Mouse Resistin Modulates Adipogenesis and Glucose Uptake in 3T3-L1 Preadipocytes Through the ROR1 Receptor

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Mouse resistin, a cysteine-rich protein primarily secreted from mature adipocytes, is involved in insulin resistance and type 2 diabetes. Human resistin, however, is mainly secreted by immune mononuclear cells, and it competes with lipopolysaccharide for the binding to Toll-like receptor 4, which could mediate some of the well-known proinflammatory effects of resistin in humans. In addition, resistin has been involved in the regulation of many cell differentiation and proliferation processes, suggesting that different receptors could be involved in mediating its numerous effects. Thus, a recent work identifies an isoform of Decorin (Δ Decorin) as a functional resistin receptor in adipocyte progenitors that may regulate white adipose tissue expansion. Our work shows that the mouse receptor tyrosine kinase-like orphan receptor (ROR)1 could mediate some of the described functions of resistin in 3T3-L1 adipogenesis and glucose uptake. We have demonstrated an interaction of mouse resistin with specific domains of the extracellular region of the ROR1 receptor. This interaction results in the inhibition of ROR1 phosphorylation, modulates ERK1/2 phosphorylation, and regulates suppressor of cytokine signaling 3, glucose transporter 4, and glucose transporter 1 expression. Moreover, mouse resistin modulates glucose uptake and promotes adipogenesis of 3T3-L1 cells through ROR1. In summary, our results identify mouse resistin as a potential inhibitory ligand for the receptor ROR1 and demonstrate, for the first time, that ROR1 plays an important role in adipogenesis and glucose homeostasis in 3T3-L1 cells. These data open a new line of research that could explain important questions about the resistin mechanism of action in adipogenesis and in the development of insulin resistance. **(*Molecular Endocrinology* 26: 110–127, 2012)**

In addition to being the largest reservoir of energy in the body, the adipose tissue secretes a number of active proteins, named adipocytokines (1). Mouse resistin is one of these adipocytokines that appears positively correlated with adiposity, and it is implicated in the development of insulin resistance, glucose intolerance, and type 2 diabetes mellitus (2). Mouse resistin mRNA is expressed almost exclusively in white adipose tissue (WAT), and the protein is detected both in adipocytes and serum, which is coherent with its autocrine and paracrine functions (3). On the other hand, human resistin is mainly produced by monocytes and macrophages, and it is involved in the development of inflammatory processes (4).

Mouse resistin was independently discovered by three different research groups that used distinct genomic techniques with different purposes. Steppan *et al.* (5) identi-

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Abbreviations: 3-AT, 3-Amino-1, 2, 4-triazole; AKT, serine/threonine-specific protein kinase; AMPK, AMP-activated kinase; DCN, Decorin; FIZZ, found in inflammatory zone; FZZ, Frizzled-like; GAL4, galactose metabolism transcription factor 4; GLUT, glucose transporter; GSK, glycogen synthase kinase; HA, hemagglutinin; KR, Kringle; P0, acidic ribosomal phosphoprotein; PI3K, phosphatidylinositol 3 kinase; RETN*, resistin lacking its signal peptide; ROR, receptor tyrosine kinase-like orphan receptor; ROR1e*, extracellular region of the ROR1 protein lacking the signal peptide; rRETN, purified recombinant resistin; shRNA, short hairpin RNA; SOCS, suppressor of cytokine signaling; TLR, Toll-like receptor; WAT, white adipose tissue; WNT, Wingless type.

fied this protein as a potential target of thiazolidinediones, which enhanced insulin action in 3T3-L1 adipocytes. These authors suggested that resistin could be a factor involved in insulin resistance. By using microarray technology, Kim *et al.* (6) identified this protein as a factor secreted by mature adipocytes and able to inhibit adipocyte differentiation, and they named it as adipose tissue-specific secretory factor. Finally, Holcomb *et al.* (7) identified resistin as a protein induced during lung inflammation, calling it found in inflammatory zone (FIZZ)3 due to its homology to FIZZ1. Resistin/adipose tissue-specific secretory factor/FIZZ3 belongs to a family of proteins named FIZZ or resistin-like molecules, and it forms homooligomers or heterooligomers with other resistin-like molecules/FIZZ proteins (3). Mouse resistin is a 114-amino acid peptide with 11 cysteines that allow the association of several resistin monomers into macromolecular complexes. In mice, resistin generally circulates in blood as an hexamer but also as trimeric forms of greater activity (8). Mouse and human resistin sequences are highly homologous at the genomic, mRNA, and protein levels (9). Nonsecretable forms of rat resistin have been also identified. These forms could regulate the fate and the function of wild-type secretable forms (10).

The role of resistin differs between normal and pathological conditions and among species (11). Several studies have shown that the expression of resistin is differentially regulated in several obesity and diabetes mouse models (3, 12). Mouse resistin is directly involved in glucose metabolism and in the development of insulin resistance in several cell types and tissues, mainly through the modulation of the insulin and the AMP-activated kinase (AMPK) signaling pathways (13). The suppressor of cytokine signaling (SOCS)3, activated by resistin (14), is considered as a mediator of the inhibitory effect of resistin on insulin-mediated signaling in adipocytes. Finally, it has been widely demonstrated that human resistin acts as a proinflammatory protein and seems to exert conflicting effects on insulin resistance and type 2 diabetes mellitus in humans (4, 15).

Resistin also participates in several cell differentiation and proliferation processes by regulating different signaling pathways through the activation of well-known signaling kinases, such as the serine/threonine-specific protein kinase (AKT), ERK1/2, and AMPK (16–18). Mouse resistin plays an active role in adipogenesis, and its expression increases during adipogenesis of 3T3-L1 preadipocytes in response to insulin (6). However, the stimulation of mature adipocytes with insulin decreases the levels of both resistin mRNA and protein. The reports describing the effects of resistin on adipogenesis are contradictory. Both an inhibitory (12, 19, 20) and an enhancing effect (21–23) of mouse resistin on this differentiation process have been reported. Moreover, it has been observed that resistin knockout mice did not show differences in fat accumulation and adipocyte size as compared with wild-type mice (24).

Many reports about the functions of resistin, including those related to modulation of glucose metabolism and adipogenesis, are contradictory. Besides, the receptor or receptors able to mediate all these functions have not been fully identified yet. Two reports related to peptides binding resistin have been published, but none of them appears to clarify fully the issue (19, 23). Tarkowski *et al.* (15) have identified the human Toll-like receptor (TLR)4, involved in macrophage activation, as a molecule that could mediate some of the well-known proinflammatory effects of human resistin. Daquinag *et al.* (20) have identified an isoform of Decorin (DCN) (ΔDCN) as a functional resistin receptor that may regulate WAT expansion in adipocyte progenitors by modulating cell migration and proliferation and adipocyte differentiation. However, these two proteins, identified as possible targets for resistin, are receptors that had already well-defined ligands or they had known functions in specific cells. In this work, by taking advantage of the information found in genomic databases and by using molecular and cellular approaches, we have identified the mouse receptor tyrosine kinase-like orphan receptor (ROR)1, expressed in 3T3-L1 preadipocytes, as a new target for resistin in these cells.

Mammalian ROR1 and its homologous, ROR2, belong to the tyrosine-kinase family of receptors (25). These receptors possess three well-defined domains in their extracellular region: an Ig-like domain, near the N-terminal end; a Frizzled (FZZ)-like domain, rich in cysteine; and a Kringle (KR) domain, cysteine rich also, close to the transmembrane region (26). The FZZ-like domain is homologous to FZZ receptors, which act as canonical Wingless type (WNT) protein receptors (27). Finally, ROR1 and ROR2 intracellular regions contain a conserved tyrosine-kinase catalytic domain. Both receptors exert very important roles during mouse embryonic development, in particular during the development of the nervous system, in which their expression patterns seem to be differentially regulated (28). ROR1 and ROR2 are involved also in osteogenesis by acting as coreceptors for some WNT proteins (29, 30). In humans, it has been suggested that ROR1 could be an oncofetal antigen in chronic lymphocytic leukemia cells and it functions as a coreceptor for the ligand Wnt5a (31), whose known specific receptor is FZZ5 (32).

In this work, we demonstrate that mouse resistin specifically interacts with the FZZ-like and the KR domains of the ROR1 extracellular region. This interac-

tion results in the inhibition of ROR1 tyrosine phosphorylation. In addition, this interaction modulates ERK1/2 phosphorylation and regulates SOCS3, glucose transporter (GLUT)4, and GLUT1 expression. We also provide data demonstrating that resistin is able to modulate adipogenesis and glucose uptake in 3T3-L1 preadipocytes through the ROR1 receptor.

Results

Identification of the ROR1 receptor as a new target for mouse resistin

Despite abundant data about the functions of resistin in different species, very little is known about the receptor or receptors able to mediate its numerous effects. As mentioned in the introductory section, mouse resistin is mainly secreted by mature adipocytes, and although it exerts its effects in other tissues, it functions also as an autocrine molecule on adipocytes. This property suggested to us that adipocytes must express a resistin receptor. We searched for orphan receptors expressed by adipocytes in the European Bionformatics Institute and the European Molecular Biology Laboratory gene databanks. The first analysis revealed six possible orphan receptors at that time. After perusing the bibliography related to the orphan receptors found, we discarded five of them, because their ligands had been recently identified. The last orphan receptor that we could not discard, at that time, was ROR1. ROR1 possesses well-defined cysteine-rich extracellular domains, which suggested to us that it could be a good candidate for a functional resistin receptor, because resistin is also a cysteine-rich protein, and many examples of cysteine-rich proteins acting as receptor-ligand pairs exist. For those reasons, we decided to study whether ROR1 could be a resistin receptor or, at least, a receptor whose activity was modulated by resistin.

Mouse resistin interacts with the extracellular region of the ROR1 receptor

To investigate the potential interaction between mouse resistin and ROR1, we first used the yeast two-hybrid system. We cotransformed *Saccharomyces cerevisiae* CG1945 cells with pAS-resistin lacking its signal peptide (RETN*) and pAC-ROR1-IG, pAC-ROR1-FZZ, or pAC-ROR1-KR plasmids and verified the correct expression of the fusion proteins by Western blot analysis (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). We analyzed the strength of the interactions by performing 3-amino-1, 2, 4-triazole (3-AT) and quantitative β-galactosidase assays. The results of 3-AT assays indicated that resistin was able to interact with the FZZ and KR domains of ROR1 but not with the Ig-like domain (Fig. 1A). The interaction between resistin and the FZZ domain was stronger (20 mM 3-AT) than that occurring with the KR domain (5–10 mM 3-AT). These results were confirmed with a β-galactosidase quantitative assay (Fig. 1B). Again, the cotransformant colonies expressing RETN* and the FZZ or the KR domains of ROR1 showed the highest levels of β-galactosidase activity, whereas nontransformed cells or cells expressing RETN* and the Ig-like domain of ROR1 showed low levels of enzymatic activity. These results demonstrate that resistin specifically interacts with the KR and FZZ extracellular ROR1 domains in the yeast two-hybrid system.

