

Dimerization of Resistin and Resistin-like Molecules Is Determined by a Single Cysteine*

Received for publication, April 9, 2001, and in revised form, May 14, 2001
Published, JBC Papers in Press, May 17, 2001, DOI 10.1074/jbc.M103109200

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Resistin is a peptide hormone secreted by adipocytes. Cysteine residues comprise 11 of 94 (12%) amino acids in resistin. The arrangement of these cysteines is unique to resistin and its recently discovered family of tissue-specific secreted proteins, which have been independently termed resistin-like molecules (RELMs) and the FIZZ (found in inflammatory zone) family. Here we show that resistin is a disulfide-linked homodimer that can be converted to a monomer by reducing conditions. The intestine-specific RELM β has similar characteristics. Remarkably, however, the adipose-enriched RELM α is a monomer under non-reducing conditions. We note that RELM α lacks a cysteine residue, closest to the cleaved N terminus, that is present in resistin and RELM β in multiple species. Conversion of this cysteine to alanine abolishes dimerization of resistin. Thus, a single disulfide bond is necessary to connect two resistin subunits in a homodimer. The additional 10 cysteines most likely participate in intramolecular disulfide bonds that define the conserved structure of the family members. The monomeric nature of RELM α suggests structural and potentially functional divergence between resistin and this close family member.

Resistin is a recently described peptide hormone belonging to a novel class of cysteine-rich secreted proteins termed the RELM¹/FIZZ (found in inflammatory zone) family (1–3). Resistin (also known as FIZZ3 and adipose tissue-specific secretory factor (1, 4)) is adipocyte-specific, down-regulated by antidiabetic drugs, and functionally antagonistic to insulin (2). RELM α (also known as FIZZ1) is expressed in white adipose tissue, tongue, and lung, where it appears to be involved in inflammatory processes (1, 3). RELM β (also known as FIZZ2) is intestine-specific and of unknown function (1, 3). The family is characterized by a highly conserved, cysteine-rich C terminus in which the spacing of the cysteines is invariant. Although resistin and the RELMs have interesting biological properties,

little is known about their structure and signaling mechanisms.

Formation of disulfide bonds, both intramolecular and intermolecular, is an important process in protein folding (5). Disulfide bonding is required for proper folding and secretion of proteins (6), and it is important for creation and maintenance of tertiary structure of polypeptide growth factors and hormones (7). Given the high percentage of cysteine residues in the amino acid sequences of resistin and the RELM family, we hypothesized that disulfide bonding might have important structural consequences in these molecules. Here we show that resistin is a disulfide-linked dimer whether expressed recombinantly or endogenously secreted by 3T3-L1 adipocytes. RELM β is also a homodimer. By contrast, RELM α is secreted as a monomer. This fundamental difference is because of a single cysteine residue that is present in resistin and RELM β in multiple species but is not conserved in RELM α . The biological functions of resistin and the RELMs are likely to be influenced by this structural diversity.

MATERIALS AND METHODS

Plasmid Constructs and Site-directed Mutagenesis—Wild type resistin, as well as C-terminal FLAG epitope-tagged resistin, RELM α , and RELM β expression vectors, were described previously (2, 3). Wild type RELM α and RELM β were subcloned into the expression vector pZac2.1 using similar methods. Site-directed mutagenesis to introduce a cysteine to alanine mutation at amino acid 26 of resistin and resistin fused to a FLAG epitope at its C terminus (resistin-F) was performed using the Stratagene QuickChange kit according to the manufacturer's directions. Constructs were sequenced to confirm they were correct.

Cell Culture and Transient Transfection—293T cells were maintained in Dulbecco's modified Eagle's medium with high glucose supplemented with 10% fetal bovine serum (Life Technologies, Inc.) (hereafter referred to as growth media) and grown at 37 °C in 5% CO₂. Cells were transiently transfected using Fugene 6 (Roche Molecular Biochemicals) according to the manufacturer's directions. Briefly, 100-mm plates of cells at 50–80% confluence were transfected with a total of 3 μ g of DNA and a DNA:Fugene ratio of 6:1. The medium was changed at 24 h post-transfection, and conditioned medium was harvested 48 h later. Harvested medium was used immediately or stored at –20 °C until use.

3T3-L1 preadipocytes were maintained in growth media and differentiated as described previously (8). At 48 h (Day 2) the differentiation medium was removed, and growth medium was added to the cells. Medium was collected from preadipocytes (Day 0) and adipocytes (Day 8) and stored at –20 °C until use.

