

NK receptor on a T cell potentially facilitates a response to small amounts of antigen, it is possible that TANK cells are important in initiating immune responses. In particular, because, by flow cytometry, NK receptors occur primarily on T cells of a memory phenotype (19, 23), costimulatory NK receptors may especially facilitate the rapid induction of secondary T cell-mediated immune responses. In addition, the expression and function of activating class I MHC receptors may allow activation of a T cell whose TCR may interact weakly with self peptide. Thus, expression of such receptors could also be significant in triggering the onset of autoimmune disease.

REFERENCES AND NOTES

1. V. A. Boussiotis, J. G. Gribben, G. J. Freeman, L. M. Nadler, *Curr. Opin. Immunol.* **6**, 797 (1994).
2. E. Ciccone *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9794 (1990); E. Ciccone *et al.*, *J. Exp. Med.* **175**, 709 (1992); V. Litwin *et al.*, *ibid.* **178**, 1321 (1993); M. Colonna and J. Samaridis, *Science* **268**, 405 (1995); A. D'Andrea *et al.*, *J. Immunol.* **155**, 2306 (1995); J. E. Gumperz and P. Parham, *Nature* **378**, 245 (1995); A. Moretta *et al.*, *Annu. Rev. Immunol.* **14**, 619 (1996).
3. N. Wagtmann *et al.*, *Immunity* **2**, 439 (1995).
4. M. Colonna, E. G. Brooks, M. Falco, G. B. Ferrara, J. L. Strominger, *Science* **260**, 1121 (1993); M. Colonna, G. Borsellino, M. Falco, G. B. Ferrara, J. L. Strominger, *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 12000 (1993).
5. O. Mandelboim *et al.*, *J. Exp. Med.* **184**, 913 (1996).
6. M. Cella *et al.*, *ibid.* **180**, 1235 (1994); J. E. Gumperz *et al.*, *ibid.* **181**, 1133 (1995).
7. L. Olcese *et al.*, *J. Immunol.* **156**, 4531 (1996); D. N. Burshtyn *et al.*, *Immunity* **4**, 77 (1996); A. M. Fry *et al.*, *J. Exp. Med.* **184**, 295 (1996); K. S. Campbell *et al.*, *ibid.*, p. 93.
8. H. Spits, L. L. Lanier, J. H. Phillips, *Blood* **85**, 10 (1995).
9. M. C. Mingari *et al.*, *Int. Immunol.* **7**, 697 (1995); C. S. Falk, A. Steinle, D. J. Schendel, *J. Exp. Med.* **182**, 1005 (1995); S. Ferrini *et al.*, *Eur. J. Immunol.* **24**, 2294 (1994); H. Nakajima, H. Tomiyama, M. Takiguchi, *J. Immunol.* **155**, 4139 (1995).
10. J. H. Phillips, J. E. Gumperz, P. Parham, L. L. Lanier, *Science* **268**, 403 (1995).
11. A. Moretta *et al.*, *J. Exp. Med.* **182**, 875 (1995); R. Biassoni *et al.*, *ibid.* **184**, 645 (1996).
12. TANK cell clones were prepared in the same manner as the NK clones used in (5), except that the CD4⁺ T cells were not depleted.
13. Cells were typed by flow cytometry for the presence of CD3, CD4, CD8, CD16, CD56, TCR $\alpha\beta$, and TCR $\gamma\delta$ (with antibodies from Becton-Dickinson), the type of TCR (with antibodies from Coulter Immunology), CD94 (with mAb HP3B1), and NK receptors [with mAbs specific for NK1 receptor (HP3E4), NK2 receptor (GL183, Coulter Immunology), and NK3 receptor (DX9)].
14. Oligonucleotides complementary to nonpolymorphic regions of the known NK receptor sequences were chosen as GATGGTACATGTCATAGGAGCTCC (at the 3' end) and GAAACCTTCCCTCCTGGCCC (at the 5' end). The resultant PCR product that was amplified from cDNA derived from the T cell was cloned into pCRII (Invitrogen). The sequence of the insert was determined by automated sequencing at the Molecular Biology Core Facilities at the Dana-Farber Cancer Institute, Boston, MA. This sequence was then aligned against sequences currently held in the GenBank database with the program BLAST. The insert obtained from the cDNA of clone TANK-1 was 100% identical to that of the NK receptor, clone 39.
15. Reverse transcriptase-PCR typing of NK receptors was performed with oligonucleotides complementary to polymorphic regions of the extracellular portions of the known NK receptors and with oligonucleotides complementary to the long and short cytoplasmic tail sequences of NK receptors (H. T. Reyburn *et al.*, in preparation).
16. TANK cells are not exclusively CD4⁺ TCR $\alpha\beta$ ⁺ because TCR $\gamma\delta$ ⁺ and CD8⁺ TCR $\alpha\beta$ ⁺ TANK cells have also been obtained (O. Mandelboim *et al.*, in preparation).
17. Y. Shimizu and R. DeMars, *J. Immunol.* **142**, 3320 (1989).
18. Proliferation assays were performed as follows: The target cells were irradiated on a cesium source for 30 min (~30 Gy). Thereafter, 50,000 T cells, 25,000 target cells, various amounts of superantigen, and any other appropriate reagents were mixed in a total volume of 200 μ l of RPMI-10% fetal calf serum in each well of a 96-well plate. After incubation at 37°C and 5% CO₂ for 2 days (or the time indicated), 1 μ Ci of [³H]thymidine was added to each well, and the cells were further incubated at 37°C overnight. The cells were then harvested (Harvester 96 Mach III M, Tomtec) and counted on a liquid scintillation counter (1450 Microbeta Plus, Wallac). In analysis of the counts per minute (cpm) from each well, the background cpm from a well in which identical reagents and target cells were placed in the absence of any T cells was subtracted.
19. O. Mandelboim, D. M. Davis, H. T. Reyburn, E. G. Sheu, unpublished data.
20. J. F. A. P. Miller and G. Morahan, *Annu. Rev. Immunol.* **10**, 51 (1992).
21. L. Pazmany *et al.*, *Science* **274**, 792 (1996).
22. This may be because HLA-G has a greater binding affinity for NK2 than for NK1 receptors or because HLA-G can interact with other inhibitory receptors on lymphocytes yet to be determined. Alternatively, the signals for inhibition of the proliferative response could be dominant over activating signals when both are triggered in this clone.
23. A. D'Andrea, *J. Exp. Med.* **194**, 784 (1996).
24. We thank M. López-Botet (mAbs HP3B1 and HP3E4) and L. Lanier (mAb DX9). Supported by EMBO and the Fullbright Commission (O.M.), The Wellcome Trust (H.T.R.), the Arthritis Foundation (L.P.), and NIH grant CA 47554.