The interaction between resistin and the extracellular region of ROR1 was confirmed by coimmunoprecipitation assays with soluble cell extracts (soluble fractions), obtained by the procedure described in *Materials and Methods*, from HEK 293T/17 cells overexpressing the complete RETN-6XHIS-hemagglutinin (HA), or the 3XFLAG extracellular ROR1 region lacking the signal peptide (ROR1e*), fusion proteins. After confirming the correct expression of the proteins used for these immunoprecipitation assays (Fig. 1, C and D), mixtures of these soluble extracts and the adequate control samples were incubated overnight, at 4 C, with an α-FLAG antibody. Immunoprecipitation samples were analyzed by Western blotting with rat α-resistin and mouse α-FLAG antibodies. As shown in Fig. 1E, resistin was detected only in the mixture containing RETN-6XHIS-HA and the FLAG-tagged ROR1 extracellular region, which was immunoprecipitated with the α-FLAG antibody. This result demonstrates and confirms the interaction between resistin and ROR1.

Because the interaction of resistin with ROR1 had not been previously reported, to test whether this interaction also occurred in living mammalian cells, we performed binding assays between mouse purified resistin and intact cells overexpressing ROR1. We first incubated HEK 293T/17 cells overexpressing, or not, the entire ROR1-FLAG protein, with or without 500 ng/ml mouse purified recombinant resistin (rRETN). After washing with PBS buffer, we lysed cells with a mild lysis buffer, as described in *Materials and Methods*, obtaining a soluble fraction and a membrane fraction. This last fraction was supposed to contain ROR1 receptor bound potentially to resistin. The membrane fractions were boiled in denaturing sample buffer and analyzed by Western blotting. Figure 2A shows that despite purified rRETN could bind to the membrane fraction of HEK 293T/17 control cells, which do not express the ROR1-FLAG protein, higher amounts of this adipocytokine were bound to the insoluble fraction

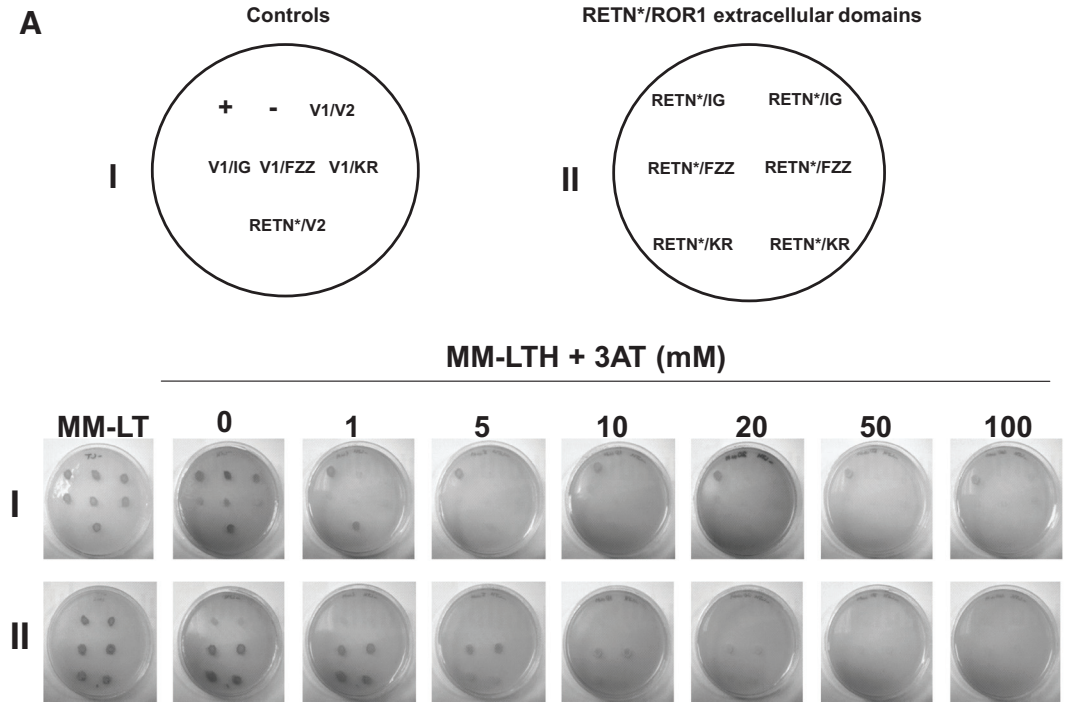

FIG. 1. Interaction of mouse resistin with the ROR1 receptor. 3-AT assay (A) and quantitative β-galactosidase activity assay, by using ortho-nitrophenyl-β-galactoside as a substrate (B), of the cotransformant colonies coexpressing mouse resistin, lacking its signal peptide (RETN*), and each one of the indicated mouse ROR1 extracellular domains (IG, KR, or FZZ). I) +, Positive control, pVA3-1/pTD1-1; −, Negative control), pLAM5′-1/pTD1-1; V1/V2, pAS2–1/pACT2 empty vectors; V1/IG, pAS2-1/pAC-ROR1-IG; V1/FZZ, pAS2-1/pAC-ROR1-FZZ; V1/KR, pAS2-1/pAC-ROR1-KR; and RETN*/V2, pAS-RETN*/pACT2. II) Two colonies of each type of cotransformant cells coexpressing RETN* and one of the extracellular domains of ROR1 are shown: RETN*/IG, RETN*/FZZ, and RETN*/KR. CG1945 NT, Nontransformed yeast cells. Western blot analysis of the overexpression of entire RETN-6XHIS-HA (C) and 3XFLAG-ROR1e* (D) fusion proteins in soluble extracts from HEK 293T/17 cells was performed with α-HA and α-FLAG antibodies, respectively. V 6XHIS-HA and V 3XFLAG: empty vectors. Both C and D Western blot analyses show the signal corresponding to a 10% of the total protein concentration used for the coimmunoprecipitation assay in E. E, Western blot analysis (WB) of the coimmunoprecipitation of ROR1 and RETN proteins. A mixture of soluble cell extracts from HEK 293T/17 cells, overexpressing, or not, 3XFLAG-ROR1e* or RETN-6XHIS-HA proteins, were immunoprecipitated (IP) with an α-FLAG antibody. The α-resistin antibody revealed the coimmunoprecipitation of the ROR1 receptor and RETN-6XHIS-HA protein pulled down with the α-FLAG antibody. MM-LTH, Minimal medium without leucine, tryptophan, and histidine; MM-LT, minimal medium without leucine and tryptophan.

of the same cells overexpressing ROR1-FLAG. After confirming the binding of resistin to the ROR1 receptor, we explored whether this binding was saturable and displaceable. For this purpose, we incubated HEK 293T/17 cells, overexpressing, or not, the entire ROR1-FLAG protein, with different concentrations (from 0 to 200 ng/ml) of purified RETN-6XHIS-HA, obtained by the procedure described in *Materials and Methods*, or of a mouse purified leptin, used as a negative control. The procedure used in this experiment was the same as the one used in the experiment presented in Fig. 2A. As Fig. 2B shows, the binding of purified RETN-6XHIS-HA to ROR1-FLAG seems to be saturable, whereas leptin was unable to bind to the insoluble fractions of HEK 293T/17 cells overexpressing ROR1-FLAG. Moreover, the binding of purified HA-tagged mouse resistin to ROR1-FLAG was displaceable by the addition of purified rRETN in approximately 100-fold excess (Fig. 2C).

Furthermore, the binding of this purified HA-tagged resistin to ROR1-FLAG was strongly diminished in the presence of an α-ROR1 antibody but not in the presence of an α-HA control antibody (Supplemental Fig. 2). The ROR1 protein signal in the α-ROR1-treated sample was also poorly detected by the α-FLAG antibody. Thus, it appears that the α-ROR1 antibody binds to the extracellular region of the ROR1 receptor, where it competes for the binding of resistin to that region.

As it is the case for ROR2, the dimerization and phosphorylation of ROR1 in specific tyrosine residues could be essential for its activation (33–35). To investigate whether the interaction of ROR1 with resistin could modulate the phosphorylation of ROR1 in tyrosine residues, transient transfectants of HEK 293T/17 cells, overexpressing, or not, ROR1-FLAG plasmid for 48 h, were incubated with 100 ng/ml purified rRETN for 15 min. Afterwards, ROR1 was immunoprecipitated with an α-FLAG antibody, and its general tyrosine phosphorylation state was analyzed by Western blotting. As shown in Fig. 2D, treatment of cells with purified rRETN strongly inhibited tyrosine phosphorylation of the ectopically ex-

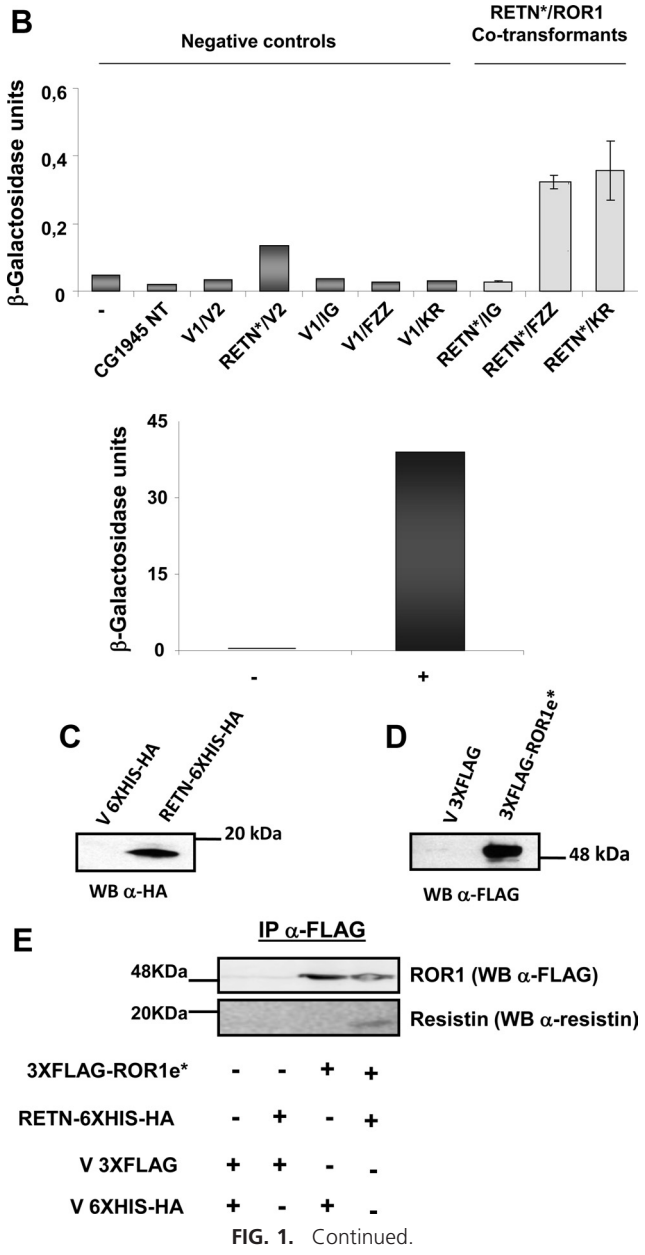

FIG. 1. Continued.

pressed ROR1-FLAG protein. Taken together, the results shown here strongly support that resistin interacts with the extracellular region of ROR1 and suggest an inhibitory effect on ROR1 receptor tyrosine phosphorylation.