Immunoblot Analysis—Proteins were separated on 15% SDS-PAGE gels. For non-reducing conditions, samples were mixed with an equal volume of 2 \times Laemmli sample buffer (Bio-Rad). For reducing conditions, samples were mixed with an equal volume of sample buffer supplemented previously with 20% β -mercaptoethanol. All samples were boiled for 5 min prior to electrophoresis. Blots were transferred to Hybond-C nitrocellulose (Amersham Pharmacia Biotech) in Tris/glycine buffer (Bio-Rad) at 4 °C for 2 h at 60 V or overnight at 20 V. Blots were probed with primary antibodies diluted in Tris-buffered saline containing 0.1% Tween 20 and 5% blotting grade nonfat dry milk (Bio-Rad) for 1 h but were otherwise performed as described previously (9). IgG purified from rabbit polyclonal antiserum to resistin was used at 1:1000 dilution as described previously (2). The mouse monoclonal

* This work was supported in part by NIDDK, National Institutes of Health Grants DK49780 and DK49210 (to M. A. L.) and by the Penn Diabetes and Endocrinology Research Center (DK19525) and the University of Pennsylvania Center for the Molecular Study of Digestive Diseases (DK50306). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: RELM(s), resistin-like molecule(s); resistin-F, resistin fused to a FLAG epitope at its C terminus; PAGE, polyacrylamide gel electrophoresis.

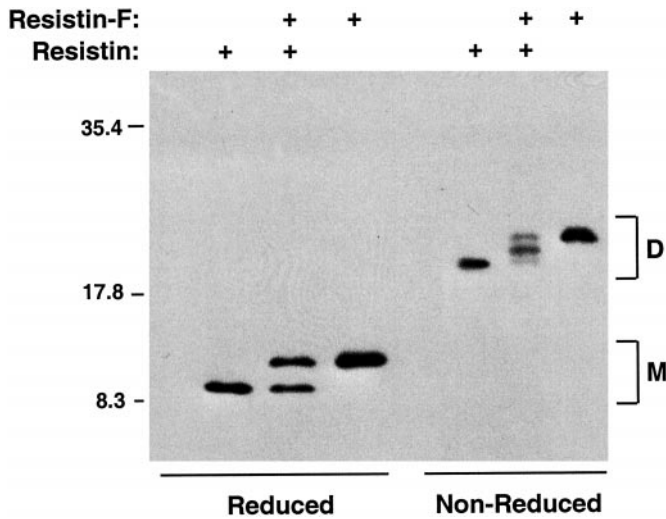


FIG. 1. Recombinant resistin is secreted as a dimer. 293T cells were transiently transfected with resistin, resistin-F, or both together as indicated. Medium from these cells was electrophoresed on 15% SDS-PAGE gels under reducing or non-reducing conditions (see "Materials and Methods") and detected by immunoblot with anti-resistin IgG. Monomeric (*M*) and dimeric (*D*) species are indicated. Migration of molecular mass markers is shown.

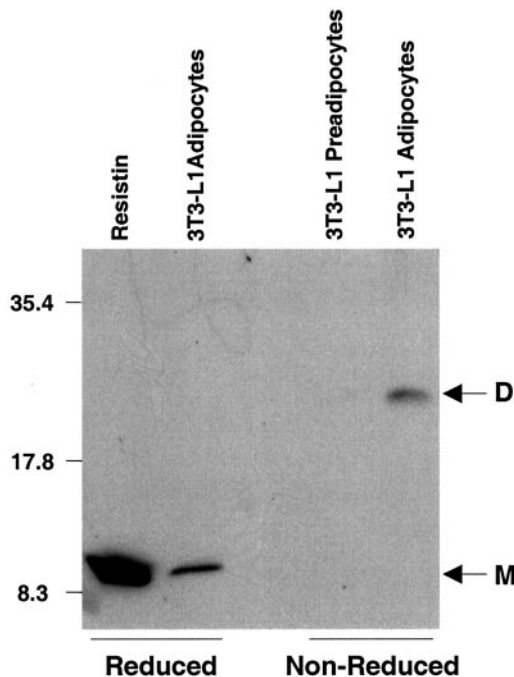


FIG. 2. 3T3-L1 adipocytes secrete dimeric resistin. Media from cultured 3T3-L1 preadipocytes and adipocytes electrophoresed on 15% SDS-PAGE gels under reducing or non-reducing conditions and detected by immunoblot with anti-resistin IgG are shown. Recombinant wild type resistin was also electrophoresed under reducing condition as a control. Monomeric (*M*) and dimeric (*D*) species are indicated. Migration of molecular mass markers is shown.

