variants were assessed by Student’s t test (two tailed, unpaired); a p value less than 0.05 was considered significant.

Colony-forming Efficiency Assay—The day after seeding, NIH-3T3 cells (104 cells/6-well plate) were transfected with 2 µg of the empty vector or plasmids containing yORF4 or PPARγ (Roche Applied Science). The cells were exposed to the FuGENE-DNA complexes for 16–20 h and then split 1:10 in 100-mm plates in the G418 selection medium (600 µg/ml Geneticin, Invitrogen). After 15 days, the plates were stained with crystal violet. Colony-forming efficiency was calculated by dividing the number of colonies obtained on plates transfected with the various PPAR constructs by that obtained with the empty vector (30). The reported numbers are the mean from at least five independent experiments and are given as mean ± S.D. Differences between controls and the variants were assessed by Student’s t test; a p value less than 0.05 was considered significant.

BrdUrd Incorporation—NIH-3T3 were transfected with the various cDNA-containing expression vectors. DNA synthesis was assayed by a 2-h pulse with 100 µM BrdUrd, and incorporation was measured as reported (34) by using the in situ cell proliferation kit FLUOS (Roche Applied Science). Experiments were performed in duplicate and repeated at least three times. Data are given as mean ± S.D. Differences were assessed by Student’s t test; a p value less than 0.05 was considered to be significant.

Immunofluorescence Analysis—NIH-3T3 and Caco2 cells were plated on glass slides and transfected with the various cDNA constructs. Coverslips were washed with phosphate-buffered saline, parafomaldehyde fixed, permeabilized with Triton X-100, and incubated 1 h with phosphate-buffered saline containing 1% v/v bovine serum albumin. The fixed cells were then stained with anti-5V, anti-HA, or anti-FLAG antibodies (Invitrogen and Sigma) and revealed with Texas red-conjugated affini-pure mouse IgG. Nuclei were stained with Hoechst 33258 (Sigma). Images were generated with an Axiopt fluorescent microscope.
RESULTS

Identification and Analysis of the γ-ORF4 Transcript—Using human genome sequencing data (UCSC Genome Browser, genome.ucsc.edu), we looked for expressed sequence tags (EST) and conserved non-coding sequences between human and mouse sequences distributed at the 3′-end of the chromosomal region spanning the entire PPARG. We identified an intriguing non-coding sequence at the 3′-end of exon 4 that extends into the intronic sequences. This non-coding sequence overlaps two ESTs initially identified in an ovary fibrothecoma (BF514811) and in an adenocarcinoma (AI476551). Both ESTs code for the same product, which we examined in detail (Fig. 1A). We next looked for the putative transcript, named γ-ORF4, in cDNAs.
from two human transformed colon cell lines and from colon and ovarian carcinoma tissues. To this aim, we used RT-PCR analysis with oligonucleotides located on exon 1 and intron 4 sequences within the EST as primers (Fig. 1B). The RT-PCR analysis confirmed production of the γORF4 transcript, and we extended the analysis to the full-length cDNA. To map the correct ends of the transcript, primers on exons A and B (for the 5′-end) were synthesized and paired with primers synthesized on the intronic sequences at different distances from the splice junction between exon 4 and intron 4 (for the 3′-end) (data not shown). Sequence analysis of the products obtained yielded two full transcripts of about 1600 nucleotides originating from exons A and B (European Molecular Biology Laboratory accession number AJ563369 and AJ563370) (Fig. 1C). No base pair changes or deletions were detected versus the wild-type sequence. It is noteworthy that there were no mutations in the exon-intron junctions that could explain the readthrough in intron 4. Only the transcript from exon A was characterized further. It contains the PPARγ coding sequences from exon 1 to exon 4 plus 63 additional in-frame nucleotides derived from intron 4 sequences. The γORF4 mRNA extends into the intron for about 1 kb up to a canonical poly-A addition site and reaches a length similar to that of the entire PPARγ transcript, which explains why it has eluded detection so far. We would expect this transcript to generate a protein containing the PPARγ sequence from amino acid 1 to 273 plus 21 new C-terminal amino acids derived from the intronic sequences. Thus, the expected protein would lack the entire ligand-binding domain (Fig. 1D).

