Wdnm1-like, a new adipokine with a role in MMP-2 activation

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Submitted 27 March 2008; accepted in final form 13 May 2008

Wu Y, Smas CM. Wdnm1-like, a new adipokine with a role in MMP-2 activation. Am J Physiol Endocrinol Metab 295: E205–E215, 2008.-White adipose tissue functions in energy storage and as an endocrine organ. DNA microarray analysis led us to identify Wdnm1-like, a distant member of the whey acidic protein/four-disulfide core (WAP/4-DSC) family, as a differentiation-dependent gene in white and brown adipogenesis. Wdnm1-like is a novel 6.8-kDa protein, and Western blot analysis reveals secretion into culture media. Wdnm1-like transcript is selectively expressed in adipose tissue and liver and is enriched ~500-fold in white adipose depots vs. brown. Cellular fractionation of WAT demonstrates Wdnm1-like transcript expression is restricted to the adipocyte population. Studies in 3T3-L1 preadipocytes, an in vitro model of white adipogenesis, indicate Wdnm1-like transcript increases within 6 h of adipogenic induction with an ~17,000-fold increase by day 7. Dramatic upregulation of Wdnm1-like also accompanies white adipogenesis of ScAP-23 preadipocytes and primary adipocytes. TNF-α treatment of 3T3-L1 adipocytes increased Wdnm1-like transcript level 2.4-fold and was attenuated by pretreatment with the p38 MAP kinase inhibitor SB203580. A number of WAP/4-DSC family members function as protease inhibitors. This, taken with the role of extracellular remodeling in adipogenesis, led us to address effects of Wdnm1-like on matrix metalloproteinase (MMP) activity. Gelatinzymography of HT1080 fibrosarcoma cells transfected with a Wdnm1-like expression construct revealed markedly increased levels of active MMP-2. Our findings identify a new member of the adipocyte “secretome” that functions to enhance MMP-2 activity. We postulate that Wdnm1-like may play roles in remodeling of the extracellular milieu in adipogenesis, as well as in tumor microenvironments where adipocytes are key stromal components.

Adipocytes make up from one-third to two-thirds of the cell population found in adipose tissue. The remaining cells, collectively referred to as the stromal-vascular fraction (SVF), include endothelial cells, nerve cells, macrophages, fibroblast-like interstitial cells, preadipocytes, and likely other cell types yet to be identified (3). In adipose tissue development, mature adipocytes arise from the differentiation of preadipocyte precursors present in adipose tissue in a process that occurs throughout the lifespan (2, 28, 39, 47). For the past decades, in vitro preadipocyte cell lines such as 3T3-L1 (26) have been used extensively to define genes central to the adipocyte phenotype (48, 57). Adipogenesis is accompanied by upregulation of genes that encode molecules central to adipose tissue function, including those critical in lipogenesis, lipolysis, lipid transport, and hormone signaling (28, 29). A variety of in vitro and in vivo studies have determined that the peroxisome proliferator-activated receptor-γ (PPARγ), a member of the ligand-activated steroid hormone receptor family, is a master transcriptional regulator of the adipogenic program (35, 41, 46, 47). Studies have also illustrated the important contribution of the CCAAT/enhancer-binding protein (C/EBP) family of transcriptional regulators and other transcriptional signals to adipogenesis (22, 46, 47). In addition to nuclear signals, signals from the extracellular environment, such as those involved in extracellular matrix (ECM) remodeling, are important in adipocyte differentiation and adipose tissue development (29, 56).

During an ongoing study aimed at uncovering novel gene expression patterns in brown adipogenesis, we identified a novel and to date uncharacterized secreted factor, Wdnm1-like, as a differentiation-dependent adipocyte-enriched transcript. Although initially it was found on the basis of its upregulation during in vitro brown adipogenesis, we have discovered that Wdnm1-like is expressed ~500-fold higher in WAT than in brown adipose tissue (BAT). Given their potential to impact adipose tissue function and systemic energy balance, there is keen interest in the characterization of novel adipocyte-secreted factors. Such factors may also have roles in the stromal microenvironment of tumor cells, wherein adipocytes frequently occur. In this study, we have characterized the expression, regulation, and function of this novel secreted factor and demonstrate a role for Wdnm1-like in enhancing matrix metalloproteinase-2 (MMP-2) activity.