anti-FLAG M2 antibody (Sigma) was used at 1:5,000. Rabbit anti-RELM β polyclonal serum was raised against mouse RELM β expressed in *Escherichia coli* as a glutathione *S*-transferase fusion protein. Rabbit anti-RELM α polyclonal serum was raised against a peptide corresponding to amino acids 32–46 of mRELM α . Mouse RELM β and RELM α antisera were used at 1:1000 dilution.

RESULTS

Resistin Is a Disulfide-linked Homodimer—Wild type resistin, as well as resistin-F, was expressed separately or together in 293T cells. When analyzed by SDS-PAGE and immunoblot

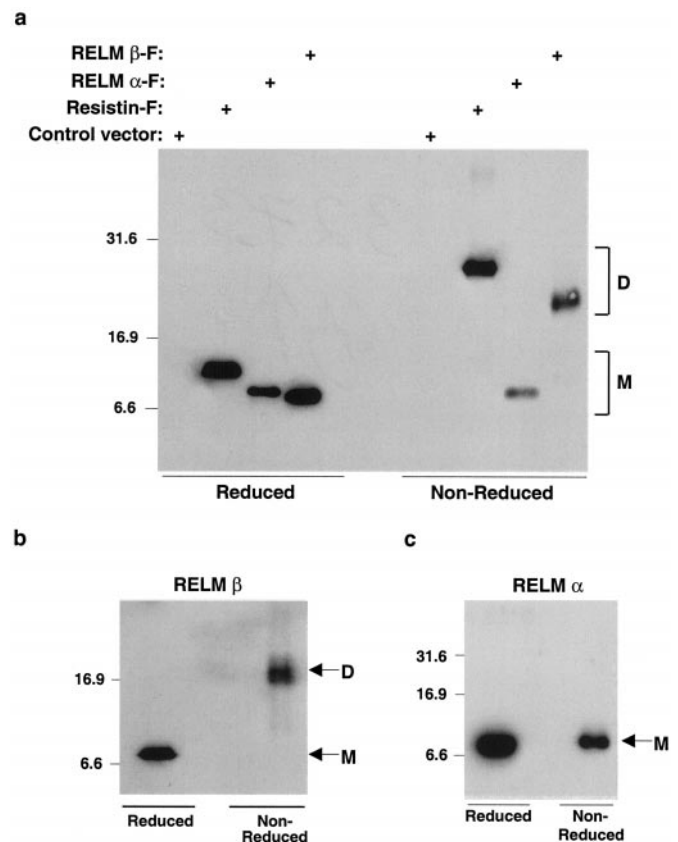


FIG. 3. Differential dimerization of resistin and resistin-like molecules. *a*, FLAG-tagged (*-F*) resistin, RELM β , and RELM α were transiently expressed in 293T cells. Medium from these cells was electrophoresed on 15% SDS-PAGE gels under reducing or non-reducing conditions and detected by immunoblot with anti-FLAG antibody. *b* and *c*, Wild type RELM β (*b*) and RELM α (*c*) were transiently expressed in 293T cells, and conditioned medium was electrophoresed on 15% SDS-PAGE gels under reducing or non-reducing conditions and detected by immunoblot with anti-RELM β (*b*) or anti-RELM α (*c*) antiserum. Monomeric (*M*) and dimeric (*D*) species are indicated, and migration of molecular mass markers is shown.

under reducing conditions, the secreted proteins migrated as monomeric species (labeled *M* in Fig. 1) with molecular masses consistent with those deduced from the cDNA sequences. However, under non-reducing conditions, both resistin and resistin-F migrated with apparent molecular masses approximately twice those of the respective monomers (labeled *D* in Fig. 1). Moreover, when resistin and resistin-F were coexpressed, a novel band migrating with an electrophoretic mobility intermediate between resistin and resistin-F appeared. This corresponds to a covalent heterodimer of resistin and resistin-F. This assignment was confirmed by immunoblotting with anti-FLAG antibody, which only detected the upper two bands (data not shown). Heterodimerization between resistin and resistin-F was not noted when the individual proteins were mixed after being expressed separately (data not shown), suggesting that homodimers were stable in non-reducing conditions.

