Inhibition of Adipocyte Differentiation by Resistin-like Molecule α

BIOCHEMICAL CHARACTERIZATION OF ITS OLIGOMERIC NATURE*

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A novel family of cysteine-rich secreted proteins with unique tissue distribution has recently been identified. One of the members, resistin (for “resistance to insulin”), also called FIZZ3, was identified in a screen for molecules that are down-regulated in mature adipocytes upon administration of thiazolidinediones. The prototypical member of this family was originally identified from bronchoalveolar lavage fluid of inflamed lungs and designated FIZZ1 (“found in inflammatory zone”). This molecule was also found to be highly expressed in adipose tissue and was named resistin-like molecule α (RELMα). Here we demonstrate that RELMα inhibits the differentiation of 3T3-L1 preadipocytes into adipocytes. RELMα has no effect on proliferation of 3T3-L1 preadipocytes. Pretreatment of 3T3-L1 preadipocytes with RELMα does not affect insulin- or platelet-derived growth factor-induced mitogenesis. IRS-1 phosphorylation and glucose transport stimulated by insulin in mature adipocytes were also unaffected by RELMα. We show that RELMα forms disulfide-linked homooligomers based on results from electrophoresis under reducing and nonreducing conditions, communoprecipitation experiments as well as by mass spectrometry. In addition, RELMα is able to form heterooligomers with resistin but not RELMβ. Since RELMα is expressed by adipose tissue and it is a secreted factor, our findings suggest that RELMα may be involved in the control of the adipogenesis as well as in the process of muscle differentiation.

Adipose tissue serves as major energy depot where energy stored in the form of triglycerides can be released during periods of fasting. In addition, adipose tissue plays an important role in the regulation of glucose levels in the blood and is one of the target sites for the insulin-dependent glucose uptake (1, 2). The development of mature adipose cells from adipocyte precursor cells is accompanied by morphological changes, rearrangement of extracellular matrix, and increased expression of genes involved in lipid and glucose metabolism (3). In order to determine the molecular mechanisms of adipocyte differentiation, several preadipocyte cell lines such as 3T3-L1 have been generated. The differentiation of these cells into adipocytes can be induced by stimulation with a combination of dexamethasone, 3-isobutyl-1-methylxanthine, and insulin (4). The process of differentiation is regulated by a coordinated expression of key transcription factors such as sterol regulatory element-binding protein, PPARγ,1 and C/EBPα that are expressed during this conversion process (1). Despite its primary function as an organ for storing surplus energy, adipose tissue is now believed to be a major secretory organ that secretes factors that influence not only adipocyte growth and differentiation but also total body homeostasis (5). The factors secreted by adipocytes control processes ranging from inflammation and angiogenesis to modulation of insulin action and appetite control (6, 7).

Recently, a member of a novel class of cysteine-rich proteins, resistin (for “resistance to insulin”) or FIZZ3, was demonstrated to be secreted by adipocytes (8, 9). The expression of resistin is induced during adipocyte differentiation and is down-regulated by thiazolidinediones that represent a class of insulin-sensitizing drugs known to serve as ligands for PPARγ (8, 10, 11). When administrated to mice, resistin impairs glucose tolerance and antagonizes insulin-stimulated glucose uptake in vitro. Therefore, resistin has been suggested to serve as a link between obesity and type II diabetes.

There are two other members of the resistin family of molecules, RELMα and RELMβ, that are 29 and 37% identical, respectively, to resistin at the protein level (9, 12). RELMα, also known as FIZZ1, and resistin are both expressed by white adipose tissue (9, 12). The biological role of RELMα has not yet been elucidated. In this report, we have produced biologically active recombinant RELMα. When added to a culture of 3T3-L1 preadipocytes that have been stimulated to undergo differentiation, RELMα has an inhibitory effect on this process. The inhibition is accompanied by a decrease in the mRNA levels of several markers of differentiation including PPARγ, C/EBPα, aP2, Acrp30, and glyceral-3-phosphate dehydrogenase (13, 14). In addition, RELMα was able to inhibit the conversion of C2C12 myoblasts into myotubes. No proliferation of 3T3-L1 preadipocytes was observed upon the addition of RELMα although a proliferative response was clearly seen both with insulin and with PDGF. This suggests that the inhibition of adipocyte differentiation by RELMα is not mediated by induction of mitogenesis, as is the case with several growth factors.