ROR1 modulates the expression of GLUT1 and GLUT4 and glucose uptake in 3T3-L1 preadipocytes

We investigated the functionality of the resistin-ROR1 interaction in adipogenesis and glucose uptake by using 3T3-L1 preadipocytes. To initiate these studies, we first confirmed the low expression levels of *Ror1* and *Retn* genes in these cells by quantitative RT-PCR (data not shown). Many published works demonstrate that mouse resistin induces insulin resistance by inhibiting the phosphorylation of AMPK and that of different members of the insulin signaling pathway, leading to the down-regulation of *Glut* genes and/or the blockade of the translocation of GLUT to the plasma membrane and, therefore, to a decrease of glucose entry into the cells (13). The fact that resistin interacts with ROR1 and can inhibit ROR1 phosphorylation suggested to us that ROR1 could mediate those resistin effects. To study this, we generated stable 3T3-L1 transfectants overexpressing *Ror1* (Fig. 3A). We then analyzed the effect of the overexpression of *Ror1* on the expression of GLUT1 and GLUT4 by using quantitative RT-PCR and Western blotting techniques. *Ror1*-overexpressing cells expressed greater GLUT1 and GLUT4 mRNA and protein levels than control cells (Fig. 3, B and C). In addition, the expression of these two GLUT diminished by treating cells with purified rRETN (Fig. 3C). We analyzed also the expression levels of these two GLUT in stably transfected 3T3-L1 cells with diminished levels of ROR1, obtained by short hairpin RNA (shRNA) knockdown (Fig. 3D). As expected, the level of expression of GLUT1 and GLUT4 diminished as compared with control cells (Fig. 3E).

To investigate whether or not the variation of GLUT1 and GLUT4 expression levels caused by ROR1 correlated with the rate of glucose uptake, we measured the remaining glucose concentrations in culture media from cells, overexpressing, or not, *Ror1*, or from cells with down-regulated *Ror1* expression levels obtained by shRNA knockdown, along different times under cell confluence conditions. As shown in Fig. 4A, cells overexpressing *Ror1* showed a higher rate of glucose uptake than control cells, determined by lower amount of remaining glucose in the culture media. On the other hand, cells with diminished *Ror1* expression levels showed a lower rate of glucose uptake than control cells (cells transfected with shRNA control plasmid) during the first hours of cell culture (Fig. 4B). These results correlate with the effect of the changes in *Ror1* expression on GLUT1 and GLUT4 expression levels and suggest that ROR1 could also participate in activating their translocation to the cell plasma membrane.

We also analyzed the effect of *Ror1* expression and resistin treatment in the glucose uptake stimulated by insulin in 3T3-L1 preadipocytes. As expected, treatment with purified rRETN inhibited glucose uptake in control cells treated with insulin (Fig. 4, C and E), but it was not able to affect glucose uptake neither in cells that overexpress *Ror1* (Fig. 4D) nor in cells with diminished levels of *Ror1* expression (Fig. 4F). These results suggest that resistin could inhibit GLUT expression and translocation through the ROR1 receptor.

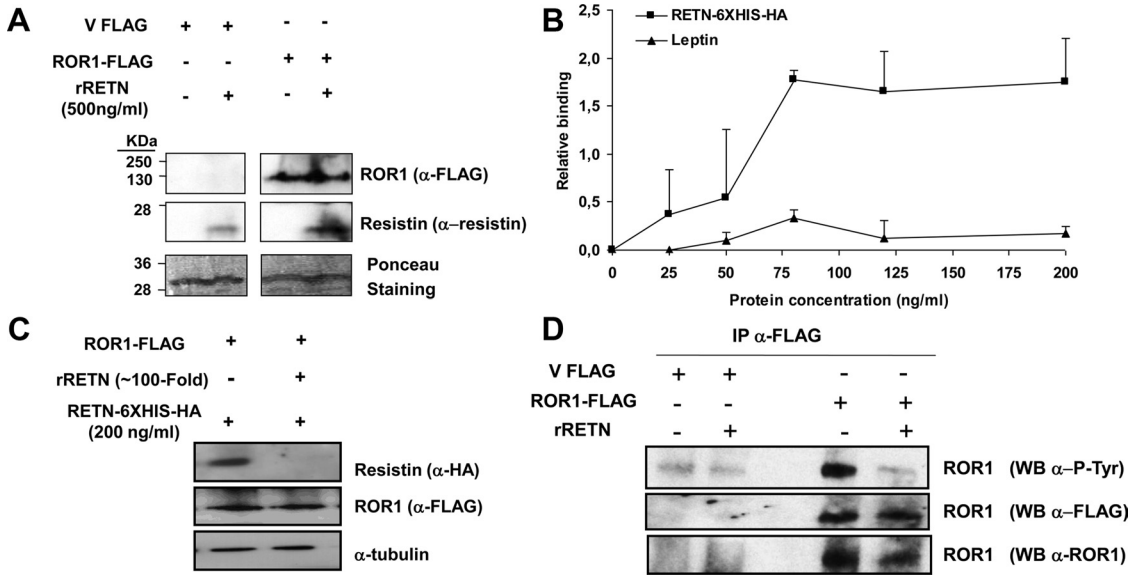

FIG. 2. Mouse resistin binds to intact HEK 293T/17 cells overexpressing the ROR1 receptor and inhibits ROR1 tyrosine phosphorylation. A, Western blot analysis for the binding of purified rRETN to HEK 293T/17 cells overexpressing, or not, ROR1-FLAG fusion protein. Resistin bound to membrane fractions was detected with an α-resistin antibody. ROR1-FLAG was detected with the α-FLAG antibody. Sample load was estimated by Ponceau staining. B, *In vitro* binding assay of resistin and ROR1. HEK 293T/17 cells overexpressing, or not, the entire ROR1-FLAG protein were incubated with different concentrations of purified HA-tagged mouse resistin (RETN-6XHIS-HA), or purified mouse leptin, used as a negative control (concentrations ranging from 0 to 200 ng/ml in both cases). Relative binding was calculated by normalizing the amount of purified RETN-6XHIS-HA bound to the membrane fractions with the amount of ROR1-FLAG in each sample. Relative amounts of these proteins were obtained by densitometric analysis of the Western blot signals of at least three different assays. C, Western blot analysis of the competition between purified RETN-6XHIS-HA and rRETN proteins for ROR1 receptor in the binding assay in B. HEK 293T/17 cells, overexpressing the entire ROR1-FLAG protein, were incubated with 200 ng/ml purified RETN-6XHIS-HA and with, or without, 100-fold excess of purified rRETN. A representative Western blot analysis is shown. RETN-6XHIS-HA and ROR1-FLAG were detected with α-HA and α-FLAG antibodies, respectively. The α-tubulin signal was used as a loading control. D, Tyrosine phosphorylation of the ROR1 receptor in ROR1-FLAG transiently transfected HEK 293T/17 cells, in the presence or the absence of 100 ng/ml purified rRETN. To detect ROR1-FLAG, α-ROR1 and α-FLAG antibodies were used. To detect P-Tyr residues, an α-P-Tyr antibody was used. Representative Western blottings (WB) are shown. V FLAG: HEK 293T/17 cells transfected with empty vector. ROR1-FLAG, HEK 293T/17 cells overexpressing the complete ROR1 protein fused to the FLAG epitope at the C-terminal end. WB, Western blot; IP, immunoprecipitation.

Mouse resistin increases the adipogenic potential of 3T3-L1 preadipocytes through the ROR1 receptor

Several contradictory reports about the effects of resistin on the adipogenesis of 3T3-L1 preadipocytes compelled us to investigate the effect of murine resistin in the adipogenesis of this cell line grown in our laboratory. We first analyzed resistin mRNA and protein expression patterns along the adipogenic differentiation of 3T3-L1 preadipocytes. Supplemental Fig. 3 shows the expected increase in resistin mRNA and protein expression, as well as its secretion to the culture medium, after insulin treatment. We also analyzed the effect of exogenously added purified rRETN in the adipogenesis potential of these cells. Purified rRETN increased the adipogenic potential of 3T3-L1 preadipocytes in response to the standard triggering treatment, as demonstrated by increased *Fabp4/aP2* expression and number of adipocytes in these cultures, 7 d after the adipogenic induction, compared with nondifferentiated cells (Fig. 5, A and B).

Until the completion of this work, nothing was known about the potential role of ROR1 in adipogenesis. The enhancing effect of resistin in 3T3-L1 adipogenesis, together with its interaction with the ROR1 receptor, suggested an active role for ROR1 in this differentiation process. To explore this, we first analyzed the expression of *Ror1* mRNA in 3T3-L1 preadipocytes along their adipogenic induction, by quantitative RT-PCR (Fig. 5C), and the expression of ROR1 protein at different times after cell confluence in preadipocytes, by Western blotting (Fig. 5D). The levels of expression of both *Ror1* mRNA and protein increased when cells were maintained under confluence for 24–48 h, but these levels decreased when cells were induced to differentiate with the adipogenic cocktail or when cells were maintained under cell confluence for longer times. Moreover, the decrease of ROR1 expression was greater in fully differentiated adipocytes (cells stimulated to differentiate with the adipogenic cocktail for a total period of 7 d under confluent conditions) (Supplemental Fig. 4, *upper panel*, *left half*). Additionally, a greater decrease of ROR1 protein expression was ob-

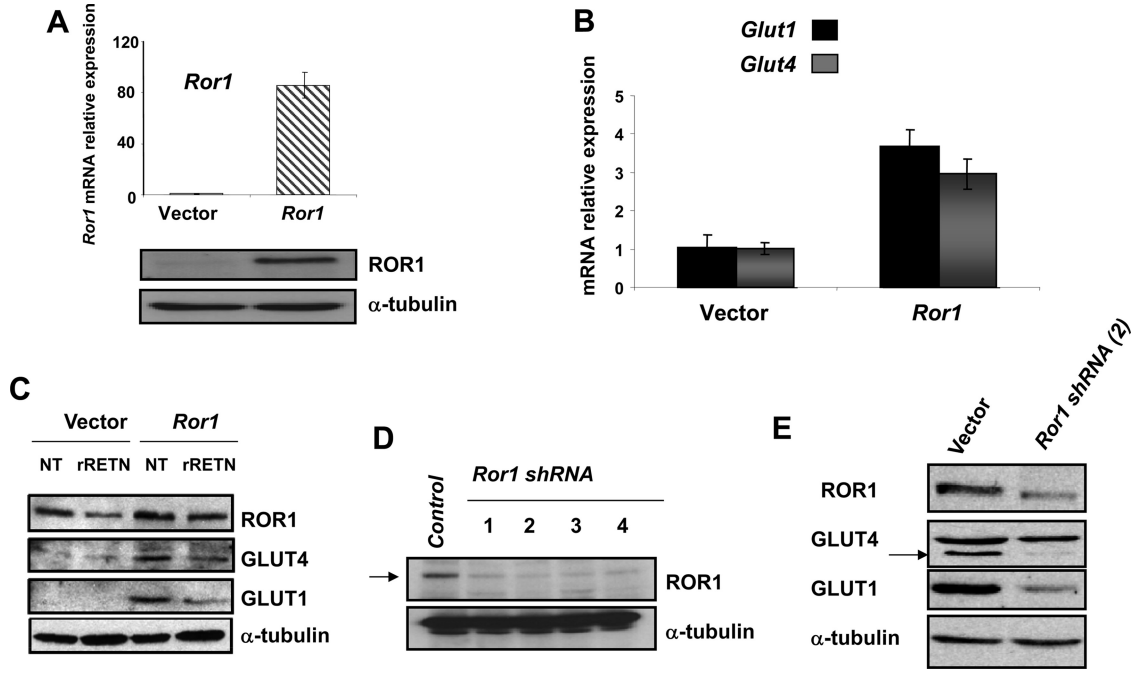

FIG. 3. Mouse ROR1 receptor modulates the expression of GLUT1 and GLUT4 in 3T3-L1 preadipocytes. Analysis of the expression of *Ror1* (A) and *Glut1*/*Glut4* (B) mRNAs in 3T3-L1 cells overexpressing, or not, *Ror1*. Data were normalized to *P0* mRNA expression levels. The *thin bars* represent the SD of RT-PCR data. C, Western blot analysis of ROR1, GLUT1, and GLUT4 proteins in stably transfected 3T3-L1cells, overexpressing, or not, *Ror1*, in the presence or in the absence of purified rRETN 100 ng/ml. Analysis of ROR1 (D) and GLUT1 and GLUT4 (E) proteins in ROR1 knockdown 3T3-L1 cells by Western blotting. To detect the expression of the proteins, rabbit α-ROR1, mouse α-GLUT4, and rabbit α-GLUT1 antibodies were used. Representative Western blottings are shown. The expression of α-tubulin was used as a loading control and to normalize data. The *bars* correspond to the SD of data from three different assays. NT, Not treated cells.