In Vitro Expression and Subcellular Localization of PPARγ and the γORF4 Isoform—To determine whether the γORF4 transcript is translated into protein in an in vitro system and to investigate its biological role, we performed transient transfections in Cos7, NIH-3T3, and Caco2 cells using expression vectors containing PPARγ1, PPARγ2, and γORF4 cDNAs (Fig. 2A). All cDNAs were fused in-frame to DNA segments coding for specific epitopes (HA, FLAG, and V5) recognized by commercially available antibodies. These antibodies reveal only the proteins synthesized by the transfected cDNAs. In fact, using anti-FLAG antibodies, specific bands corresponding to the tagged PPARγ or γORF4 proteins (~60 and 40 kDa, respectively) were obtained in Western blots of transfected cell extracts (Fig. 2B). Their intensity was similar, indicating that the two proteins are synthesized at equivalent levels (data not shown). Similar bands were detected with anti-PPARγ antibodies (data not shown). PPARγ and γORF4 proteins were also localized by immunofluorescence. Using an epitope-specific antibody (anti-FLAG) on transiently transfected NIH-3T3 and Caco2 cells, PPARγ showed diffuse cell staining, whereas the isoform showed mainly nuclear staining (Fig. 2C). To rule out that this result was due to the high expression of the protein in a limited number of transfected cells, we did the same experiments with cells stably expressing PPARγ or γORF4 and obtained a similar result (data not shown). Thus, the isoform has a more evident nuclear localization than PPARγ.

Transcriptional Activity of PPARγ and γORF4 in Vitro—We evaluated the transactivation capacity of PPARγ and γORF4 by cotransfecting into Cos7 cells expression vectors carrying their corresponding cDNAs and a reporter gene. This construct contains three copies of a PPRE fused upstream to the minimal herpes simplex TK promoter that in turn drives transcription of a luciferase reporter gene. The cells were exposed to increasing concentrations of troglitazone, a PPARγ-selective ligand belonging to the thiazolidinedione family (5). PPARγ-transfected cells had a strong ligand-dependent transcriptional response, whereas γORF4 resulted in a negligible response, even at the highest ligand concentrations used (Fig. 2A). Indeed, luciferase activity was even lower (~50%) in γORF4-transfected cells than in cells transfected with the empty expression vector, which probably reflects inhibition of the endogenous receptor (Fig. 3A, inset). Cells were also transfected with the PPRE-luciferase reporter gene and increasing amounts of PPARγ or γORF4 expression vectors in the presence of a fixed concentration of the ligand (1 μM troglitazone). PPARγ caused a proportional increase of luciferase activity, whereas γORF4 did not, even at the highest ratios used (Fig. 3B). Therefore, the isoform does not promote transcription from a reporter gene and can conceivably interfere with PPARγ receptor activity. To investigate this possibility, we cotransfected Cos7 cells with the PPRE-TK-luciferase reporter gene and equal amounts of PPARγ and γORF4 expression vectors (ratio 1:1) in the presence of increasing concentrations of troglitazone. At all
whereas the transactivation activity is reported relative to the maximum obtained with PPARγ empty vector (light gray) or PPARγ expression vector alone. In all transfections, 200 ng of the CMV-β-galactosidase control plasmid were cotransfected to normalize for different transfection efficiency. The reporter/expression vector ratios used are indicated, obtained with PPARγ empty vector (light gray) in the presence of increasing concentrations of troglitazone. Transcriptional repression is reported relative to the maximal activation obtained with PPARγ expression vector alone. In all transfections, 200 ng of the CMV-β-galactosidase control plasmid were cotransfected to normalize for different transfection efficiency. The results reported are the mean of at least five different experiments carried out in duplicate with different DNA preparations and were assessed by Student’s t test and was significant for all combinations. TZD, troglitazone.