1 The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; Acrp30, adipocyte complement-related protein 30; aP2, adipocyte fatty acid-binding protein; C/EBP, CCAAT/enhancer-binding proteins; RELM, resistin like molecule; TBP, TATA-binding protein; PDGF, platelet-derived growth factor; IRS-1, insulin receptor substrate-1; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium.

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such as epidermal growth factor and PDGF. The addition of RELMα had no effect on insulin- or PDGF-induced mitogenesis. Furthermore, RELMα did not alter insulin-stimulated glucose uptake by mature adipocytes. Since the resistin family of molecules is rich in cysteine residues, the migration of RELMα was examined by SDS-PAGE under reducing and nonreducing conditions. It was found that although RELMα migrated as a monomer under reducing conditions, several forms were detected under nonreducing conditions, suggesting formation of homooligomers. This was confirmed by coimmunoprecipitation experiments using FLAG and V5-tagged versions of RELMα. In order to confirm the presence of higher order oligomers, we subjected reduced and unredced samples of RELMα to mass spectrometry and observed only monomers under reducing conditions, whereas trimers, dimers, and monomers were detected under nonreducing conditions. The existence of oligomeric forms of RELMα was confirmed in mouse serum using an antibody directed against the endogenous protein. Finally, we observed heterooligomerization of RELMα with resistin but not with RELMβ, suggesting that the formation of homo- or heterooligomers may serve to regulate the biological activity of this family of molecules.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Growth Factors, and Antibodies—**3T3-L1 preadipocytes were grown in DMEM with 10% calf serum plus antibiotics in 5% CO₂. C2C12 cells and 293T cells were grown in DMEM supplemented with 2 mM L-glutamine and 10% fetal bovine serum plus antibiotics in 5% CO₂. PDGF and anti-phosphotyrosine mouse monoclonal antibody (4G10) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-V5 monoclonal antibody was from Invitrogen, anti-IRS-1 antibody (C-20) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-FLAG monoclonal antibody was from Sigma. Recombinant mouse resistin was from R & D Systems (Minneapolis, MN). A keyhole limpet hemocyanin-conjugated peptide, ENKVKELLANPANYP, was used to generate a rabbit polyclonal antibody against murine RELMα (Biocarta, Carlsbad, CA).

**cDNAs and Constructs—**Expressed sequence tag clones with GenBank™ accession numbers AI528963, AA712003, and AA518288 were used as templates to amplify the open reading frames for resistin, RELMα, and RELMβ, respectively. For carboxyl terminus FLAG-tagged ligands, polymerase chain reaction fragments were subcloned into pCMV-Tag4a vectors (Stratagene, La Jolla, CA). RELMα fragment was also subcloned into pCDNA8/V5-His vector (Invitrogen). Constructs were confirmed by sequencing. A cDNA encoding RELMα without a signal sequence was subcloned into pHadG/H vector (Invitrogen) to produce recombinant ligand from bacteria. The predicted average molecular mass of mature recombinant RELMα is 11,214.8 in its completely reduced state.

**Purification of Recombinant Ligands—**293T cells were transfected with an empty vector or an expression vector encoding FLAG-tagged RELMα 24 h after transfection, the medium was changed to serum-free medium, and the cells were allowed to grow for a further 48 h. The supernatant from transfected dishes was then harvested, pooled, and concentrated using Amicon Centriprep centrifugal filtration devices with a 3,000-Dalton cutoff. For production of recombinant bacterial RELMα, competent Escherichia coli were transformed with FLAG-tagged RELMα in pBAD/gH vector. The bacteria were then grown to log phase, and protein expression was induced by 0.04% arabinose. 4 h after induction, the cells were pelleted and subjected to osmotic shock by first treating them with 20% sucrose, 20 mM Tris, pH 8.0, 2.5 mM EDTA and then with 20 mM Tris, pH 8.0, 2.5 mM EDTA for releasing recombinant RELMα from the periplasmic space. The shock fluid was then mixed with anti-FLAG immunoprecipitation buffer containing 0.5 mg/ml/FLAG peptide in phosphate-buffered saline (PBS) followed by extensive dialysis against PBS.