served when these terminally differentiated 3T3-L1 adipocytes had been treated with exogenous purified rRETN, as compared with nontreated cells (Supplemental Fig. 4, *upper panel*, *right half*). These data indicate that both the induction of adipogenesis and extended cell confluence inhibit the increased expression of *Ror1*, reached at 24–48 h under cell confluence, and that the addition of resistin could negatively modulate further its expression.

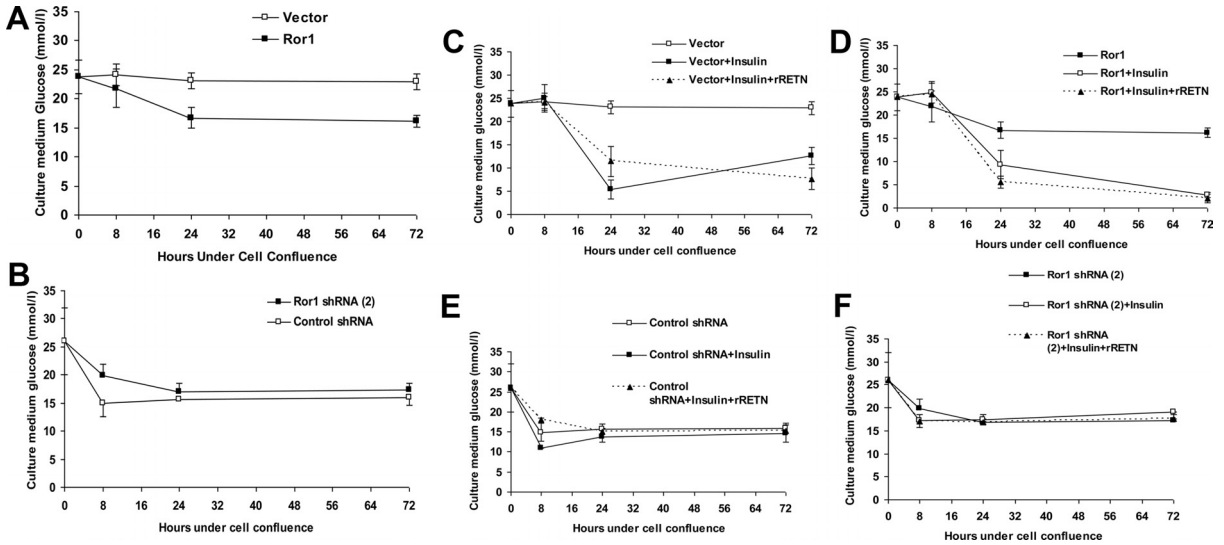

FIG. 4. Mouse resistin modulates glucose uptake stimulated by insulin in 3T3-L1 preadipocytes through ROR1 receptor. A, Culture medium glucose (mmol/liter) in control 3T3-L1 cells and cells overexpressing *Ror1* at different times (hours) under cell confluence. B, Culture medium glucose (mmol/liter) at different times (hours) under cell confluence in control 3T3-L1 cells or 3T3-L1 cells with diminished ROR1 expression levels, obtained by shRNA methodology. Culture medium glucose (mmol/liter) in 3T3-L1 cells overexpressing, or not, *Ror1* (C and D), or in control shRNA and *Ror1* shRNA stable transfectants of these cells with diminished levels of ROR1 expression (E and F). These cells were incubated in the presence of 1 μM insulin or 1 μM insulin plus 100 ng/ml purified rRETN, at different times (hours) under cell confluence and compared with not treated cells. *Curves* represent the remaining glucose in culture medium at the indicated times. The data correspond to the average of three different assays.

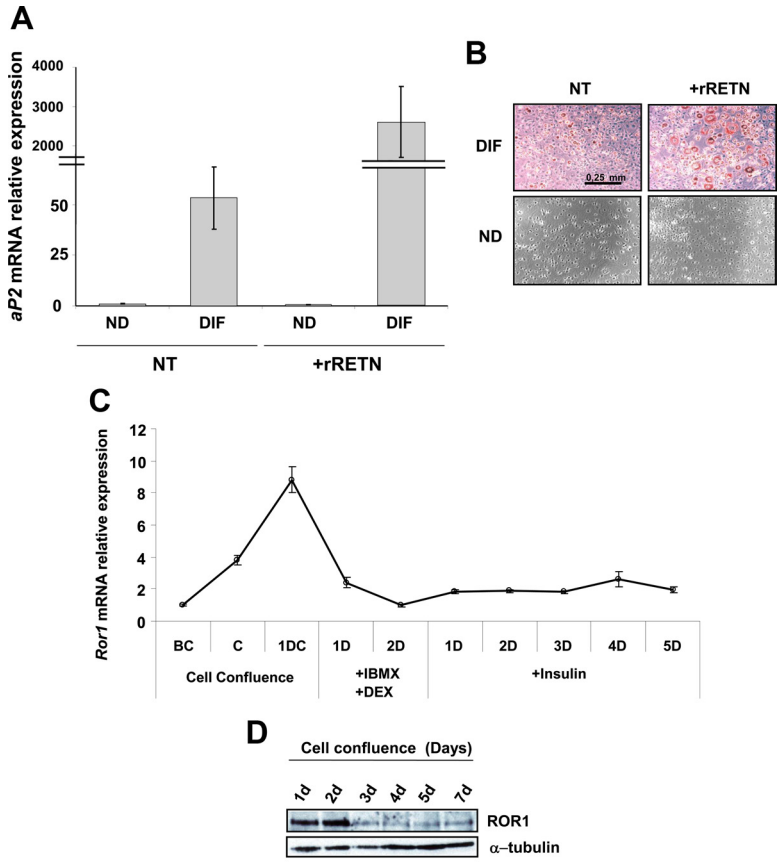

FIG. 5. Mouse resistin enhances adipogenesis of 3T3-L1 preadipocytes through the ROR1 receptor. A, Quantitative RT-PCR analysis of *Fabp4/aP2* gene expression levels in differentiated (DIF) and not differentiated (ND) 3T3-L1 preadipocytes, in the presence or in the absence of purified rRETN. B, Red Oil O staining analysis of differentiated and not differentiated 3T3-L1 preadipocytes, in the presence or in the absence of purified rRETN. Representative images of the adipocyte differentiation level of these cells are shown. Analysis by quantitative RT-PCR of *Ror1* expression of 3T3-L1 preadipocytes along adipogenesis (C), and at different times under cell confluence, by Western blotting (D). BC, Before cell confluence; C, cell confluence; 1DC, 1-d confluent cells; D, days of cell confluence or treatment; DEX, dexamethasone; IBMX, 3-isobuthyl-1-methylxanthine. The expression of α-tubulin was used as a loading control. To detect the expression of ROR1, a rabbit α-ROR1 antibody was used. A representative Western blotting is shown. E, Quantitative RT-PCR analysis of *Fabp4/aP2* expression in differentiated or not differentiated 3T3-L1 preadipocytes, overexpressing *Ror1*, in the presence or in the absence of purified rRETN. Quantitative RT-PCR analysis of *Fabp4ABP4/aP2* expression (F) and analysis of the level of adipogenesis visualized by direct microscope observation, without adipocyte staining (G), in differentiated or not differentiated 3T3-L1 preadipocytes incubated with α-ROR1 or α-HA antibodies, and in the presence or in the absence of purified rRETN. The expression of P0 mRNA was used to normalize data from RT-PCR assays. The expression values obtained in not differentiated cells were used as a reference. The *thin bars* represent the SD of data from three different assays. NT, Not treated cells.

Moreover, these results suggest that the expression of *Retn* and *Ror1* could be inversely coordinated along adipogenesis.

These observations prompted us to investigate the possible effect of *Ror1* overexpression on the adipogenic potential of 3T3-L1 preadipocytes. The overexpression of *Ror1* in these cells resulted in a drastic decrease of their adipogenic potential determined 7 d after adipogenesis induction, as indicated by the decrease in *Fabp4/aP2* expression levels (Fig. 5E) and the number of adipocytes (data not shown). Moreover, resistin was able to rescue partially the inhibitory effect of *Ror1* overexpression in these cells, suggesting again an inhibitory role for resistin on ROR1 activity. To investigate whether the inhibitory effect of resistin on ROR1 ability to block adipogenesis was due to a direct interaction of resistin with ROR1, we performed the same adipogenic assays with 3T3-L1 preadipocytes in the presence or the absence of a specific α-ROR1 antibody and purified rRETN (Fig. 5, F and G). An α-HA antibody was used as a control. The potential blockage of the endogenous ROR1 protein by a specific antibody directed against its extracellular region resulted in an increase of the adipogenic response of 3T3-L1 preadipocytes, at the end of the adipogenesis induction, as compared with the differentiated cells incubated with the control α-HA antibody. This result demonstrates that the inhibitory effect of ROR1 in this process is probably due to the binding of its extracellular region to an external factor. Moreover, the treatment of these cells with exogenous purified rRETN was able to increase the expression of *Fabp4/aP2* and the number of adipocytes in α-HA-treated cells but failed to do so in the α-ROR1-treated cells. Taken together, these results indicate that the α-ROR1-specific antibody used competes with resistin for ROR1 binding and inhibition, and they strongly suggest that the ROR1 receptor mediates the proadipogenic effect of resistin in 3T3-L1 preadipocytes.