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Inhibition of Adipogenesis Through MAP Kinase-Mediated Phosphorylation of PPAR γ

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Adipocyte differentiation is an important component of obesity and other metabolic diseases. This process is strongly inhibited by many mitogens and oncogenes. Several growth factors that inhibit fat cell differentiation caused mitogen-activated protein (MAP) kinase-mediated phosphorylation of the dominant adipogenic transcription factor peroxisome proliferator-activated receptor γ (PPAR γ) and reduction of its transcriptional activity. Expression of PPAR γ with a nonphosphorylatable mutation at this site (serine-112) yielded cells with increased sensitivity to ligand-induced adipogenesis and resistance to inhibition of differentiation by mitogens. These results indicate that covalent modification of PPAR γ by serum and growth factors is a major regulator of the balance between cell growth and differentiation in the adipose cell lineage.

Adipose differentiation is influenced by a large number of mitogens and growth factors (1). In general, polypeptides that stimulate cell growth block fat cell differentiation. Platelet-derived growth factor, epidermal growth factor (EGF), fibroblast growth factor, and tumor promoters all inhibit fat cell differentiation in culture or in vivo (2). Various cytokines, including tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), IL-6, transforming growth factor- β , and interferon- γ also inhibit adipogenesis (3). Insulin has a prominent and complex role in the development of adipose cells, serving as a growth or differentiation factor depending on the specific cell type. Adipose cell precursors (preadipocytes), which express small amounts of insulin receptors, generally require insulin or insulinlike growth factor-1 for optimal differentiation (4). Adipose cells, which contain large numbers of insulin receptors but are postmitotic, respond to

insulin with a lipogenic response as a result of the activation of lipogenic enzymes and the stimulation of Glut4-mediated glucose transport (5). In contrast, fibroblasts that express ectopically large amounts of insulin receptors usually respond to insulin with cell growth rather than differentiation (6).

Two families of factors are especially prominent in the transcriptional control of adipogenesis: the PPARs and C/EBPs. PPAR γ is a member of the nuclear hormone receptor family that is expressed preferentially in adipose tissue (7). It is expressed in small amounts in preadipocytes, and its synthesis is increased during the process of adipogenesis (8). PPAR γ binds specific ligands, including synthetic antidiabetic thiazolidinediones and 15-deoxy- $\Delta^{12,14}$ prostaglandin J₂ (9), resulting in a full and powerful adipogenic response. Thus, PPAR γ appears to be a key component in the determination and differentiation process in vivo (9, 10).