**Immunoprecipitation and Western Blotting—**293T cells in 10-cm dishes were transfected with 15 μg of plasmid encoding a FLAG-tagged RELMα alone or 7.5 μg of each plasmid if V5-tagged RELMα was co-transfected with FLAG-tagged RELMα. 48 h after transfection, the supernatants were harvested and immunoprecipitated with anti-FLAG antibodies. The immunoprecipitates were washed three times in PBS, and the eluates were resolved by SDS-PAGE (Novex, San Diego, CA) followed by transfer onto nitrocellulose.

The membranes were Western blotted with anti-V5 or anti-FLAG antibody. The membrane shown in the top panel in Fig. 5 was stripped after Western blotting with anti-V5 and reprobed with anti-FLAG antibody to confirm equal expression. The supernatants were also directly Western blotted with anti-V5 antibody to check equal expression of V5-tagged molecules. Detection was performed by enhanced chemiluminescence method according to the manufacturer’s instructions (Amersham Biosciences).

For the experiments shown in Fig. 3C, 3T3-L1 adipocytes in 6-cm dishes were serum-deprived for 18 h and left untreated or pretreated with 1 μM RELMα for the indicated times, stimulated with 50 nM insulin for 5 min, and processed as described previously (15). Cleared cell lysates were incubated with anti-IRS-1 antibody coupled to agarose (New England BioLabs, Inc., Santa Cruz, CA) at 4 °C. Immunoprecipitates were then washed in lysis buffer, boiled in sample buffer, resolved by SDS-PAGE, and transferred onto nitrocellulose. The membrane was first probed with 4G10 anti-phosphotyrosine monoclonal antibody and then stripped and reprobed with anti-IRS-1 antibody to confirm an equal loading.

For in vivo studies of RELMα (Fig. 7), 50 μl of mouse serum was filtered through a 100,000-Dalton cut-off Microflex filter device (Millipore Corp., Bedford, MA) and resolved by SDS-PAGE under reducing or nonreducing conditions. Proteins from the gel were then transferred onto nitrocellulose, and Western blotting was performed using rabbit anti-RELMα immune serum.

**Differentiation Assays—**Mouse 3T3-L1 preadipocytes were differentiated essentially as previously described (16). Briefly, cells were grown to confluence in DMEM with 10% calf serum plus antibiotics in 5% CO₂. Two days after the cells were confluent (day 0), the cells were induced to differentiate by changing the medium to DMEM containing 10% fetal bovine serum plus 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) and 1 μM dexamethasone (Sigma). After 48 h (day 2), the medium was replaced with DMEM containing only 10% fetal bovine serum. The medium was changed every second day. In cases where supernatant was used, cells were cultured in medium containing 50% supernatant and 50% DMEM containing 10% fetal bovine serum (plus dexamethasone and 3-isobutyl-1-methylxanthine for the first 2 days). 250 nM, 500 nM, and 750 nM of purified recombinant RELMα was used in experiments shown in Fig. 2B.

To examine a possible effect of RELMα on muscle differentiation, C2C12 cells were grown to complete confluence in 6-cm dishes, and the medium was then changed to differentiation medium containing 2% horse serum instead of 10% bovine serum. Purified RELMα (1 μM) was added, and cells were fed with fresh medium every second day.

**Thymidine Incorporation Assays—**3T3-L1 cells were grown in complete medium until they were ~60% confluent. The medium was then changed to serum-free medium containing purified RELMα (1 μM), insulin (50 nM), and PDGF (4 nM) alone or in combination, as indicated in Fig. 3A, and the cells were grown for an additional 2 h. During the labeling period, [3H]thymidine (Amersham Biosciences) was added to the medium, and the cells were stimulated for 15 min with 15 nM insulin (see Fig. 3B). Subsequently, cells were washed twice with PBS and trypsinized for 10 min at 37 °C and then transferred onto glass microfiber filters. Subsequently, the filters were washed twice with PBS, twice with 5% trichloroacetic acid, and once with absolute ethanol. The incorporated radioactivity was measured using liquid scintillation mixture on a Tri-Carb 2100TR liquid scintillation counter (Packard Instrument Co.).