Mouse ROR1 modulates SOCS3 expression levels and the phosphorylation of different kinases involved in glucose metabolism and adipogenesis in 3T3-L1 preadipocytes

Resistin affects glucose metabolism through different mechanisms involving the modulation of AMPK and other kinases activated by insulin (36, 37). This effect seems to be generally mediated by the increase of SOCS3 expression and activation (14). We have shown above that treatment of cells with purified rRETN inhibits ROR1 tyrosine phosphorylation. The potential effects of ROR1 on the phosphorylation of the kinases involved in the regulation of glucose metabolism and adipogenesis were unknown. We decided to study the

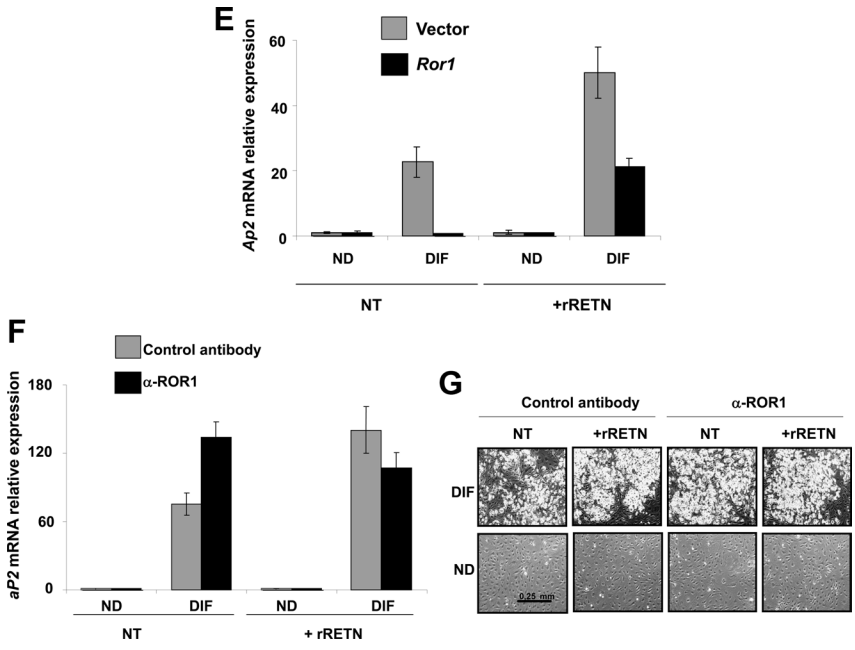

FIG. 5. Continued.

effects of ROR1 on the phosphorylation of AMPK and other kinases activated by insulin that are involved in glucose metabolism and adipogenesis, such as phosphatidylinositol 3 kinase (PI3K)-AKT, glycogen synthase kinase (GSK)3β, ERK1/2, and p38MAPK, and whether, or not, this was affected by resistin. For this purpose, we used 3T3-L1 stable transfectants, overexpressing, or not, *Ror1*, or stable transfectants with diminished levels of ROR1 expression. Figure 6A shows that the overexpression of *Ror1* produced an increase of ERK1/2, AKT-Thr308, GSK3β, and AMPK phosphorylation and a decrease of AKT-Ser473 and p38MAPK phosphorylation. We also investigated the effects of ROR1 overexpression on SOCS3 expression. As Fig. 6, B and C, shows, the overexpression of *Ror1* inhibited SOCS3 mRNA and protein expression, and this effect was reversed by treatment with purified rRETN. On the contrary, down-regulation of ROR1 expression produced the opposite effects on the phosphorylation level of these kinases, except for that of AKT in Ser473, and it activated the expression of SOCS3 (Fig. 6D). These data indicate, for the first time, that the receptor ROR1 is able to differentially regulate the phosphorylation of different proteins that are involved in adipogenesis, glucose metabolism, and the development of insulin resistance and that resistin could reverse ROR1 effects on SOCS3 expression.

Among the kinases activated by insulin and involved in adipogenesis, ERK1/2 MAPK has been the most extensively studied (38, 39). We have studied whether changes in the phosphorylation of ERK1/2 MAPK could explain the observed effects of ROR1 and resistin on adipocyte differentiation. To investigate this hypothesis, 3T3-L1 preadipocytes, overexpressing, or not, *Ror1*, were treated with 1 μM insulin and/or 100 ng/ml purified rRETN for the indicated times. When control cells were treated with purified rRETN (Fig. 6E) at different times, we could observe an activation of ERK1/2, 5–10 min after treatment, which is consistent with the observed enhancing effect of resistin on adipogenesis. This activation was abrogated when cells overexpressed *Ror1*, which correlates with the inhibitory effect of ROR1 overexpression on adipogenesis. These results also provide further evidence about the inhibition of ROR1 activity by resistin. Thus, ROR1 overexpression and associated hyperactivation appear to prevent resistin from stimulating ERK1/2 phosphorylation (Fig. 6E). In addition, overnight treatment of control cells with 100 ng/ml purified rRETN, before insulin treatment, led to an increase of ERK1/2 phosphorylation, compared with insulin-treated control cells, which is again compatible with the enhancing effect of resistin on 3T3-L1 adipogenesis in response to insulin (Fig. 6F). Finally, insulin treatment of cells overexpressing *Ror1* was not able to increase the basal level of ERK1/2 phosphorylation (Fig. 6F), which is again consistent with the inhibition of adipogenesis caused by *Ror1* overexpression. However, the activation pattern of ERK1/2 phosphorylation was partially restored when cells were previously treated with purified rRETN overnight, which suggest again the regulatory effect of resistin on ROR1 activity, and the observed effects on adipogenesis.

Discussion

The adipose tissue was traditionally regarded as a mere fat store, with few active functions. However, due to the dramatic increase in the incidence of obesity over the past years, and its pathologic consequences, adipose tissue has attracted considerable scientific interest. Many works have demonstrated that, in addition to regulate body fat and nutritional homeostasis, the adipose tissue secrets a wide range of active molecules, named adipocytokines. These adipocytokines are secreted from mature adipocytes and other cells and are involved in several physiological processes (1). One of these adipocytokines is resistin, mainly secreted by adipocytes in mice and by

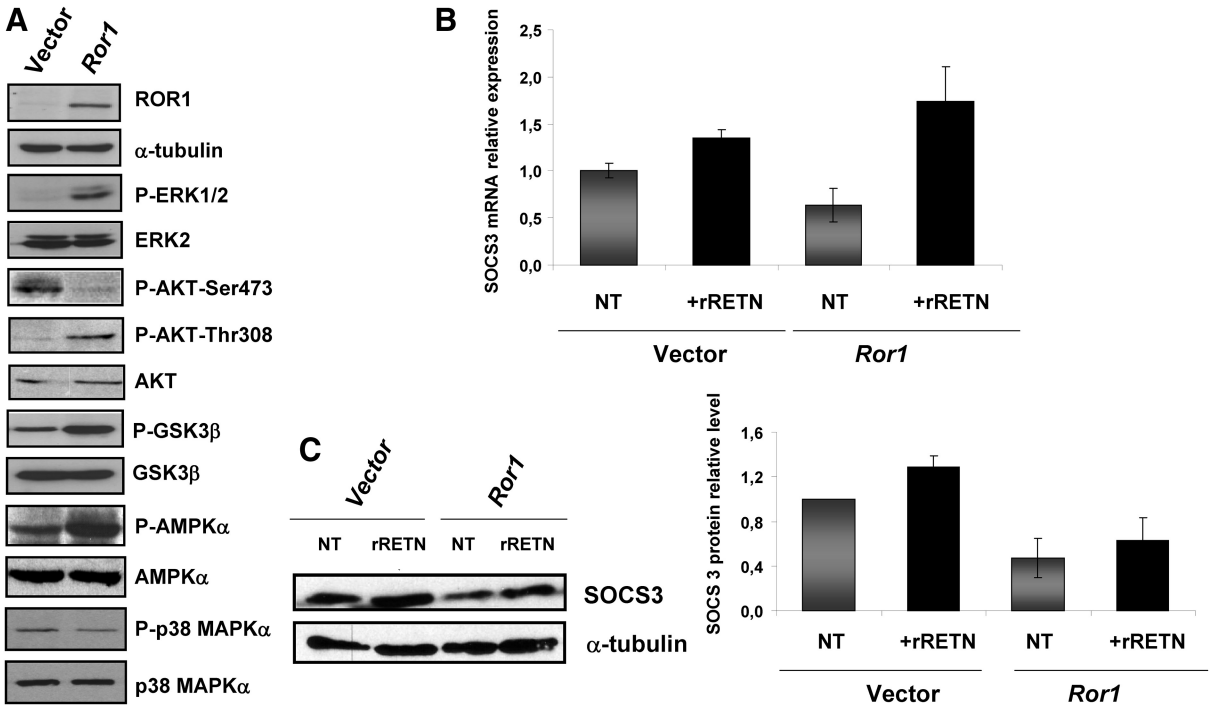

FIG. 6. Mouse ROR1 receptor modulates SOCS3 expression and the phosphorylation of different kinases involved in glucose metabolism and adipogenesis in 3T3-L1 preadipocytes. A, Analysis of ERK1/2 MAPK, AKT, GSK3β, p38MAPK, and AMPK phosphorylation in stably 3T3-L1 transfectants overexpressing, or not, *Ror1*. Analysis of SOCS3 mRNA and protein expression levels in stable 3T3-L1 transfectants overexpressing, or not, *Ror1*, in the presence or the absence of purified rRETN, by quantitative RT-PCR (B), and by Western blotting (C). The *right panel* in C shows the densitometric analysis of the Western blot signals corresponding to three different assays. D, Analysis of SOCS3 expression and ERK1/2 MAPK, AKT, GSK3β, p38MAPK, and AMPK phosphorylation in a stable 3T3-L1 transfectant with diminished levels of ROR1 receptor (*Ror1* shRNA) (2). Analysis of the expression and phosphorylation of ERK1/2 MAPK, by Western blotting, in stable 3T3-L1 transfectants overexpressing *Ror1*, in response to purified rRETN (E), or insulin treatments (F), at the indicated times and concentrations. Cells in F were previously treated overnight with 100 ng/ml purified rRETN. To detect the expression and phosphorylation of the different kinases analyzed, we used the antibodies described in *Materials and Methods*. The expression of α-tubulin and the expression of nonphosphorylated kinases were used as a loading control and to normalize data in densitometric analysis. The expression of P0 mRNA was used to normalize the data from the RT-PCR assays. The *bars* correspond to the SD of data. *Ror1*, Stably transfected 3T3-L1cells overexpressing *Ror1*; vector, 3T3-L1 cells transfected with empty vector; NT, not treated cells.

mononuclear cells in humans (3, 4, 12). The functions attributed to resistin are numerous, and they seem to differ between mice and humans. Secreted resistin acts on organs and tissues distinct from the ones that have secreted it, but it also exerts an autocrine function on secretory cells (40). Despite the numerous studies about the function of resistin in several biological processes, few aspects about the receptor or receptors mediating all of these functions are known. Besides the two peptides that seem to interact with resistin (19, 23), other two reports have identified two distinct potential resistin receptors. While the present manuscript was being revised, an isoform of mouse DCN (ΔDCN) that binds resistin and may regulate WAT expansion was identified in adipocyte progenitors (20). DCN is a small cellular or pericellular matrix proteoglycan on average 90–140 kDa in size, belonging to the small leucine-rich proteoglycan family, and consisting of a protein core containing leucine repeats with a glycosaminoglycan chain. This protein is a component of connective tissue, binds to type I collagen fibrils, among several other proteins, and plays a role in matrix assembly (41–47). Resistin only binds to the DCN isoform lacking the glycanation site (20), which suggests that its glucidic moiety does not bind resistin or it binds it poorly. As a glycosylated secreted protein, mature DCN lacks any possibility of directly sending a signal to the interior of the cell, such as the interaction of resistin with its receptor should trigger. Therefore, although nonglycosylated DCN may be a cofactor allowing a better interaction of resistin with its receptor in the extracellular matrix, it is unlikely that it acts as a *bona fide* resistin receptor. The second report, also recently published, identified the human TLR4 receptor, involved in lipopolysaccharide binding, macrophage activation, and inflammatory processes, as a molecule mediating some of the well-known proinflammatory effects of human resistin by competing with bacterial lipopolysaccharide (15). These two resistin targets are proteins with well-defined ligands and known functions. Although resistin can interact with them in specific cells and tissues, it is likely that other receptor or receptors, besides TLR4 or DCN, must exist to mediate the numerous functions of resistin, in-