**FIG. 3.** γORF4 has impaired transactivation ability and interferes with PPARγ function. A, γORF4 does not activate a reporter gene. Cos7 cells were transfected with 250 ng of the reporter gene and 100 ng of PPARγ ( ), γORF4 ( ) expression vectors (exp. vector) or the empty vector ( ) in the presence of increasing concentrations of troglitazone. Transcriptional activity is reported relative to the maximum obtained with PPARγ. Inset, basal transcriptional activity (see “Results”). B, Cos7 cells exposed to a single dose of troglitazone were transfected with 250 ng of the PPRE-TK-luciferase reporter gene and increasing amounts of PPARγ (light gray) or γORF4 (dark gray) expression vectors. The reporter/expression vector ratios used are indicated, whereas the transactivation activity is reported relative to the maximum (100%) obtained with PPARγ receptor. C, γORF4 interferes with the transactivation activity of PPARγ. 250 ng of the PPRE-TK-Luc reporter gene were cotransfected with equal amounts of PPARγ and γORF4 expression vectors (ratio 1:1) (dark gray) or with PPARγ expression vector alone (light gray) in Cos7 cells exposed to increasing concentrations of troglitazone as indicated. Transcriptional repression is reported relative to the maximal activation obtained with PPARγ receptor. D, Cos7 cells exposed to vehicle or 1 μM troglitazone were cotransfected with 250 ng of the reporter gene and a fixed amount (100 ng) of PPARγ and increasing concentrations of γORF4 expression vector. Transcriptional repression is reported relative to the maximal activation obtained with PPARγ expression vector alone. In all transfections, 200 ng of the CMV-β-galactosidase control plasmid were cotransfected to normalize for different transfection efficiency. The results reported are the mean of at least five different experiments ± S.E. carried out in duplicate with different DNA preparations and were obtained with the FLAG-containing expression vectors. p ≤ 0.05 was assessed by Student’s t test and was significant for all combinations. TZD, troglitazone.

**TABLE I.**

| Clinical-pathological features of the patients examined | Number of patients % |
|------------------------------------------------------|----------------------|
| Age (years)                                           |                      |
| 20–40                                                | 0                    |
| 40–60                                                | 24                   |
| >60                                                  | 76                   |
| Gender                                               |                      |
| Men                                                   | 68                   |
| Women                                                 | 32                   |
| Localization                                         |                      |
| Colon                                                 | 52                   |
| Sigmoid colon                                         | 32                   |
| Rectum                                                | 16                   |
| Cytological grading (Broder’s classification)         |                      |
| Well differentiated (1–2)                            | 16                   |
| Moderately differentiated (3)                         | 68                   |
| Poorly differentiated (4)                             | 16                   |