3T3-L1 Adipocytes Secrete a Disulfide-linked Resistin Homodimer—We next investigated whether endogenous resistin is a disulfide-linked homodimer. 3T3-L1 cells were differentiated into adipocytes over 8 days. Preadipocytes do not express the resistin gene (2, 4) and do not secrete resistin (Fig. 2). Resistin secreted by 3T3-L1 adipocytes migrates as a monomer under reducing conditions (labeled *M* in Fig. 2). By contrast, 3T3-L1 adipocyte-derived resistin was revealed to be a dimer when electrophoresed under non-reducing conditions (labeled *D* in Fig. 2). Thus both recombinant and 3T3-L1 adipocyte-

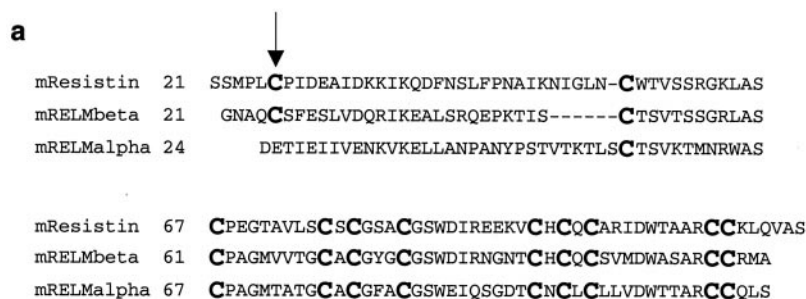
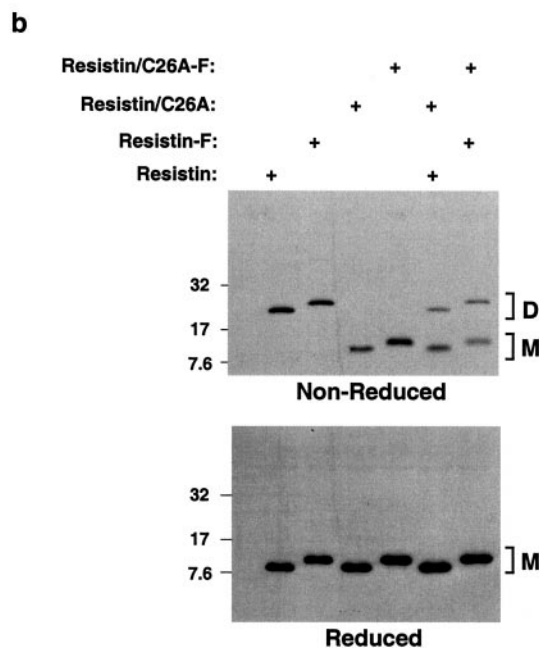


FIG. 4. Cys26Ala mutation abolishes resistin dimerization. *a*, alignment of cleaved resistin and RELMs with conserved cysteines highlighted in *bold*. The *arrow* points to the cysteine conserved in resistin and RELM β but not RELM α (Cys²⁶ in resistin). Numbering is consistent with the propeptide amino acid sequence (3). *b*, resistin and resistin-F, wild type or containing the C26A mutation, were transiently expressed in 293T cells singly or together as indicated. Medium was collected and electrophoresed on 15% SDS-PAGE gels under non-reducing (*upper*) or reducing (*lower*) conditions and detected by immunoblot with anti-resistin IgG. Monomeric (*M*) and dimeric (*D*) species are indicated, and migration of molecular mass markers is shown.



derived resistin are disulfide-linked homodimers.

RELM β Is a Disulfide-linked Dimer, but RELM α Is a Monomer—Given the similar sequences of resistin and the RELMs, we next compared the electrophoretic mobilities of RELM β and RELM α under reducing and non-reducing conditions. We first compared the FLAG-tagged proteins visualized with anti-FLAG antibody. Consistent with the behavior of resistin-F, the RELM-F proteins migrated true to their predicted monomeric molecular masses when electrophoresed under reducing conditions (labeled *M* in Fig. 3*a*). On the same gel, but under non-reducing conditions, RELM β -F migrated as a dimer (labeled *D* in Fig. 3*a*). In stark contrast, the electrophoretic mobility of RELM α -F was identical under non-reducing and reducing conditions, corresponding to the monomeric species in both cases. To ensure that the differing gel mobilities of non-reduced RELM β -F and RELM α -F were not because of the FLAG epitopes, wild type proteins were studied using specific antibodies. Under non-reducing conditions, wild type RELM β was a dimer (Fig. 3*b*) whereas RELM α behaved as a monomer. Thus, resistin and RELM β exist as disulfide-linked dimers whereas RELM α is a monomer.