MATERIALS AND METHODS

Adipocyte differentiation and cell culture treatments. 3T3-L1 cells (American Type Culture Collection, Manassas, VA) were propagated in DMEM containing 10% calf serum. For adipocyte differentiation, 3T3-L1 cells were treated at 2 days postconfluence with DMEM...
containing 10% FBS in the presence of the adipogenic inducers 0.5 mM methylisobutylxanthine (MIX) and 1 μM dexamethasone (Dex) for 48 h. Adipogenic agents were then removed, and growth of cultures continued in DMEM containing 10% FBS. For treatment of 3T3-L1 adipocytes with TNF-α and various pharmacological inhibitors following serum starvation for 6 h, 3T3-L1 adipocytes were pretreated with either 20 μM SB203580, 100 nM wortmannin, 1 μM rapamycin (Sigma-Aldrich, St. Louis, MO), or DMSO vehicle for 1 h and stimulated with 10 ng/ml of TNF-α for 16 h in the presence of the indicated inhibitors or incubated with inhibitors only for the same time period.

ScAP-23 cells were maintained in DMEM containing 10% calf serum and passaged before reaching confluence. For adipocyte differentiation, preconfluent ScAP-23 preadipocytes were cultured in DMEM containing 10% FBS in the presence of the adipogenic inducers 0.5 mM MIX, 1 μM Dex, 17 nM insulin, and 0.2 mM indomethacin for 70 h. These agents were then removed, and cultures were maintained in DMEM containing 10% FBS and 17 nM insulin for an additional 4 days.

For culture and differentiation of primary white adipocytes, WAT was collected from C57BL/6 mice and digested with 1 mg/ml of type I collagenase for 40 min with shaking at 37°C. Following digestion, material was filtered through a 300-μm-pore-size nylon mesh (Sefar America, Depew, NY) and filtrate centrifuged at 2,000 revolution/min for 5 min. Floating adipocyte fraction was removed, and the pellet of stromal-vascular cells was resuspended in DMEM containing 10% FBS and plated. Upon confluence, cells were either harvested as preadipocytes or cultured in differentiation media consisting of DMEM containing 10% FBS, 0.1 μM Dex, 0.25 mM MIX, and 17 nM insulin for 3 days, at which time differentiation media was removed and cultures were maintained in DMEM containing 10% FBS and 17 nM insulin for an additional 4 days.

For differentiation of the brown preadipocyte cell line, referred to herein as WT-BAT and obtained from C. R. Kahn (Joslin Diabetes Foundation, Harvard Medical School, Boston, MA), the method was as previously described (32, 33). Briefly, cells were cultured to confluence in DMEM containing 10% FBS, 20% calf serum, and 1 nM triiodothyronine [differentiation medium per Kahn and colleagues (33)] for 1 wk. The medium was then replaced with 0.5 mM MIX, 0.5 μM Dex, and 0.125 mM indomethacin for 48 h, at which point cultures were switched to differentiation medium for an additional 5 days.

RAW264.7 murine macrophages were plated at a density of 4 × 10⁶ cells/well of a six-well plate in DMEM with 10% FBS medium (growth medium), and 24 h postplating medium was changed to growth medium with or without 100 ng/ml LPS. After a 4-h incubation, cells were harvested for total RNA preparation.