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We next analyzed 25 sporadic colorectal cancers to determine whether the newly identified isoform is expressed in vivo. We excluded inherited tumors on the basis of the personal and familial history of each patient and the absence of such gene mutations as APC (38). PPARγ expression was monitored at the mRNA and protein level on extracts from tumor specimens and on the adjacent mucosa removed during surgery from patients affected by this malignancy. The characteristics of the patients and tumors are reported under “Materials and Methods” and in Table I. PPARγ mRNA expression was evaluated by semiquantitative RT-PCR on total RNA extracted from tumors and paired adjacent mucosa samples, as control, using primers located on exons 1 and 6. A band of the expected size (~1600 bp), corresponding to the mature transcript, was detected in all samples, and its intensity was higher in tumor samples than in control adjacent mucosa samples (Fig. 4A). Relative amounts were normalized using primers specific for cyclophilin A (PPIA), the expression of which is not altered in colorectal tumors (39). Similarly, there were no variations in the expression of β-catenin or PPARγ6/6 genes that are involved in other pathogenetic pathways (38). Strikingly, in these samples, the exon 1 and intron 4 primer pair identified a transcript identical in size to γORF4 (Fig. 4A). In 12 of the 25 specimen pairs tested, the specific band appeared only in the tumor sample (Fig. 4A, samples 1–3). Interestingly, in four samples, it was detected in both the tumor and the paired adjacent mucosa
FIG. 4. PPARγ and γORF4 expression in tumor specimens and paired adjacent control mucosa. A, RT-PCR analysis of total RNA extracted from tumors (T) and paired adjacent control mucosa (C) samples. Semiquantitative RT-PCR was performed by co-amplifying the cDNAs of interest with primers for PPIA (cyclophilin A) as control. The cDNAs amplified are indicated on the left and correspond to PPARγ, γORF4, β-catenin, and PPARβ/δ, respectively. The size of the amplified products are indicated on the right. Only some representative samples of the tumors and paired adjacent mucosa analyzed are reported. Samples 1–3 are representative of the 12 specimens in which γORF4 was detected exclusively in the tumor tissue. Samples 5 and 6 are representative of the four samples in which γORF4 was detected also in the adjacent control mucosa, although at lower levels. Lane N shows the results obtained with normal colon mRNA (Clontech). B, Western blot analysis of protein extracts (50 μg) from tumors (T) and paired adjacent mucosa used as control (C). The proteins immunodetected are shown on the left, and the size of the detected bands is on the right. The results refer to the same samples analyzed at RNA level (panel A). The N lane shows the results obtained with extracts from a normal subject with no malignancies. The Cos transf. lane shows the size of the band corresponding to the transfected γORF4. C, validation of an anti-γORF4-specific antibody (Ab). In a dot blot assay, increasing amounts of the antibody specifically bound to a fixed concentration (50 μg) of protein extracts from a tumor sample. The signal intensity increased proportionally to the amount of the peptide added to the reaction (Fig. 4C, top row). The intensity of the signal increased proportionally. Displacement experiments were also carried out by adding increasing concentrations (from 20 to 100 μg) of the synthetic peptide used for immunization. Signal intensity decreased proportionally to the amount of the peptide added to the reaction (Fig. 4C, bottom row). These results were confirmed in Western blot assays on two tumor specimens and paired control mucosa; the anti-γORF4 antibody produced a major band only in tumor tissues (Fig. 4D, top). The addition of the synthetic peptide specifically displaced the band in both tumor samples (Fig. 4D, bottom). Growth Inhibitory Effects of PPARγ and γORF4—PPARγ has been reported to inhibit cell growth (12, 13, 41, 42). We wondered whether this function could be impaired in γORF4. To address this issue, we used the colony-forming efficiency assay to test the ability of γORF4 to restrain cell growth (33). NIH-3T3 cells do not express PPARγ or at least do not express PPARγ at levels detectable with the methods currently available as control (Fig. 4A, samples 4 and 5). Of note, when the RT-PCR assay was performed with cDNAs from 10 normal human colon samples, the γORF4 transcript was not detected, even at saturating PCR conditions (i.e. between 35 and 40 cycles). A representative sample is reported in Fig. 4A as N. The results at mRNA level were paralleled at protein level. We monitored PPARγ expression using an antibody against the C-terminal region of the mature protein and found a major band with an apparent kDa of 55, corresponding to the mature receptor. After normalization of extracts with an anti-β-actin antibody, band intensity in tumor samples was similar to or higher than that in the paired adjacent mucosa as control (Fig. 4B), as reported elsewhere (40). We analyzed the same panel of tumors with anti-PPARβ/δ and anti-β-catenin antibodies and found no differences between tumor specimens and the paired adjacent mucosa, thereby confirming the results obtained at mRNA level (Fig. 4B). The anti-PPARγ antibody did not recognize the γORF4 protein product because the C-terminal part of the protein is missing. We raised a polyclonal antibody against a selected peptide from the new open reading frame and used it to recognize the isoform in the tumors analyzed. This antiserum should recognize only the γORF4 protein. In fact, 12 out of 25 tumor samples showed a band that had an apparent kDa of 35–40, which matches the band detected in extracts from γORF4-transfected Cos7 cells (Fig. 4B). In four other cases, the band appeared also in the adjacent mucosa extract, albeit at a lower intensity. Interestingly, no immunoreactive bands were detected in extracts from colonic mucosa samples of normal individuals (Fig. 4B). We verified the specificity of the interaction in dot blot experiments in which 50 μg of protein extract from a tumor specimen were challenged with increasing concentrations of the γORF4 antibody (Fig. 4C, top row). The intensity of the signal increased proportionally. Displacement experiments were also carried out by adding increasing concentrations (from 20 to 100 μg) of the synthetic peptide used for immunization. Signal intensity decreased proportionally to the amount of the peptide added to the reaction (Fig. 4C, bottom row). Of note, when the RT-PCR experiment, the addition of increasing concentrations of the synthetic peptide specifically displaced the band detected in extracts from two tumor and adjacent control mucosa pairs with the anti-γORF4 antibody. The specific signal was detected only in the tumor samples. The addition of the blocking peptide specifically reduced or abolished any signal.
able. Therefore, we used them as recipient cells, and the results obtained were exclusively referred to the exogenously transfected cDNAs and synthesized proteins. PPARγ/H9253 reduced colony-forming efficiency by about 50% with respect to the cells transfected with the empty vector alone, whereas /H9253 ORF4 did not (Fig. 5A). This experiment confirmed that PPARγ/H9253 inhibited cell growth and that /H9253 ORF4 has lost this property. To determine whether /H9253 ORF4 plays a role in cell proliferation, either alone or in association with the weak oncogene c-Myc, we examined BrdUrd incorporation in cells transiently expressing PPARγ or /H9253 ORF4 constructs. PPARγ reduced cell proliferation by 40% (15% versus 25%), whereas /H9253 ORF4 increased BrdUrd incorporation with respect to the control (58% versus 25%) (Fig. 5B). Interestingly, these effects were even more pronounced in cotransfection experiments with c-Myc. BrdUrd incorporation in cells transfected with c-Myc alone was about 45%. It was similar to the control when PPARγ was cotransfected (about 25%), but it increased to 88% with /H9253 ORF4. Moreover, /H9253 ORF4 counteracted the inhibitory effect of PPARγ when cotransfected at a 1:1 ratio. These experiments imply that not only has /H9253 ORF4 lost growth suppressor properties but it can act as a growth inducer by interfering with the inhibitory function of PPARγ.