**Glucose Transport Assay—**To test if RELMα has an effect on glucose uptake by adipocytes, 3T3-L1 cells were differentiated as previously described (4), and fully differentiated adipocytes were then incubated in serum-free medium in the presence or absence of purified RELMα or resistin (1 μM). After 20 h, 1 μCi/ml [14C]-labeled 2-glucose (Amersham Biosciences) was added to the medium, and the cells were stimulated for 15 min with 15 nM insulin (see Fig. 3B). Subsequently, cells were washed two times with PBS, harvested in water, and lysed by freezing and thawing. Measurements were done using a liquid scintillation counter.

**Reverse Transcription-PCR Analysis—**Total RNA was prepared as described previously (17). Reverse transcription reactions were performed in a 25-μl volume containing 1 μg of total RNA, 3 μg of random hexamers (Amersham Biosciences), 50 μM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 40 units of RNA-guard (Amersham Biosciences), 0.9 μM dNTPs, and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) for 1 h at 37 °C. cDNA synthesis reactions were diluted with 50 μl of water. Multiplex reverse transcription-PCR was performed as described with minor modifications (18). All reactions contained the TBPl primer
Generation of recombinant RELMα. A, generation of conditioned media containing ligands from 293T cells. 293T cells were transfected with empty vector or an expression vector encoding FLAG-tagged RELMα. The supernatants were harvested after 48 h and resolved by SDS-PAGE followed by Western blotting with anti-FLAG. B, purification of RELMα from bacteria. RELMα was expressed in bacteria followed by purification on an anti-FLAG immunoaffinity column. After elution with FLAG peptide, the sample was dialyzed against PBS. The gels in the panel show RELMα resolved by SDS-PAGE under reducing or nonreducing conditions followed by silver staining.

RESULTS AND DISCUSSION

Generation of Recombinant RELMα—To determine biological properties of RELMα, we generated an epitope-tagged version with a FLAG tag at the C terminus. We chose a C-terminal tag, because a C-terminally FLAG-tagged version of resistin has previously been shown to have functional activity (8). 293T cells were then transfected with the cDNA constructs, and the supernatants were tested for expression of the respective recombinant proteins. As shown in Fig. 1A, we were able to detect RELMα at the expected molecular weights by SDS-PAGE under reducing conditions. We also produced recombinant RELMα in bacteria followed by immunoaffinity purification on an anti-FLAG column. Fig. 1B shows a silver-stained gel of purified recombinant RELMα under reducing and nonreducing conditions. We found that although RELMα migrated strictly as a monomer under reducing conditions, it migrated as at least two additional forms with higher apparent molecular weights under nonreducing conditions. The molecular weight of these two higher forms was consistent with their being a dimer and a trimer of RELMα. It is possible that even higher order multimers are formed.

Inhibition of Adipogenesis by RELMα—RELMα is expressed highly in white adipose tissue and the lungs (9, 12). Since it is a secreted protein, it seems plausible that it may have the capacity to modulate the adipogenic process. To test this, we subjected 3T3-L1 preadipocytes to the standard differentiation program in the presence of mock-conditioned medium or RELMα-conditioned medium. As shown in Fig. 2A, the conversion of preadipocytes into adipocytes was significantly inhibited by treatment with RELMα. We also tested the purified bacterially expressed recombinant RELMα for activity in this assay. As shown in Fig. 2B, we again observed a significant inhibition of adipocyte differentiation. Since the program of differentiation involves up-regulation of a subset of genes that are specific for mature or mature adipocytes, we tested whether the expression of mRNAs of these genes correlated with the inhibition of differentiation. A multiplex reverse transcription-PCR using mRNA isolated from untreated and RELMα-treated cells was performed for this purpose. As seen in Fig. 2C, the expression of C/EBPα, glycerol-3-phosphate dehydrogenase, Acrp30, aP2, and PPARγ2 was down-regulated, whereas the expression of a control gene, TBP, was unaffected, confirming that the differentiation of preadipocytes into adipocytes is indeed inhibited by RELMα. We also observed a similar inhibition when differentiation was induced by rosiglitazone, a thiazolidinedione (data not shown).