RNA preparation, Northern blot analysis, and real-time PCR. RNA was purified using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instruction. For studies of Wdnm1-like expression in murine tissues, 8-wk-old C57BL/6 or oopho male mice were utilized, with all animal treatments conducted with approval of the University of Toledo Health Science Campus Institutional Animal Care and Use Committee. Fractionation of whole adipose tissue into adipocyte and SVF was via collagenase digestion and differential centrifugation, as previously described (32). For Northern blot analysis, 5 μg of RNA was fractionated in 1% agarose-formaldehyde gels in MOPS buffer and transferred to Hybond-N membrane (GE Healthcare, Piscataway, NJ). Blots were hybridized in ExpressHyb solution (BD Biosciences Clontech, Palo Alto, CA) with the indicated 32P-labeled murine cDNA probes. After being washed, membranes were exposed at −80°C to Kodak Biomax film with a Kodak Biomax intensifying screen. All lanes shown in a single image were run on the same blot; however, in some instances lanes were rearranged or removed for economy and/or clarity of presentation.

For real-time PCR analysis, total RNA was subject to purification with an RNasy RNA purification kit with DNase I treatment (Qiagen, Valencia, CA) for elimination of all sources of DNA, and 5 μg were used for first strand cDNA synthesis with SuperScript II RNase H-reverse transcriptase (Invitrogen) and an oligo(dT)-22 primer. Real-time PCR was conducted with an ABI 7500 real-time PCR System. Target cDNA levels were analyzed by SYBR Green-based real-time PCR in 25-μl reactions containing 1× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 100 nM each forward and reverse primers, and 10 ng of cDNA. PCR was carried out over 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 40 s, with initial cycle of 50°C for 2 min and 95°C for 10 min to activate AmpliTaq Gold DNA polymerase. Sequences of the real-time PCR primers used in this study, their annealing positions to the respective GenBank RefSeq sequences, the PCR product sizes, and intron information are provided in Supplemental Table 1. Supplemental material for this article is available at the American Journal of Physiology Endocrinology and Metabolism website. Primers were designed by using Accelrys DS Gene software and whenever possible amplified from two different exons. In the few cases wherein primers could not be designed in two separate exons, we conducted reverse transcriptase control reactions. For these, we did not observe any PCR product signal but only an extremely low degree of background signal the same as that for water-only reactions. A dissociation curve over the range of 60−95°C was generated after the 40th PCR cycle; in every case a sharp melting peak was observed indicative of a single unique species of PCR product. Expression of each gene was normalized against Gapdh transcript level. In all cases, the same amount of input RNA/cDNA was used in side-by-side comparisons, and, in most cases, the Gapdh level for the samples under comparison differed by one or fewer cycles; in no case was the difference greater than two cycles. The cycle threshold (Ct) value was generated using ABI PRISM 7500 SDS software version 1.2 and exported to an Excel spreadsheet to calculate fold differences. In samples where transcript expression was not evident after 40 PCR cycles, a value of 40 cycles was assigned to calculate a ΔCt value and estimate fold differences. Such instances are noted in the respective figure legend. Analyses were performed in triplicate.

Affymetrix DNA array studies. Affymetrix 430A Gene Chips were hybridized with material prepared from 5 μg of total RNA. Duplicate RNA samples from brown preadipocytes or brown adipocytes at 7 days postinduction of differentiation were used for transcriptional profiling studies. The Keck Microarray Facility at Yale University (New Haven, CT) carried out processing of DNA arrays according to standard protocols. An Excel file containing the complete set of fold changes generated using the R program in the Affymetrix affylmGUI package and employing robust multiarray averaging is provided as supplemental information.