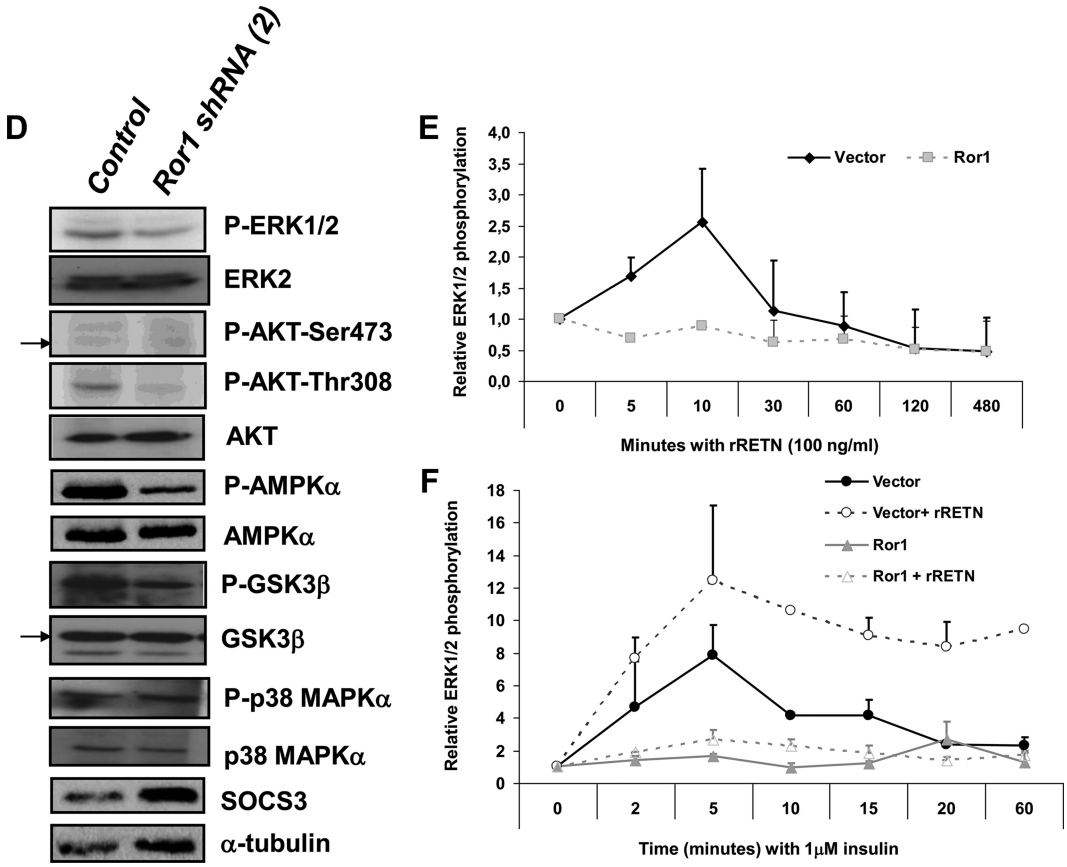

FIG. 6. Continued.

cluding those related with adipogenesis and the development of insulin resistance in mice.

The autocrine function of mouse resistin on adipocytes justified trying to identify a receptor for this adipocytokine in these cells. Database searches for orphan receptors expressed in adipocytes led to the identification of the tyrosine-kinase receptor ROR1 as the only receptor lacking a known ligand at that time. Both ROR1 and resistin are cysteine-rich proteins, a superfamily of proteins frequently involved in extracellular protein-protein interactions. We were aware that the odds of ROR1 interacting with resistin were slim. Nevertheless, we decided to study whether ROR1 could be the resistin receptor or, at least, one potential target for the action of this adipocytokine. The results obtained in the yeast two-hybrid system assays revealed that, indeed, resistin and ROR1 physically interact. 3-AT and quantitative β-galactosidase assays revealed that the interaction between these two proteins is specific, involving only the FZZ-like and KR domains of the ROR1 extracellular region but not its extracellular Ig-like domain. These results were confirmed by coimmunoprecipitation assays carried out with soluble cell extracts overexpressing the ROR1 extracellular region or the complete resistin protein. In addition, purified resistin was able to bind intact HEK 293T/17 cells overexpressing the complete ROR1 protein at the cell plasma membrane. This binding was saturable and displaceable, which are expected features of a true receptor. As it has been described for the receptor ROR2 (33–35), the dimerization and phosphorylation of ROR1 on specific tyrosine residues could be essential for its activation. Our results showed that treatment with resistin inhibits ROR1 tyrosine phosphorylation in HEK 293T/17 cells. These data suggested to us that resistin could exert a negative modulatory effect on ROR1 receptor activity by acting as an inhibitory ligand for ROR1.

The specific interaction between resistin and ROR1 does not rule out, however, other proteins as ROR1 ligands, because resistin could simply function as one of the regulators of ROR1 activity, similarly to ROR1 functioning as a coreceptor for WNT5a (31). In any case, we have demonstrated that the interaction between ROR1 and resistin is functional *in vivo* and that ROR1 modulates some of the known effects exerted by resistin, such as adipogenic differentiation and glucose metabolism. The effects of resistin on the expression and localization of GLUT, and in the development of insulin resistance, have been extensively studied (13). For that reason, we decided to study, for the first time, the role of ROR1 in the expression of GLUT1 and GLUT4 and in the glucose uptake

in 3T3-L1 preadipocytes. Our analysis revealed that the modification of *Ror1* expression levels in these cells regulates the expression of *Glut1* and *Glut4*, as well as the glucose uptake rates. Moreover, treatment of cells overexpressing ROR1 with resistin reversed these effects, suggesting, again, that resistin exerts a negative modulatory effect on ROR1 receptor activity.

As already mentioned, resistin plays a regulatory role, albeit very controversial, in the process of adipogenic differentiation (12, 19, 21–23). As expected, our results showed that resistin mRNA and protein expression, as well as its secretion to the culture medium, increased along adipogenic differentiation of 3T3-L1 cells (6). In this regard, resistin could be considered as a late marker of adipocyte differentiation. Moreover, the higher level of resistin expression at the end of adipogenesis suggested two possible roles for resistin in this differentiation process. On the one hand, resistin could promote this differentiation process, and its expression and accumulation could be necessary for adipogenesis to occur. On the other hand, resistin could act as an inhibitory molecule, by functioning as a sensor of the amount of adipocytes. Our studies demonstrated that exogenous recombinant mouse resistin, present during the entire adipogenesis induction process, increased the level of adipogenesis of 3T3-L1 cells. These results are in agreement with those previously reported by other authors (21–23, 48) and confirm that resistin not only fails to inhibit adipogenesis of 3T3-L1 cells but it enhances this differentiation process in response to adipogenic inductors. However, other authors have reported that resistin acts as an inhibitor of adipogenesis. The different reported effects of resistin on 3T3-L1 adipogenesis might be explained by the different source and bioactivity of the resistin used in the specific assays. Being a cysteine-rich molecule, proper resistin folding might be an issue affecting the results. In addition, the cellular state and the normal genetic variation of the same cell line cultured in different laboratories could be also an important factor affecting resistin effects on adipogenesis.

Because of the unveiled interaction between the ROR1 receptor and resistin, we also decided to address, for the first time, the potential role of ROR1 on adipogenesis. Differently to resistin expression, the expression of *Ror1* in 3T3-L1 cells peaks 1 or 2 d after cell confluence, and it decreases to background levels both with longer times under cell confluence and with treatment with adipogenic inducers. The apparently inversely coordinated expression of *Ror1* and *Retn* genes could be a result of the modulation of the activity and/or expression of ROR1 by resistin, or *vice versa*, although it could be an independent effect caused by the confluence state and the adipogenic inducer agents on the expression of these genes. In any case, our results support an inhibitory role for ROR1 in 3T3-L1 adipogenesis, because its overexpression was able to decrease drastically the expression of the differentiation marker *Fabp4/aP2* in cell cultures induced to differentiate. Moreover, treatment with resistin was able to reverse partially this inhibition, suggesting again an inhibitory effect of resistin on ROR1 activity. The inhibitory effect of ROR1 overexpression on adipogenesis was confirmed by the addition of an α-ROR1 antibody binding to the extracellular region of ROR1 to 3T3-L1 preadipocytes induced to differentiate, which resulted in increased differentiation levels as compared with the control. More importantly, treatment with resistin was able to increase the adipogenic response of the cells in the presence of the control α-HA antibody but not in the presence of the α-ROR1 antibody, which strongly indicates that resistin enhances adipogenesis of these cells by interacting with the ROR1 receptor and inhibiting its activity.

Until completion of this work, very little was known about the signal transduction pathways modulated by ROR1. The interaction of resistin with ROR1 suggested that this receptor could regulate different signaling pathways modulated by resistin. In addition, the modulation of *Glut1* and *Glut4* expression by ROR1 suggested that, in some way, this tyrosine kinase receptor could function similarly to the insulin receptor. In this work, we have shown that variations in *Ror1* expression levels lead to the modulation of phosphorylation of several kinases involved in glucose metabolism and adipogenesis, including AMPK, PI3K-AKT, GSK3β, ERK1/2, and p38MAPK. The modulation of ERK1/2 MAPK and p38 MAPK activation is involved in the expression and translocation of the GLUT proteins (49, 50). Therefore, the increased levels of *Glut1* and *Glut4* expression and the highest rate of glucose uptake by cells overexpressing *Ror1* could be, at least partially, the result of the modulation of the phosphorylation status of these kinases by ROR1. PI3K-AKT signaling plays also a very active role in glucose metabolism (51). It is therefore possible that the increase in the AKT phosphorylation at Thr308 in 3T3-L1 preadipocytes overexpressing *Ror1*, despite the observed inhibition of AKT Ser473 phosphorylation, is responsible, at least in part, for the increased expression and translocation of these GLUT. Consistently with this hypothesis, some studies show that only the phosphorylation of the AKT Thr308 residue, but not that of the Ser473 residue, is directly involved in glucose transport (52). Another kinase regulated by insulin and AKT, and implicated in glycogen synthesis, is GSK3β (36). Our results indicate that overexpression of *Ror1* leads to an increase in

GSK3β phosphorylation, which would favor glycogen synthesis, consistently with the increased glucose uptake promoted by ROR1. Activation of AMPK by phosphorylation also plays an important role in glucose homeostasis. AMPK activation promotes the expression and translocation of GLUT4 and GLUT1 (53). Our results show that overexpression of *Ror1* increases basal phosphorylation of AMPK. This effect may help explaining also the increase in the levels of expression of GLUT1 and GLUT4 and the increased glucose uptake rate caused by ROR1. Finally, SOCS3, activated by resistin and other hormones, is considered as a possible mediator of the inhibitory effect of resistin on insulin-mediated signaling in adipocytes (14). According to our data, overexpression of *Ror1* decreased SOCS3 mRNA and protein levels, and the down-regulation of ROR1 receptor increased the level of this factor. More importantly, treatment with resistin produced the expected increase in the expression of this factor, both in control cells and in cells overexpressing *Ror1*, suggesting, once again, a negative regulatory effect of resistin on ROR1 activity.