Ectopic expression of C/EBP- β and C/EBP- δ stimulates adipogenesis in fibroblasts as well (11, 12). This occurs through

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the C/EBP-mediated expression of PPAR γ (12). Adipogenesis induced by C/EBP- β and C/EBP- δ requires a PPAR γ ligand (13). Expression of large amounts of C/EBP- α also promotes fat cell differentiation (14). However, when expressed at more physiological amounts, C/EBP- α can synergize with PPAR γ in the promotion of fat cell differentiation of fibroblasts or myoblasts (10, 15). Cross-regulation between PPAR γ and the C/EBPs may be crucial in maintaining the differentiated state of adipocytes (16).

Growth factor inhibition of adipogenesis might occur through effects on PPAR γ . To investigate this, we treated cells with various mitogens and examined their effects on PPAR γ ectopically expressed in two established lines of fibroblasts: NIH 3T3 cells, which have small amounts of insulin receptors, or Rat-1 cells that ectopically express the insulin receptor (Rat-IR cells). Rat-IR cells respond to insulin with a mitogenic response but no adipogenesis, whereas insulin promotes the differentiation of the NIH-PPAR γ cells (10). PPAR γ migrates as two closely spaced bands on an SDS-polyacrylamide gel, with the lower form being the predominant species (Fig. 1A). Stimulation of Rat-IR cells with EGF, 12-O-tetradecanoylphorbol-13-acetate (TPA), serum, or insulin for 30 min caused a reduction in the amount of the lower migrating species and an increase in the amount of the upper species (Fig. 1A, top panel). TNF- α treatment did not cause this mobility shift. An identical mobility shift was seen in NIH-PPAR γ cells treated with TPA or serum, but not with insulin (Fig. 1A, bottom panel). The mobility shift could be detected within 5 min after treatment of cells with insulin in Rat-IR cells and persisted for at least 4 hours (Fig. 1B). Treatment of cell extracts with calf intestinal alkaline phosphatase uniformly converted PPAR γ into the form of higher mobility (Fig. 1B), suggesting that the protein in the upper band is phosphorylated. To confirm directly that PPAR γ is phosphorylated, we metabolically labeled cells with [³⁵S]methionine and ³²PO₄ and immunoprecipitated PPAR γ . Phosphate was preferentially associated with the upper form of PPAR γ , and the intensity of the upper form was increased upon insulin treatment (Fig. 1C).

Preliminary mapping of the phosphorylation site associated with this mobility shift was carried out by examination of a series of NH₂-terminal deletions (17). The key region was localized near a serine residue (Ser¹¹²) that is present in a sequence (PASP) that matches a consensus sequence for MAP kinase (PXSP), where X represents neutral or basic amino acids (18, 19). Indeed, treatment of wild-type PPAR γ in vitro with MAP kinase (specifically, Erk1, also called p44)

caused a mobility shift of wild-type PPAR γ , but it did not alter the mobility of an allele containing a serine-to-alanine mutation at position 112 (S112A, Fig. 2A). This mutation

also blocked the ability of insulin, EGF, TPA, or serum to cause this mobility shift in vivo (Fig. 2B). In metabolic labeling experiments, this mutation blocked the ability of insulin

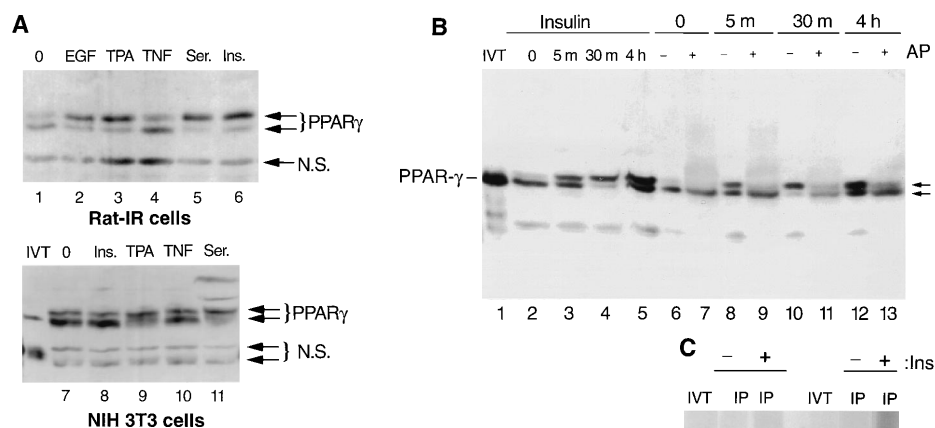
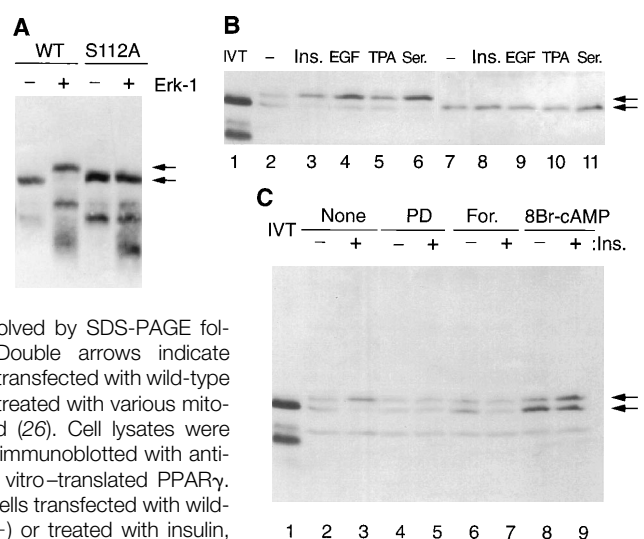