Preparation of a murine Wdnm1-like expression construct. For preparation of a murine Wdnm1-like expression construct, termed Wdnm1-like-HA, in which a COOH-terminal hemagglutinin (HA) epitope tag was fused in frame to the Wdnm1-like coding sequence, a full-length sequence-verified mouse Wdnm1-like cDNA L.M.A.G.E clone (American Type Culture Collection) was used as template for PCR-based cloning. Primer sequences for PCR cloning were: 5′ PCR primer (5′-TCCGAGATTTCCCCCTGATCCCTGACGACGATG-AA-3′) and the 3′ PCR primer (5′-GTCGATGATCTGGGCTTAATCTGACGAGATGATGAC-3′) and the 3′ PCR primer (5′-GTCGATGATCTGGGCTTAATCTGACGAGATGATGAC-3′) and the 3′ PCR primer (5′-GTCGATGATCTGGGCTTAATCTGACGAGATGATGAC-3′). A 5′ EcoRI site and a 3′ EcoRV site were incorporated into respective primers to facilitate directional cloning into pcDNA3.1 vector (Invitrogen).

Transfections and Western blot analysis of Wdnm1-like protein expression. For assessment of secretion of Wdnm1-like into culture media, 293T cells were transfected with the Wdnm1-like-HA expression construct or empty vector with the use of Lipofectamine 2000 (Invitrogen). Culture media and cell lysates were collected for analysis at 48 h posttransfection. Medium was centrifuged at 5,000 revolution/min for 3 min, and cells were harvested by lysis in TNN(+)-buffer containing 10 mM Tris, pH 8.0, 120 mM NaCl, 0.5%
lysate (6/11011 of total media (20% v/v) determined (Bio-Rad Laboratories, Hercules, CA). A sample (1/150) of total media (20 µl of a total 3 ml) or 1/150 of total cell lysate (~6 µg of 900 µg total protein yield) were size fractionated on 15% SDS-PAGE gels for Western analysis and proteins transferred onto Immobilon P-seq membranes (Millipore, Billerica, MA). For signal detection, membranes were blocked by a 1-h incubation in 5% nonfat milk/0.5% Tween 20 in PBS and then incubated with a 1:2,000 dilution of anti-HA primary antibody (Covance Research Products, Princeton, NJ) for 1 h followed by three 10-min washes. Secondary antibody was goat anti-mouse at a dilution of 1:2,000 for 1 h followed by three 10-min washes. All washes were in 0.5% Tween 20 in PBS. Signal was detected by ECL Plus enhanced chemiluminescence (GE Healthcare) and exposure to X-ray film.

In vitro translation. A TNT quick-coupled transcription/translation kit (Promega, Madison, WI) was employed for in vitro transcription and translation. Reactions programmed with either the Wdnm1-like HA construct or with empty vector were incubated at 30°C for 90 min, and 10 µl or 2 µl of the respective reaction was analyzed on 10% SDS-PAGE Tricine gels, followed by overnight transfer to Immobilon P-Seq membrane (Millipore). Membranes were processed for Western blot analyses with anti-HA antibody as described in Transfections and Western blot analysis of Wdnm1-like protein expression.

Gelatin zymography assay. HT1080 cells were plated at a density of 6.25 × 10^4 per 60-mm dish. The day after plating, cells were transfected with the Wdnm1-like-HA expression construct or empty pCDNA3.1 vector using Lipofectamine and Plus Reagent (Invitrogen). At 4 h posttransfection, medium was removed and 2.5 ml of serum-free media added. Medium was harvested at 48 h after transfection, centrifuged for 3 min at 5,000 revolution/min to remove cell debris, and used fresh for zymography. For zymograph analysis (30), 15 µl of medium was mixed with 7.5 µl of 3× sample buffer (125 mM Tris·HCl, pH 6.8, 8% SDS, 50% glycerol, and 0.02% Coomassie blue), and gel lanes were loaded without prior heat denaturation of samples. Samples were fractionated in 10% SDS-PAGE zymograph gels that contained 0.1% (wt/vol) gelatin. After electrophoresis, gels were treated for renaturation by incubation in 2.5% Triton X-100 for 30 min at room temperature and then transferred to development solution (0.05 M Tris·HCl, pH 8.8, 5 mM CaCl_2, and 0.02% NaCl) and incubated overnight at 37°C. Gels were incubated in staining solution (40% methanol, 10% acetic acid, and 0.5% Coomassie blue) for 4 h and destained in fixed/staining solution (40% methanol and 10% acetic acid) until bands of gelatinase activity were clearly visible. For each experiment, transfections and assays were carried out on a minimum of duplicate independent samples, and the overall study from transfection through zymography was conducted three times. Data was documented with digital photography, and representative data are shown.