**DISCUSSION**

A hallmark of the human genome is its plasticity, *i.e.* its coding capacity exceeds the number of genes detected. The generation of proteins with distinct functions from a single transcript is frequently due to alternative and aberrant splicing, events that are implicated in physiological activities as well as in tumors and other diseases (25). PPARγ is a paradigm of such plasticity. In fact, differential promoter usage and chromosomal translocations occur at this locus (4, 5, 8). Our data confirm that alternative splice junctions at PPARγ result in different transcripts. We also report a new PPARγ transcript, /H9253 ORF4, which results from a readthrough that juxtaposes the coding sequences from exon 1 to exon 4 to the adjacent sequences of intron 4. The /H9253 ORF4 transcript results in the synthesis of a protein containing the first 273 amino acids of

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2 L. Sabatino, A. Casamassimi, G. Peluso, M. V. Barone, D. Capaccio, C. Migliore, P. Bonelli, A. Ciccodicola, and V. Colantuoni, unpublished data.

3 M. V. Barone, unpublished data.
the mature PPARγ plus an additional 21 amino acids coded for by intron 4 sequences. As a consequence, the whole ligand-binding domain encoded for by exons 5 and 6 is deleted. Moreover, like other PPARγ mutants (43), γORF4 appears to be predominantly nuclear, probably because it lacks the C-terminal part of the protein that contains a nuclear export signal. In contrast, PPARγ is shuttled between the cytoplasm and the nucleus in response to the availability of specific ligands. Despite its nuclear localization, γORF4 has lost the ability to activate transcription from a responsive promoter, and it has become dominant negative to PPARγ. Natural and artificial PPARγ mutants in the ligand-binding domain have been reported (35–37) and associated to diabetes and the metabolic syndrome or to the inability to promote adipocyte differentiation. All have impaired transactivation ability and act in a dominant negative manner. Since γORF4 lacks the ligand-binding domain, the transcriptional impairment could be because it cannot bind the ligand and thus cannot recruit coactivators and undergo the structural changes necessary for chromatin remodeling and gene activation (1, 5). However, γORF4 retains the DNA-binding domain and the heterodimerization interface and can thus recognize the cognate-response elements of target genes upon heterodimerization with the retinoid X receptor (1, 5). The resulting heterodimers are, however, transcriptionally inactive and could function by sequestering the retinoid X receptor partner to the heterodimers of the wild-type receptor and/or by competing in binding to the DNA. This mechanism of action is speculative. However, we have already evidence that some PPARγ target genes are no longer activated in cells overexpressing γORF4. However, other alternative mechanisms cannot be ruled out.

Strikingly, γORF4 was expressed in vivo in sporadic colorectal cancers. We chose to examine this neoplasia because one of the original ESTs was identified in a colon adenocarcinoma in sporadic colorectal cancers (19, 20), which implies that mutations are exceedingly rare (13, 44). Recent studies show that PPARγ is involved in cancer development in animal models (21, 22). Expression of γORF4 in some of our tumor specimens implied that it may be related to tumorigenesis. Our data suggested that the mechanism by which the new transcript can affect tumorigenesis. In fact, we showed that γORF4 has lost the capacity to inhibit cell proliferation and has acquired the capacity to promote cell division. Interestingly, the oncogene c-Myc enhances this property, and the interference with PPARγ takes place at a 1:1 ratio, a condition found in the living cell. These data supported the idea that γORF4 is a cell proliferator agonist and, in concert with a weak oncogene such as c-Myc, it further stimulates cell proliferation. These data suggest that the new isoform may play a role in the tumorigenic process.

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A Novel Peroxisome Proliferator-activated Receptor γ Isoform with Dominant Negative Activity Generated by Alternative Splicing

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