Fig. 1. Modification of PPAR γ in response to mitogenic stimulation. **(A)** Transfection of PPAR γ 2 into Rat-IR and NIH 3T3 cells, growth factor stimulation, SDS-polyacrylamide gel electrophoresis (PAGE), and protein immunoblots were performed as described (26). PPAR γ 2 (shown with two arrows) migrates as two closely spaced bands with a molecular mass of \sim 55 kD. Lanes 1 to 6 are extracts from Rat-IR cells and lanes 7 to 11 are from NIH 3T3 cells. Treatments with the indicated mitogens were for 30 min. Ser., serum; Ins., insulin; IVT, in vitro-translated PPAR γ 2; N.S., nonspecific bands. **(B)** Transfected Rat-IR cells were treated with insulin (5 μ g/ml) and harvested at different time points as in (A). Lysates were treated with calf intestine alkaline phosphatase (AP) as described (27). Proteins in treated (+) and untreated (-) lysates along with the original lysate were separated by SDS-PAGE and immunoblotted. Lane 1 (IVT), 1 μ l of in vitro-translated PPAR γ 2 (TNT kit, Promega). Lanes 2 to 5 are undialyzed lysates stimulated with insulin for 0, 5 min (m), 30 min, and 4 hours (h), respectively. Lane pairs 6-7, 8-9, 10-11, and 12-13 are dialyzed samples at different time points with (+) or without (-) AP treatment. **(C)** Transfected Rat-IR cells were metabolically labeled with [³⁵S]methionine (1 mCi/ml) or ³²P (2 mCi/ml) for 4 hours and were then stimulated with insulin (5 μ g/ml) for 30 min. Cells were lysed with RIPA as described and immunoprecipitations (IPs) were done in RIPA buffer as standard procedure. ³⁵S-labeled in vitro-translated PPAR γ (1 μ l) was used as positive control for IP (IVT, lanes 1 and 5). Lanes 2 and 3 are IPs from ³⁵S-labeled extracts without (-) or with (+) insulin stimulation. Lanes 6 and 7 are IPs from ³²P-labeled extracts without or with insulin stimulation. IPs with preimmune serums did not contain any bands in the 40- to 60-kD range.

Fig. 2. Phosphorylation of PPAR γ by MAP kinase in vitro and in vivo. **(A)** Mutant PPAR γ was constructed by overlapping polymerase chain reaction (PCR) and verified by sequencing. In vitro-translated wild-type (WT) and mutant (S112A) PPAR γ 2 were treated with active bacterial-synthesized glutathione-S-transferase (GST)-MAP kinase (Erk-1) fusion protein and resolved by SDS-PAGE followed by immunoblotting. Double arrows indicate PPAR γ 2. **(B)** Rat-IR cells were transfected with wild-type or S112A mutant PPAR γ and treated with various mitogens for 30 min as described (26). Cell lysates were separated by SDS-PAGE and immunoblotted with antibody to PPAR γ . Lane 1 is in vitro-translated PPAR γ . Lanes 2 to 6 are lysates from cells transfected with wild-type PPAR γ 2 left untreated (-) or treated with insulin, EGF, TPA, or 30% serum, respectively. Lanes 7 to 11 are lysates from cells transfected with mutant PPAR γ 2 left untreated or treated with the indicated mitogens. **(C)** Rat-IR cells transfected with wild-type PPAR γ 2 were treated with PD98059 (PD) (50 μ M), forskolin (For.) (10 μ M), or 8Br-cAMP (2 mM) for 30 min before being stimulated (+) with insulin (5 μ g/ml) or left unstimulated (-), and cell lysates were immunoblotted as described (26). Lane 1 is 1 μ l of in vitro-translated PPAR γ 2. Lanes 2 and 3 are control lysates without or with insulin stimulation.



and TPA to induce phosphorylation of PPAR γ in Rat-IR cells (17). We also tested several agents that inhibit MAP kinase activity. PD98059 inhibits MAP kinases through direct inhibition of MAP kinase kinase (MEK) and completely prevented the mobility shift (Fig. 2C). Forskolin and 8-bromo-adenosine 3',5'-monophosphate (8Br-cAMP), although not specific agents, also inhibit activation of MAP kinases. No mobility was seen when cells were incubated with them. These data indicate that multiple growth factors all cause a phosphorylation of PPAR γ on a MAP kinase consensus site at Ser¹¹² that can be mediated, at least in some instances, by the classical MAP kinases.