Effect of RELMα on Proliferation, Glucose Transport, and IRS-1 Tyrosine Phosphorylation—Inhibition of differentiation may be brought about by an induction of proliferation in 3T3-L1 preadipocytes, as is the case with other antiadipogenic factors such as epidermal growth factor and PDGF (20). Therefore, we performed a thymidine incorporation assay to examine the effect of purified RELMα on DNA synthesis in 3T3-L1
Inhibition of Adipocyte Differentiation by RELMα

Since secreted factors such as tumor necrosis factor-α and endothelin-1 modulate insulin-stimulated tyrosine phosphorylation of IRS-1 (21, 22), we decided to check whether RELMα has any effect on IRS-1 phosphorylation. Fig. 3C shows that RELMα alone does not induce any tyrosine phosphorylation of IRS-1, and short or long-term pretreatment with RELMα does not alter the tyrosine phosphorylation of IRS-1 induced by insulin.

RELMα Inhibits Differentiation of C2C12 Myoblasts into Myotubes—RELMα was originally identified as a protein expressed in the lung and adipose tissues. Since it is a secreted factor that is able to inhibit adipocyte differentiation, we decided to test whether it can affect differentiation of other cell types as well. For this purpose, C2C12 myoblasts were subjected to a differentiation protocol in the presence or absence of RELMα. It was observed that the addition of RELMα was able to inhibit the differentiation of C2C12 myoblasts into myotubes (Fig. 4).

Assessment of Oligomerization of RELMα by Coinmunoprecipitation Studies—The resistin family of molecules is quite rich in cysteines, with 10 cysteine residues being conserved among all members. Resistin and RELMβ contain one additional cysteine residue at their N termini. The structure of these molecules is distantly reminiscent of epidermal growth factor repeats found in other soluble factors. Although it is possible that these cysteine residues solely mediate the intramolecular disulfide bond, we reasoned that some of these residues might mediate intermolecular disulfide bridges, leading to formation of higher order oligomers.

We cotransfected 293T cells with V5 epitope-tagged RELMα and FLAG-tagged resistin or RELMβ cDNAs. 2 days later, the supernatants were collected and immunoprecipitated with an anti-FLAG antibody. Fig. 5 shows that V5-tagged RELMα was coimmunoprecipitated with both FLAG-tagged RELMα and resistin, indicating that they formed oligomers. FLAG-tagged RELMβ served as a negative control, since it did not bind to RELMα. This experiment shows that RELMα forms a homooligomeric complex that is a dimer at a minimum. This is consistent with the migration of RELMα as a dimer and trimer as shown in Fig. 1B. Since assessment of higher order multimers is difficult using such an epitope-tagging approach, we resorted to mass spectrometry to determine whether RELMα could form higher order oligomers than a dimer (see below).

Interestingly, coimmunoprecipitation of RELMα with resistin shows that they are able to form heterooligomers (Fig. 5). This may serve to regulate their biological activity in vivo, since they are both secreted by adipocytes.

Assessment of Oligomerization of RELMα by Mass Spectrometry—Mass spectrometry has been successfully used to investigate disulfide bond formation in proteins. We subjected recombinant purified RELMα to nanoelectrospray mass spectrometry on a quadrupole time-of-flight mass spectrometer before and after reduction of the sample. As shown in Fig. 6A, the unreduced sample showed several peaks corresponding to mul-
Multiple charge states of RELMα. The molecular masses derived from the spectrum were 11,203.6 ± 0.7, 22,406.7 ± 1.5, and 33,611.8 ± 1.9, which are in agreement with monomeric, dimeric, and trimeric forms of RELMα. Reduction of the sample abolished the dimeric and trimeric species from the mass spectrum, since only a single charge series with a derived molecular mass of 11,212.9 ± 0.2 was observed (Fig. 6B). These molecular masses correspond to the calculated mass of RELMα with all 10 cysteine residues in their reduced state. These findings confirm the oligomerization pattern that was predicted for RELMα based on its migration on electrophoretic gels under reducing and nonreducing conditions (Fig. 1B).