The modulation of ERK1/2, AKT, or p38MAPK phosphorylation by insulin seems to be important for adipocyte differentiation. The results presented here show that overexpression of *Ror1* in 3T3-L1 preadipocytes increases basal phosphorylation levels of ERK1/2 and inhibites its time-dependent phosphorylation increase in response to resistin treatment. Moreover, the increase of ERK1/2 phosphorylation by insulin treatment is abrogated when ROR1 is overexpressed. Although the activation of ERK1/2 is essential for adipogenesis, it has also been shown that its hyperactivation can lead to an inhibition of this differentiation process (54). The effects of ROR1 overexpression in basal ERK1/2 phosphorylation and in the pattern of phosphorylation of this kinase in response to insulin could explain the inhibitory effect of ROR1 in adipogenesis. The overexpression of ROR1 also activates AKT at Thr308, but it inhibited basal phosphorylation of p38MAPK and AKT at Ser473, which may be compatible also with the inhibition of adipogenesis caused by ROR1 in these cells. As described above, our data demonstrate that treatment of 3T3-L1 cells with resistin enhances adipogenesis. In this context, the inhibition of ROR1 activity and signaling by resistin could explain the observed enhancing effect of resistin on the adipogenesis of these cells.

It is generally accepted that chronic exposure to resistin adversely affects the entry of glucose into cells, thus counteracting the effect of insulin. As mentioned above, we have observed that overexpression of *Ror1* induces the expression of GLUT1 and GLUT4 in 3T3-L1 preadipocytes, and this effect can be reversed by resistin. On the other hand, overexpression of *Ror1* blocks adipogenesis and resistin is able to enhance this differentiation process by inhibiting the activity of ROR1. Some works demonstrate that AMPK activation exerts an antiadipogenic effect and promotes glucose uptake in 3T3-L1 preadipocytes (55). The observed increase of AMPK phosphorylation by *Ror1* overexpression could explain the inhibition of adipogenesis *vs.* activation of glucose uptake.

Taken together, our results demonstrate a specific interaction of mouse resistin and the receptor ROR1 and an effect of resistin on ROR1 activity, which position the ROR1 receptor as a molecule involved in adipogenesis and glucose metabolism that is negatively modulated by resistin. The interaction between these two proteins opens a new line of research that could lead to explaining important open questions about the mechanism of action of resistin in adipogenesis, inflammation, and the development of insulin resistance.

Materials and Methods

Yeast plasmids and yeast two-hybrid methods

The yeast plasmids used to study the protein-protein interactions between mouse resistin and ROR1 protein domains in the yeast two-hybrid system derive from pACT2 and pAS2–1 vectors (CLONTECH, Mountain View, CA). Plasmid pAS-RETN* expresses RETN*, fused to the C-terminal end of the galactose metabolism transcription factor 4 transcription factor binding domain. RETN* cDNA was amplified by standard RT-PCR with the oligonucleotides 5′-TGG GGG AAT TCC TGC TGG GCT CCA GCA TGC CAC TGT GT-3′, as the upper primer, and 5′-ATG GGG GGA TCC GTT CTC AAC TGA CCG ACA TCA GG-3′, as the lower primer. The amplified fragment was digested with *Eco*RI and *Bam*HI restriction enzymes and cloned into the pAS2–1 vector. Plasmids pAC-ROR1-IG, pAC-ROR1-FZZ, and pAC-ROR1-KR express the Ig-like, the FZZ-like, and the KR extracellular domains of ROR1, respectively, all of them fused to the C-terminal end of the GAL4 activation domain. The cDNA corresponding to these domains were also amplified by standard RT-PCR. The oligonucleotides used to amplify the Ig-like domain were: 5′-AGG TTG GAA TTC GGG TGC CTA CCT CGT CCT GGA AC-3′, as the upper primer, and 5′-AGG GGG CTC GAG GAT TAA CCA GTG GTA GAC ACC AC-3′, as the lower primer. The oligonucleotides used to amplify the FZZ-like domain were: 5′-GGG GGG AAT TCG AGA AGA AGA TGG ATT CTG TCA GCC G-3′, as the upper primer, and 5′-AGG GGG CTC GAG GAG GGT TAG CAT TTG TGA TTT TTA TTT ATA GGA TCC GC-3′, as the lower primer. Finally, the oligonucleotides used to amplify the KR domain were: 5′-GGG GGG AAT TCG ACA CAA ATG CTA CAA TAG CAC GGG TG-3′, as the upper primer, and 5′-GGG GGC TCG AGG GTT AAT CTT TGG AAT CAC ATG CTG G-3′, as the lower primer. The amplified cDNA were digested with *Eco*RI and *Xho*I restriction enzymes and cloned into the pACT2 vector. We also used empty vectors and other plasmids expressing proteins known to inter-

TABLE 1. Yeast and mammalian plasmids constructed in this work

Plasmid	Sequence properties	Markers	Cloning
*pAS-RETN**	GAL4-BD fused to RETN*	*TRP, amp*^{r}	*Eco*RI-*Bam*HI
pAC-ROR1-IG	GAL4-AD- fused to ROR1 IG	*LEU2, amp*^{r}	*Eco*RI-*Xho*I
pAC-ROR1-FZZ	GAL4-AD fused to ROR1 FZZ	*LEU2, amp*^{r}	*Eco*RI-*Xho*I
pAC-ROR1-KR	GAL4-AD fused to ROR1 KR	*LEU2, amp*^{r}	*Eco*RI-*Xho*I
pLN-ROR1	Complete ROR1 cDNA	*Neomycin, amp*^{r}	*Hin*dIII-*Sal*I
*p3XFLAG-ROR1e**	ROR1e* fused to N-terminal 3XFLAG	*Neomycin, amp*^{r}	*Hin*dIII-*Xba*I
pLP-RETN-HA	Complete RETN cDNA fused to C-terminal HA	*Puromicin, amp*^{r}	*Hin*dIII-*Bam*HI
pLP-RETN-6XHIS-HA	Complete RETN cDNA fused to C-terminal 6XHIS-HA	*Puromicin, amp*^{r}	*Hin*dIII-*Sal*I

BD, Binding domain; AD, activation domain.

act, or not, with each other, as positive or negative controls, respectively, for the yeast two-hybrid assays (CLONTECH). Table 1 lists the yeast expression plasmids used in this work.

S. cerevisiae CG1945 strain was used for two-hybrid system assays. Cotransformations with different plasmid combinations were performed with one microgram of each individual plasmid. Transformant colonies were selected in a minimal medium lacking leucine and tryptophan. To search for potential protein-protein interactions, colonies able to grow in minimal medium lacking leucine and tryptophan were grown again in a minimal medium lacking histidine, leucine, and tryptophan, in the presence of increasing concentrations of 3-AT, ranging from 0 to 100 mM. We also performed quantitative β-galactosidase activity (β-galactosidase units) assays of the cotransformant colonies, by using ortho-nitrophenyl-β-galactoside (Sigma, St. Louis, MO) as a substrate. These assays were performed by following the manufacturer's instructions (CLONTECH) and were repeated at least three times.

Mammalian plasmids

Plasmid pC-ROR1-FLAG was kindly provided by Minami's group (Department of Physiology and Cell Biology, Graduate School of Medicine, Kobe University, Kobe, Japan). This plasmid derives from vector pCDNA-3 and contains the complete cDNA encoding for mouse ROR1 fused to the FLAG epitope at its C-terminal end. Plasmid pLN-ROR1 derives from vector pLNCX2 (CLONTECH) and expresses the entire mouse ROR1 protein. The cDNA encoding for the entire ROR1 protein was isolated from vector pFLCI-ROR1 (clone PX00941O12; ImaGenes, Berlin, Germany) by digestion with *Not*I-*Kpn*I restriction enzymes and then cloned into vector p3XFLAG-CMV-23 (56) to generate the plasmid p3XFLAG-ROR1. The complete *Ror1* cDNA was isolated from this plasmid by digestion with *Hin*dIII and *Sal*I restriction enzymes and cloned into vector pLNCX2 to generate plasmid pLN-ROR1. Plasmid p3XFLAG-ROR1e* expresses the extracellular region of mouse ROR1, lacking its signal sequence, fused to three copies of the FLAG epitope at its N-terminal end. The cDNA corresponding to the extracellular region of mouse ROR1 was amplified by standard RT-PCR by using pLN-ROR1 plasmid as template. We used the oligonucleotides ROR1eUP: 5′-TCT AAG CTT GGA GGA GGA GAG TTG TCA GTC AGT GCT GAG CTG G-3′, as the upper primer; and ROR1eLOW: 5′-ACG GCG TCT AGA TCA CTT GGA ATC ACA TGC TGG GAT GTC ACA CAG G-3′, as the lower primer. The amplified fragment was digested with *Hin*dIII and *Xba*I restriction enzymes and cloned into p3XFLAG-CMV-23, generating the plasmid p3XFLAG-ROR1e*.

Plasmid pLP-RETN-HA expresses the entire resistin protein fused to the HA epitope at its C-terminal end. The cDNA encoding for the entire resistin protein was amplified by standard RT-PCR with the oligonucleotides: Retn_*Hin*dIII_up, 5′-ATG GTT AAG CTT CCT CTG CCA CGT ACC CAC GGG-3′, as the upper primer; and Retn_*Bam*HI_low, 5′-ATG GTT GGA TCC GGA AGC GAC CTG CAG CTT ACA GC-3′, as the lower primer. The amplified DNA fragment was digested with *Hin*dIII and *Bam*HI restriction enzymes, and cloned into the vector pLPCX-HA (56). Plasmid pLP-RETN-6XHIS-HA expresses the entire resistin protein fused to 6XHIS and HA epitopes at its C-terminal end. This cDNA was isolated from vector pLP-RETN-HA by digestion with *Hin*dIII and *Sal*I restriction enzymes and subsequent cloning into vector pLPCX-6XHIS-HA (56).

The plasmids corresponding to the mouse ROR1 shRNA (ROR1 shRNA1, shRNA2, shRNA3, and shRNA4) and the control shRNA were purchased from Superarray Bioscience Corp. (SA Biosciences, Frederick MD). Finally, plasmid pEGFP-N1 (CLONTECH) expresses a variant of the green fluorescent protein. Table 1 lists some of the mammalian expression plasmids used in this work.

Sequencing, amplification, and purification of plasmid DNA

TOP10 *Escherichia coli* competent cells were used for transformation and plasmid amplification, following the manufacturer's recommendations (GIBCO, Rockville, MD). For bacterial culture, liquid or solid (agar plates) Luria Bertani media were supplemented with 100 μg/ml ampicillin (Sigma). Plasmid DNA was isolated and purified by using the GenElute Plasmid Miniprep kit (Sigma) or ionic exchange columns (QIAGEN, Inc., Hilden, Germany), according to the protocols recommended by the manufacturers.