To address the consequences of the Ser¹¹² phosphorylation on PPAR γ function, we examined transcriptional and adipogenic activity of the wild-type and mutant alleles. Mutation of Ser¹¹² to Ala¹¹² had no effect on the nuclear localization,

affinity for retinoid X receptor α (RXR α), or DNA binding activity of PPAR γ (17). However, differences were observed between wild-type and mutant PPAR γ in transactivation assays. Without ligand treatment, wild-type and S112A PPAR γ stimulated similar low levels of transcriptional activity (Fig. 3). A thiazolidinedione

ligand, pioglitazone, stimulated a large increase in the activity of both alleles. Addition of the tumor promoter (TPA) caused an 80% decrease in the effect of pioglitazone on wild-type PPAR γ but only a 20% reduction in ligand stimulation of the S112A allele. Thus, the mutation at position 112 reduces the negative effect of TPA

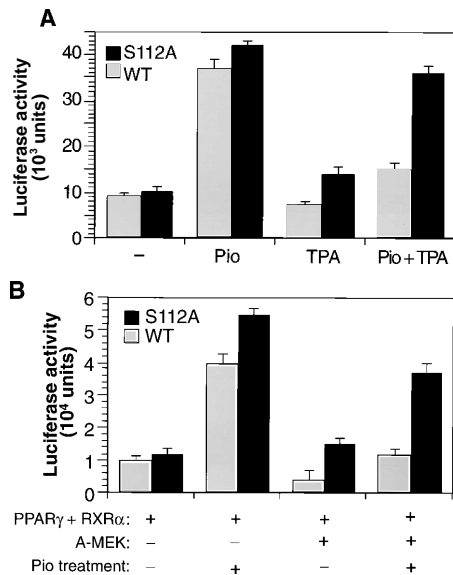


Fig. 3. Effects of TPA and activated MEK on the transcriptional activity of wild-type and S112A PPAR γ . **(A)** Rat-IR cells were transfected with a reporter gene PPRE₃-luciferase (9) (2 μ g) along with PPAR γ and RXR α expression vectors (1 μ g of each) (SV-sport-PPAR γ and SV-sport-RXR α) (8). After 12 hours, cells were washed and re-fed with DMEM medium containing 0.5% bovine serum albumin. After 24 hours, cells were stimulated with pioglitazone (Pio) (5 μ M), TPA (100 ng/ml), or both, or left unstimulated (-). Cells were harvested 18 hours later, and luciferase activity was assayed according to standard procedures. **(B)** Cells were transfected with PPRE₃-luciferase, PPAR γ , and RXR α expression vectors along with an expression vector containing activated MEK-1 (A-MEK) (2 μ g) (28). Cells were treated with or without pioglitazone, and luciferase activities were measured. Transfection efficiency was monitored and normalized by cotransfecting cells with 2 μ g of pCH110 (Pharmacia) vector for β -galactosidase. Error bars represent the standard deviation.