**Oligomerization of RELMα in Vivo**—Since our studies regarding oligomeric nature of RELMα were performed using the recombinant form, we decided to check whether such oligomerization also occurs in vivo. For this purpose, we generated rabbit polyclonal antibody against mouse RELMα. A synthetic peptide corresponding to 15 amino acids at the amino terminus was used as an immunogen as described under “Experimental Procedures.”

Equal amounts of mouse serum were resolved on SDS-PAGE under reducing or nonreducing conditions followed by Western blotting with anti-RELMα antibody. As shown in Fig. 7 (left panel), only a single band with an approximate size of 10 kDa was observed when the electrophoresis was performed under reducing conditions. When the same experiment was performed under nonreducing conditions (right panel), two extra bands in the region between 28 and 38 kDa were detected. All of these experiments clearly show that RELMα is capable of forming higher order oligomers that are mediated by covalent bonds between cysteine residues.

**CONCLUSION**

Resistin and Related Molecules, RELMα and RELMβ, belong to a new family of cysteine-rich secreted proteins. Although...
their precise physiological role is not yet understood, resistin has been shown to be up-regulated during the process of adipocyte conversion, and its expression is down-regulated by thiazolidinediones. Administration of resistin to mice results in impaired glucose tolerance, suggesting that it may predispose to type II diabetes. More recently, resistin expression was shown to be greatly induced by refeeding of normal mice or administration of insulin to diabetic mice, and the addition of resistin inhibited conversion of preadipocytes into adipocytes (23). This is analogous to leptin, which is also up-regulated dramatically by refeeding and by diabetes/insulin.

Although resistin has been somewhat characterized, nothing is known about the biological functions of RELMa, which is also expressed by adipocytes. Therefore, we produced conditioned media as well as purified RELMa. Our results show that RELMa inhibits conversion of preadipocytes to adipocytes and that this effect is not accompanied by an increase in proliferation of preadipocytes. Transforming growth factor-β is a factor that inhibits the adipocyte differentiation process, and although it induces proliferation of preadipocytes, this effect is not required for its antiadipogenic effect (24). Therefore, it is likely that RELMa functions through a nonmitogenic pathway to mediate this effect. Cloning of the receptor(s) for this family of molecules will provide a better understanding of the downstream signal transduction events.

Leptin and Acrp30 are only two of many examples for proteins secreted by adipocytes and acting on the adipose tissue as well as other tissues and organs (7, 25, 26). Thus, our observations that RELMa affects the process of myotube formation is not entirely surprising. These results suggest possible involvement of the resistin family of ligands not only in adipose and muscle tissues but also in other distant tissues in the body.

Finally, we have also shown that RELMa forms disulfide-linked dimers and trimers in addition to formation of heterooligomers with resistin. In fact, under native conditions, we do not observe any low molecular weight species at all, suggesting that these different oligomeric species (monomers and disulfide-linked multimers) are possibly capable of binding in a noncovalent manner to form even higher order complexes (data not shown). Such a phenomenon has been reported in the case of Acrp30 as well. Other cysteine-rich ligands are also known to form disulfide-bonded oligomers. In the case of activin A, which predominantly exists as a homodimer, dimerization is required for its biological activity (27, 28). Similarly, angionpeptiotin-1 forms homotrimers as well as higher order multimers, whereas angionpeptiotin-2 predominantly exists as dimers and forms some higher order oligomers (29). Whether oligomerization of RELMa is required for its biological activity will require detailed mutagenesis studies due to the presence of numerous cysteine residues. Since RELMa and resistin are the only known members of this family that are secreted by adipocytes, our findings suggest that they may together serve to regulate the adipogenic process.

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