All the yeast and mammalian plasmids constructed were sequenced to confirm the correct cloning of the fragments and the absence of mutations. For this purpose, 100–500 ng of the plasmids were sent for sequencing (Macrogen, Seoul, South Korea). Oligonuleotides for cloning and sequencing were purchased from Bonsai Technologies (Madrid, Spain). The oligonucleotides used to sequence the plasmids are indicated in Table 2.

Mammalian cell culture, cell transfections, and gene expression analysis

Mammalian cell lines were cultured at 37 C in a 5% CO_2 humidified atmosphere, in DMEM (Life Technologies, Carls-

TABLE 2. Oligonucleotides used for DNA sequencing

Plasmid/cDNA	Oligonucleotides
Yeast	MATCHMAKER 5′ primer (CLONTECH) MATCHMAKER 3′ primer (CLONTECH)
pLNCX2/ pLPCX	Primer 5′, 5′-agc tcg ttt agt gaa ccg tca gat c-3′ (CLONTECH) Primer 3′, 5′-acc tac agg tgg ggt ctt ttc att ccc-3′ (CLONTECH)
pCDNA3	T7, 5′-taa tac gac tca cta tag g-3′ (Invitrogen) Sp6, 5′-att tag gtg aca cta tag-3′ (Invitrogen)
Ror1 specific	ROR1_SEQ1, 5′-gtg cac gat tta ttg gca acc g-3′ (Bonsai Technologies) ROR1_SEQ2, 5′-tgc caa gtg tgg cca ttc c-3′ (Bonsai Technologies) ROR1_SEQ3, 5′-gaa cat gta att ggg aaa tcg ggg-3′ (Bonsai Technologies) ROR1_SEQ4, 5′-gta aga aat cac cat ggt cca gg-3′ (Bonsai Technologies)

bad, CA) containing 10% fetal bovine serum. The cell lines used were 3T3-L1 (CCL-92.1; American Type Culture Collection, Manassas, VA) and HEK 293T/17 (CRL-11268; American Type Culture Collection). Transient and stable transfectants were obtained by transfecting 50% confluent cells with different amounts of the plasmids, depending on the culture plates and the transfection reagent used. The plasmid pEGFP-N1 (CLONTECH) was used to determine the transfection efficiency. Stable transfectants were generated by selecting the cells in the presence of 420 μg/ml G418 antibiotic (Sigma).

Cell cultures were treated with 100 ng/ml purified rRETN (Calbiochem, San Diego, CA) (as other authors have described, this concentration of rRETN was the most effective to get the observed effects) and/or 1 μM insulin (Sigma) for different times. For expression analysis, confluent cell monolayers were collected by centrifugation (180 $\times$ *g* for 5 min at 4 C), and total RNA was isolated by using the RNeasy kit (QIAGEN, Inc.). cDNA were obtained by using a cDNA synthesis kit (Fermentas, Madrid, Spain) and employed as templates in quantitative PCR experiments. Quantitative PCR assays were performed by using the Fast SYBR Green Master Mix and the ABI PRISM 7500 (Applied Biosystems, Carlsbad, CA) detection system. The mRNA expression of the gene *36B4* encoding for the acidic ribosomal phosphoprotein P0 was used as the expression and quality control (57). These assays were repeated at least three times. The oligonucleotides used for gene expression analysis by quantitative RT-PCR are shown in Table 3.

Affinity chromatography

HEK 293T/17 cells were transiently transfected with empty vector pLPCX-6XHIS-HA or with pLPRETN-6XHIS-HA plasmid. Protein expression was analyzed by Western blotting of the cell culture supernatants, by using an anti-HA antibody (Covance, Princeton, NJ). Culture supernatants from cells expressing RETN-6XHIS-HA were filtered and concentrated with 3-kDa centricons (Millipore, Billerica, MA). Control sample, corresponding to the empty vector, was processed in the same way as follows: after filtering, the protein and control samples were

TABLE 3. Oligonucleotides used for quantitative RT-PCR analysis

Gene	Oligonucleotides
P0	5′-aagcgcgtcctggcattgtct-3′ 5′-ccgcaggggcagcagtggt-3′
Ror1	5′-ctgcagaactgcactgcaaagtgt-3′ 5′-tagttggttgcccggaaggagatt-3′
Retn	5′-acccacgggatgaagaacctttca-3′ 5′-atggcttcatcgatgggacacagt-3′
aP2	5′-agtcacatggtccagggcatctt-3′ 5′-aaccttcgaggaggagctgtct-3′
Glut1	5′-gggcatgtgcttccagtatgt-3′ 5′-acgaggagcaccgtgaagat-3′
Glut4	5′-gattctgctgcccttctgtc-3′ 5′-attggacgctctctctccaa-3′
Socs3	5′-gaacctacgcatccagtgtgag-3′ 5′-gcttgagtacacagtcgaagcg-3′

purified from the concentrated solution by using the Ni-NTA His Bind Resin (Novagen, Darmstadt, Germany). The purified samples were dialyzed and concentrated with binding buffer (Novagen) lacking imidazole. Protein concentration was determined by Dot blot and Western blot analyses with an anti-HA antibody, using known concentrations of a purified HA epitope (Alpha Diagnostic International, San Antonio, TX).

Protein sample preparations, immunoprecipitation assays, and Western blot analysis

To obtain soluble extracts from yeast cells, liquid cultures were first centrifuged at 1500 $\times$ *g* for 10 min, and then cells were vigorously vortexed in 1$\times$ Laemly sample buffer for 5 min, followed by boiling for another 5 min. Yeast protein extracts were loaded and electrophoresed into 10–15% polyacrylamide gels and analyzed by Western blotting. Mammalian cells were resuspended in a mild lysis buffer [50 mM Tris-HCl (pH 7.4), 1% Igepal, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, and 10 μl/ml protease inhibitor cocktail; Sigma], maintained on ice for 30 min, and centrifuged at 16,000 $\times$ *g* for 10 min at 4 C. In the case of kinase phosphorylation analysis, phosphatase inhibitor cocktails I/II (Sigma) were added also to the lysis buffer. Total soluble extracts were collected, and the samples' protein concentrations were determined by using the Protein Assay Dye Reagent (Bio-Rad, Munich, Germany), following the manufacturer's recommendations. Fifty to 100 micrograms of total protein extracts were loaded and electrophoresed into 10–15% polyacrylamide gels and analyzed by Western blotting.

Coimmunoprecipitation of resistin and ROR1 proteins was achieved by incubating, overnight at 4 C, mixtures of soluble extracts from HEK 293T/17 cells overexpressing 3XFLAG-ROR1e* or RETN-6XHIS-HA proteins with an α-*FLAG* antibody. After overnight incubations were performed, protein A+G Agarose (Pierce, Rockford, IL) was added, and the samples were incubated for 2 h at room temperature. Bound complexes were washed three times in PBS and then resuspended in Laemly sample buffer. In all the cases, immunoprecipitation complexes were boiled and loaded into 10–15% (wt/vol) polyacrylamide gels and analyzed by Western blotting.

For resistin and ROR1 binding assays, HEK 293T/17 cells, overexpressing, or not, the entire ROR1-FLAG protein, were

treated with 500 ng/ml of purified rRETN (Fig. 2A) or with different concentrations purified RETN-6XHIS-HA (Fig. 2, B and C, and Supplemental Fig. 2) for 2 h at 4 C. After washing with PBS, cells were lysed with the mild buffer described above, the membrane fractions were boiled in sample buffer, and both the ROR1-FLAG and RETN-6HIS-HA proteins were detected by Western blot analysis. Ponceau staining was used to determine the load of the samples in Fig. 2A. The capacity of resistin to bind to the ROR1 receptor in Fig. 2B was estimated by normalizing the amount of purified RETN-6XHIS-HA bound to the membrane fraction with the amount of ROR1-FLAG present in each sample. Mouse leptin (Sigma), added at the same concentrations range as resistin, was used as the negative control in these binding experiments. To analyze the tyrosine phosphorylation of the ROR1 receptor, we immunoprecipitated ROR1-FLAG protein by adding an α-FLAG antibody to HEK 293T/17 control cells or HEK 293T/17 cells that overexpress ROR1-FLAG that had been treated, or not, with 100 ng/ml purified rRETN for 15 min.

Western blot analyses were performed by using the appropriated dilutions of the primary and the corresponding horseradish peroxidase-secondary antibodies. The primary antibodies used in this work were: mouse α-FLAG-M2 (Sigma), mouse α-HA 16B12 (Covance), rabbit α-ROR1 (Cell Signaling, Beverly, MA), rat α-resistin (Chemicon, Billerica, MA), rabbit α-leptin (Chemicon), rabbit α-p-AKT Ser473 (Cell Signaling), rabbit α-p-AKT Thr308 (Cell Signaling), rabbit α-AKT (Cell Signaling), mouse α-P-ERK E4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit α-ERK2 C14 (Santa Cruz Biotechnology, Inc.), rabbit α-P-p38 MAPKα T180/182 3D7 (Cell Signaling), rabbit α- p38MAPKα C-20 (Santa Cruz Biotechnology, Inc.), rabbit α-p-AMPKα(Thr172) (Cell Signaling), mouse α-AMPKα (Cell Signaling), rabbit α-p-GSK3β (Ser9) (Cell Signaling), rabbit α-GSK3β (27C10) (Cell Signaling), rabbit α-SOCS3 (Cell Signaling), mouse α-phosphotyrosine, clone 4G10 (Upstate, Lake Placid, NY), rabbit α-GLUT1 H43 (Santa Cruz Biotechnology, Inc.), and mouse α-GLUT4 1F8 (Cell Signaling). Mouse α-tubulin antibody (Sigma) was generally used as the loading control. Densitometric analyses were made by using Quantity One, 1-D analysis software (Bio-Rad).

Adipogenic and glucose uptake assays

For adipogenic assays, 24-h confluent cell cultures of control or *Ror1*-overexpressing 3T3-L1 preadipocytes were treated with 1 μM dexamethasone and 0.5 mM 3-isobuthyl-1-methylxanthine (Sigma) for 48 h. Then, cells were treated with 1 μM insulin (Sigma) for 5 d. In addition, all these cell cultures were treated, or not, with 100 ng/ml purified rRETN (Calbiochem) during the whole differentiation process. We performed also adipogenic assays with 3T3-L1 preadipocytes in the presence, along the whole adipogenic process, of a 1:500 dilution of a *rabbit α-ROR1* antibody (Cell Signaling) that recognizes the amino-terminal region of ROR1, or the same dilution of the mouse *α-HA 16B12* (Covance) antibody, used as a control. We determined the adipogenic conversion at 7 d of treatment by quantitative RT-PCR analysis of the expression levels of the late adipogenic maker *Fabp4/aP2*. In some cases, the resultant terminally differentiated adipocytes were stained by using the standard Red Oil O (Sigma) staining protocol. Stained or nonstained adipocytes were visualized with a Motic AE31 microscope. Representative images were acquired with a Moticam 2300 camera by using Motic Images Plus 2.0 ML software. These assays were repeated at least three times.

To analyze glucose uptake rate in 3T3-L1 preadipocytes in different conditions, we used the Biovision Glucose assay kit II (BioVision Research Products, Mountain View, CA). We directly measured the remaining glucose concentrations, at different growing times after cell confluence, in the supernatants of culture cells. The assay was performed following the manufacturer's recommendations. These assays were repeated at least three times.

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