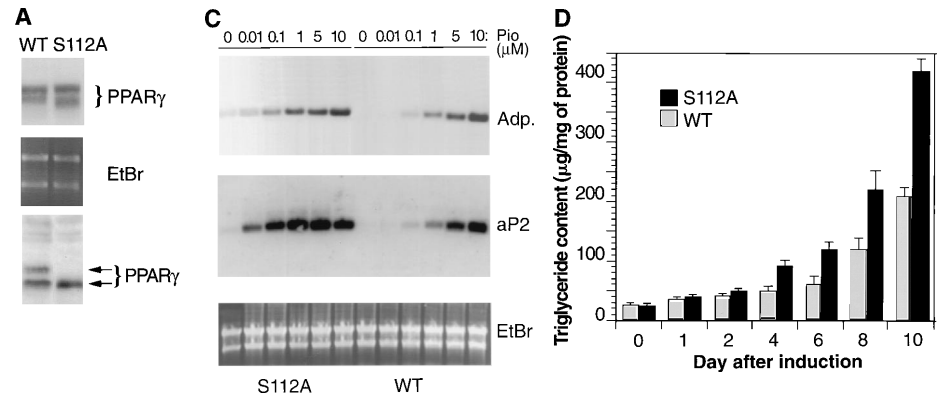


Fig. 4. Adipocyte differentiation induced by wild-type and S112A PPAR γ in NIH 3T3 cells. **(A)** Expression of wild-type and mutant PPAR γ in NIH 3T3 cells. The pBabe retroviral vector (29) was used to express wild-type and S112A mutant PPAR γ in NIH 3T3 cells. Viral infection and cell selections were done as described (10). Expression of viral PPAR γ mRNA (top two panels) and protein (bottom panel) is shown. No endogenous PPAR γ mRNA or protein was detected in NIH 3T3 cells (17). EtBr, ethidium bromide-stained gel. **(B)** Adipose differentiation of NIH 3T3 cells expressing wild-type or mutant PPAR γ with various doses of pioglitazone. Differentiation conditions were essentially as described (10). Ten days after induction, dishes were stained with Oil-Red-O and photographed. **(C)** Total RNA was isolated from NIH 3T3 cells expressing wild-type or mutant PPAR γ that had been treated with the indicated concentration of pioglitazone and probed with the adipocyte-specific cDNAs adipisin (Adp.) and aP2. Equal loading of RNA was ensured by ethidium bromide staining (bottom panel). **(D)** Triglyceride accumulation in cells expressing wild-type or mutant PPAR γ . NIH 3T3 cells expressing wild-type or mutant PPAR γ were induced to differentiate with insulin (5 μ g/ml), 1 μ M dexamethasone, and 5 μ M of pioglitazone in DMEM containing fetal bovine serum (10%). Total triglyceride content of the cells was measured with a triglyceride (GPO-Trinder) kit (Sigma) at various times during the differentiation process. The triglyceride content per milligram of protein for each time point was plotted.

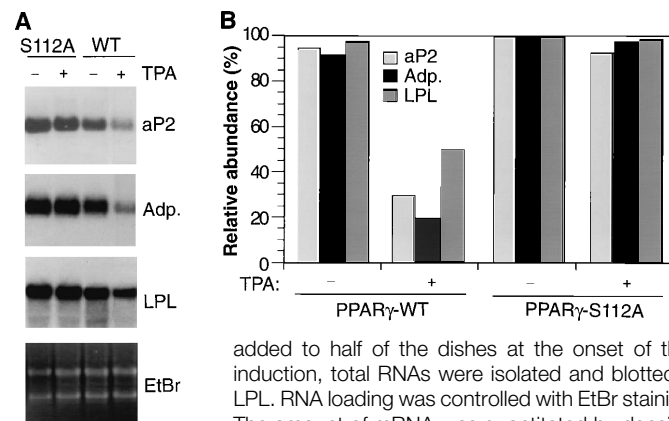


Fig. 5. Effects of TPA on adipocyte differentiation in NIH 3T3 cells expressing wild-type and S112A mutant. **(A)** NIH 3T3 cells expressing wild-type and mutant PPAR γ were induced to differentiate with insulin (5 μ g/ml), dexamethasone (1 μ M), and pioglitazone (5 μ M) as described in Fig. 4D. TPA (100 ng/ml) was added to half of the dishes at the onset of the induction. Ten days after induction, total RNAs were isolated and blotted with aP2, adipisin (Adp.), or LPL. RNA loading was controlled with EtBr staining. **(B)** Quantitation of mRNA. The amount of mRNA was quantitated by densitometry scanning (LKB Pharmacia). One representative experiment is shown. The expression level of these three genes in NIH 3T3 cells expressing the PPAR γ mutant was defined as 100%.

on PPAR γ -mediated transcription, suggesting that the common growth factor-mediated phosphorylation may negatively modulate PPAR γ activity. To specifically examine the role of MAP kinase, we also examined the effects of an activated allele of MEK on PPAR γ activity. Activated MEK suppressed the transcriptional activity of wild-type PPAR γ but had only a small effect on the S112A mutant PPAR γ (Fig. 3B). These data add further support to the role of a MAP kinase in mediating this suppression.

To assess the effects of the S112A mutation on differentiation, we expressed this mutant allele or the wild-type PPAR γ in NIH 3T3 cells with retroviral vectors. Differentiation was assessed in the differentiation medium containing serum, insulin, or various amounts of pioglitazone. These cells have been used extensively to investigate the role of PPAR γ and C/EBPs in adipogenesis (10–12, 20). Similar amounts of both wild-type and mutant PPAR γ mRNAs and proteins were expressed in these cells (Fig. 4A). However, a large increase in differentiation was observed in cells expressing the S112A allele as revealed by lipid accumulation (Fig. 4B) or expression of the differentiation-linked mRNAs adipin and aP2 (Fig. 4C). The dose-response curve for the differentiation response to pioglitazone was shifted 10- to 100-fold in these two assays. Expression of adipin and aP2 mRNAs was observed in cells expressing the S112A mutant without the addition of exogenous ligand, whereas these RNAs were not observed in the wild-type cells (Fig. 4C). The time course of differentiation in the presence of 5 μ M pioglitazone was followed by glycerol accumulation (Fig. 4D). Cells containing the S112A allele differentiated 2 to 4 days before cells bearing the wild-type allele.