Dimerization of Resistin Requires a Single Cysteine That Is Not Present in RELM α —Alignment of mouse resistin and the RELMs revealed that although all three share the 10 most C-terminal cysteines, RELM α lacks the most N-terminal cysteine that is present in both resistin and RELM β (indicated by an *arrow* in Fig. 4*a*). This cysteine is also conserved in human resistin and RELM β (as well as rat and bovine resistin; data not shown). However, rat RELM α (which is also secreted as a

monomer) also lacks this 11th cysteine. We hypothesized that the 10 conserved C-terminal cysteine residues present in resistin and all RELMs could form five intramolecular disulfide bonds, whereas the additional cysteine in resistin and RELM β would be paired by an intermolecular disulfide bond, resulting in the observed secretion of homodimers. To determine the role of the most N-terminal cysteine (Cys²⁶) in resistin dimerization, we mutated this residue to alanine (C26A). Unlike wild type resistin, the C26A mutant was found to migrate as a monomer under both reducing and non-reducing conditions (Fig. 4*b*). No intermediate species were observed when the wild type and C26A mutant resistin were coexpressed, indicating that Cys²⁶ of one subunit of the dimer interacts with Cys²⁶ in the other subunit. This experiment shows that Cys²⁶ is required for resistin dimerization. The equivalent mutation in RELM β also abolishes its disulfide-linked dimerization (data not shown). Thus, in all members of the RELM family each of the 10 conserved cysteines participates in creating what is likely to be a conserved structure of the signature region of the molecule. In resistin and RELM β , an 11th cysteine mediates covalent dimerization via disulfide bonding. This cysteine is missing from RELM α , which is therefore a monomer.

DISCUSSION

The RELM/FIZZ family has only been described recently (1, 3). We and others have observed previously that this family is characterized by a signature C-terminal sequence containing 10 C-terminal cysteine residues (1, 3). We have shown that an 11th cysteine residue present in resistin (Cys²⁶), as well as

RELM β but not RELM α , is necessary for homodimerization. It is likely that dimerization is because of a single disulfide bond between Cys²⁶ residues in each of two monomers. The remaining 10 cysteines that are conserved in all members of the RELM/FIZZ family are likely to be involved in intramolecular disulfide bonding, creating a structure that may contribute to a common function such as receptor binding. The precise intramolecular disulfide bonding remains to be determined. We cannot exclude the possibility that cysteine residues other than Cys²⁶ participate in intermolecular disulfide bridging in the resistin dimer. However, our results clearly show that a cysteine in position 26 is required for any such additional intermolecular disulfide bonds to form.

The cysteine residue required for resistin dimerization is contained within the highly variable N-terminal domain of the RELMs. This variability in primary sequence and dimerization potential, together with the tissue-specific expression patterns, suggests functional differences in the actions of resistin and the RELMs. Although the signaling mechanisms of these proteins is not yet known, it is likely that these secreted proteins act via cell surface receptors. There is precedent for oligomerization status affecting the signaling function of a ligand, particularly in receptor binding and activation. For example, oligomerization of tumor necrosis factor α is required for ligand-induced receptor activation (10). More relevant to resistin and the RELMs are dimeric signaling molecules in which intermolecular disulfide bonds have functional significance. Activin, a growth factor in the transforming growth factor- β superfamily, is a disulfide-bonded dimer containing nine cysteines. Mutation of a single cysteine results in monomeric activin with essentially no ability to activate the activin receptor (11, 12).

Another example is intestinal trefoil factor, which contains seven cysteines, one of which is involved in dimer formation (13, 14). Dimerization is required for some but not all functions of the trefoil peptides. Future identification of the receptors for members of the RELM/FIZZ family will shed light on the biological significance of their differential dimerization.

Acknowledgments—We are extremely grateful to Claire Steppan and Elizabeth Brown for antibodies to resistin and the RELMs. We thank Jim Cunningham, Myles Brown, David Speicher, and members of the Lazar laboratory for helpful discussions.

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