Finally, the ability of a mitogen to interfere with differentiation driven by wild-type and mutant PPAR γ was examined. TPA blocks adipogenesis of standard preadipocyte cell lines (2). TPA inhibited (50 to 80%) the expression of three differentiation-linked genes in cells expressing wild-type PPAR γ : aP2, adipin, and lipoprotein lipase (LPL) (Fig. 5). In contrast, morphological adipogenesis (17) and expression of the differentiation-linked genes in cells expressing mutant PPAR γ were basically unaffected by the presence of TPA throughout the differentiation protocol. These results demonstrate that mutation of Ser¹¹² of PPAR γ strongly suppresses the ability of TPA to inhibit adipogenesis.

The ability to balance cell growth and differentiation is critical in the development of multicellular organisms. The data shown here illustrate a rather clear and simple mechanism for interaction between these two processes; MAP kinase, a central regulator of cell

growth, modifies PPAR γ in a way that significantly reduces its transcriptional activity and ability to promote adipogenesis. MAP kinase may be particularly suitable for this purpose because, among the signal transduction machinery linked to the cell cycle, MAP kinase can enter the nucleus to modify transcription factors (21). It is interesting to note that MAP kinase has been implicated in the phosphorylation of another nuclear receptor, the estrogen receptor, although this correlates with an increase in transcriptional activity (22).

These data may have implications for insulin resistance as well as for adipogenesis. The demonstration that PPAR γ is the high-affinity receptor for the thiazolidinedione class of insulin-sensitizing drugs suggests that this receptor is involved in systemic insulin action. This conclusion would imply that peptides or hormones that cause MAP kinase-mediated Ser¹¹² modification of PPAR γ could cause resistance to insulin. In this regard, it is notable that TPA and insulin itself can cause this modification of endogenous PPAR γ (17).

Finally, it will be important to determine the mechanisms by which phosphorylation of Ser¹¹² reduces the activity of PPAR γ . This could occur by direct interference with the binding of ligands, although the region around Ser¹¹² is not near the ligand-binding domain, which resides at the COOH-terminus. Alternatively, this modification could control interactions between PPAR γ and co-repressors or coactivators that have been described to interact with many members of the nuclear receptor family (23). Whether such interactions and their regulation by MAP kinase-mediated phosphorylation contribute to PPAR γ function in adipogenesis *in vivo* remains to be studied.

REFERENCES AND NOTES

- C. M. Smas and H. S. Sul, *Biochem. J.* **309**, 697 (1995); G. Ailhaud, P. Grimaldi, R. Negrel, *Annu. Rev. Nutr.* **12**, 207 (1992); P. Cornelius, O. A. MacDougald, D. M. Lane, *ibid.* **14**, 99 (1994); O. A. MacDougald and D. M. Lane, *Annu. Rev. Biochem.* **64**, 345 (1995).
- H. Hauner, K. Rohrig, T. Petruschke, *Eur. J. Clin. Invest.* **25**, 90 (1995); G. Serrero and D. Mills, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3912 (1991); G. Serrero, *Biochem. Biophys. Res. Commun.* **146**, 194 (1987); M. Navre and G. M. Ringold, *J. Cell Biol.* **109**, 1857 (1989).
- F. M. Torti, S. V. Torti, J. W. Larrick, G. M. Ringold, *J. Cell Biol.* **108**, 1105 (1989); D. Ron, A. R. Brasier, R. E. McGehee, J. F. Habener, *J. Clin. Invest.* **89**, 223 (1992); M. Berg, D. L. Fraker, H. R. Alexander, *Cytokine* **6**, 425 (1994).
- P. J. Smith, L. S. Wise, R. Berkowitz, C. Wan, C. S. Rubin, *J. Biol. Chem.* **263**, 9402 (1988); W. Schmidt, G. Poll-Jordan, G. Löffler, *ibid.* **265**, 15489 (1990); H. Hauner, *Endocrinology* **127**, 865 (1990).
- J. C. Lawrence, *Annu. Rev. Physiol.* **54**, 177 (1992); M. Rodbell, *J. Biol. Chem.* **239**, 375 (1964); C. S. Rubin, E. Lai, O. M. Rosen, *ibid.* **252**, 3554 (1977); J. H. Exton *et al.*, *ibid.* **247**, 3579 (1972).
- A. Ando *et al.*, *J. Biol. Chem.* **267**, 12788 (1992); C. Hofmann, I. D. Goldfine, J. Whittaker, *ibid.* **264**, 8606 (1989); R. S. Thies, A. Ullrich, D. A. McClain, *ibid.*, p. 12820.
- P. Tontonoz, E. Hu, B. M. Spiegelman, *Curr. Opin. Genet. Dev.* **5**, 571 (1995).
- P. Tontonoz, E. Hu, R. A. Graves, A. I. Budavari, B. M. Spiegelman, *Genes Dev.* **8**, 1224 (1994).
- J. M. Lehmann *et al.*, *J. Biol. Chem.* **270**, 12953 (1995); B. Forman *et al.*, *Cell* **83**, 803 (1995); S. A. Klewer *et al.*, *ibid.*, p. 813.
- P. Tontonoz, E. Hu, B. M. Spiegelman, *Cell* **79**, 1147 (1994).
- W. C. Yeh, Z. Cao, M. Classon, S. L. McKnight, *Genes Dev.* **9**, 168 (1995).
- Z. Wu, Y. Xie, N. Bucher, S. R. Farmer, *ibid.*, p. 2350.
- Z. Wu, N. L. Bucher, S. R. Farmer, *Mol. Cell Biol.* **16**, 4128 (1996).
- F. T. Lin and M. D. Lane, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8757 (1994); S. O. Freytag, D. L. Paielli, J. D. Gilbert, *Genes Dev.* **8**, 1654 (1994).
- E. Hu, P. Tontonoz, B. M. Spiegelman, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9856 (1995).
- B. M. Spiegelman and J. Flier, *Cell* **87**, 377 (1996).
- E. Hu and B. M. Spiegelman, unpublished data.
- Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
- F. A. Gonzalez, D. L. Raden, R. J. Davis, *J. Biol. Chem.* **266**, 22159 (1991); E. Alvarez *et al.*, *ibid.*, p. 15277; I. Clark-Lewis, J. S. Sanghera, S. L. Pelech, *ibid.*, p. 15180; A. K. Erickson *et al.*, *ibid.* **265**, 19728 (1990).
- R. Brun *et al.*, *Genes Dev.* **10**, 974 (1996).
- R. Treisman, *Curr. Opin. Cell Biol.* **8**, 205 (1996), and references therein.
- S. Kato *et al.*, *Science* **270**, 1491 (1995); G. Bunone *et al.*, *EMBO J.* **9**, 2174 (1996).
- S. A. Oñate, S. Y. Tsai, M.-J. Tsai, B. W. O'Malley, *Science* **270**, 1354 (1995); S. Halachmi *et al.*, *ibid.* **264**, 1455 (1994); Y. Kamei *et al.*, *Cell* **85**, 403 (1996); A. Horlein *et al.*, *Nature* **377**, 451 (1995); J. D. Chen and R. M. Evans, *ibid.*, p. 454.
- T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, ed. 2, 1989); F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Wiley, New York, 1995).
- A. Vidal-Puig *et al.*, *J. Clin. Invest.* **97**, 2553 (1996).
- Subconfluent Rat-IR or NIH 3T3 cells were transiently transfected with PPAR γ 2 expression vector (SV-sport-PPAR γ 2) as described (8, 10). Twenty-four hours after transfection, cells were washed and fed with Dulbecco's minimal essential medium (DMEM) supplemented with 0.5% bovine serum albumin. Twenty-four hours later, cells were stimulated with EGF (20 ng/ml), TPA (100 ng/ml), TNF (50 ng/ml), insulin (5 μ g/ml), or 30% serum in DMEM for 30 min. Untreated and treated cells were harvested into RIPA lysis buffer (24) supplemented with sodium vanadate (5 mM), NaF (100 mM), phenylmethylsulfonyl fluoride (2 mM), aprotinin (5 μ g/ml), pepstatin (5 μ g/ml), and leupeptin (5 μ g/ml). Soluble proteins were separated by SDS-PAGE (10% gel, acrylamide:bis-acrylamide ratio of 100 with 5 M urea). Protein immunoblots were performed as described (25).
- Rat-IR cells were transfected with expression vector for PPAR γ and cell lysate harvested as described (26). After RIPA lysis, protein (200 μ g) from each time point was dialyzed in buffer (50 mM Tris, pH 8.5, 0.1 mM EDTA) in a microdialyzer (Pierce), and half of the dialyzed lysates were subsequently treated with calf intestine alkaline phosphatase (50 units) for 1 hour at 37°C.
- S. Cowley, H. Paterson, P. Kemp, C. J. Marshall, *Cell* **77**, 841 (1994).
- J. P. Morgenstern and H. Land, *Nucleic Acids Res.* **18**, 3587 (1990).
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