RESULTS

Wdnm1-like is a novel secreted protein of adipocytes. We initially identified upregulation of murine Wdnm1-like in adipogenesis during an unrelated DNA microarray study designed to determine the effects of retrovirally driven ectopic expression of a novel gene, SMAF1, on brown adipocyte conversion of WT-BAT preadipocytes. For this we had generated an empty vector control sample set of preadipocytes that had undergone G418 drug selection for the PLNCX2 retroviral vector and a sample set following their differentiation to brown adipocytes. To our knowledge, global gene expression assessment on the adipogenic conversion of WT-BAT cells has not been previously determined. Kahn and colleagues (63), the source of the WT-BAT cell line, however, have reported on the differentiation-dependent expression of similarly derived brown preadipocyte cell lines, which were null for distinct insulin receptor substrate genes (63). In the course of our analyses, we compared gene expression data for brown preadipocytes and adipocytes. We found that 173 genes of the ~22,000 on the array were upregulated tenfold or greater during brown adipogenesis in this in vitro model. We chose several well-characterized adipogenic genes and 20 other genes that had not yet been assessed in either brown or white adipogenic conversion and validated their respective fold increases upon adipogenesis using real-time PCR. These data are presented in Table 1. We note here that in the time since we initially carried out PCR validation of gene expression

Table 1. PCR validation of select genes upregulated during in vitro brown adipogenesis

<table>
<thead>
<tr>
<th>Fold Increase</th>
<th>Symbol</th>
<th>Gene Name</th>
<th>Unigene</th>
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<tr>
<td>12</td>
<td>Ero1l</td>
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<tr>
<td>13</td>
<td>Arf4a</td>
<td>ADP-ribosylation factor-like 4A</td>
<td>Mm.12723</td>
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<td>14</td>
<td>Itga6</td>
<td>Integrin-α6</td>
<td>Mm.225096</td>
</tr>
<tr>
<td>19</td>
<td>Scd1</td>
<td>Stearoyl CoA-desaturase 1</td>
<td>Mm.267377</td>
</tr>
<tr>
<td>19</td>
<td>Sh2b2</td>
<td>SH2B adaptor protein 2</td>
<td>Mm.425294</td>
</tr>
<tr>
<td>24</td>
<td>Bnap3</td>
<td>BCL2/adenovirus E1B interacting protein 1, NIP3</td>
<td>Mm.378890</td>
</tr>
<tr>
<td>24</td>
<td>Selenbp1</td>
<td>Selenium-binding protein 1</td>
<td>Mm.196558</td>
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<tr>
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<td>Laminin-α4</td>
<td>Mm.258065</td>
</tr>
<tr>
<td>53</td>
<td>Rgs2</td>
<td>Regulator of G protein signaling 2</td>
<td>Mm.28262</td>
</tr>
<tr>
<td>55</td>
<td>Cebpα</td>
<td>CCAAT enhancer-binding protein-α</td>
<td>Mm.349667</td>
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<tr>
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<td>Aldhba1</td>
<td>Aldehyde dehydrogenase family 6, subfamily A1</td>
<td>Mm.247310</td>
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<tr>
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<td>Lamb3</td>
<td>Laminin-β3</td>
<td>Mm.435441</td>
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<td>1,040</td>
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<td>Retinoic acid receptor responder 2</td>
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<td>1,510</td>
<td>Fabp4</td>
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<td>45,000</td>
<td>Adipoq</td>
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changes, the adipocyte-related function of several of these genes has been reported. The endoplasmic reticulum oxidoreductase Ero1-like has been demonstrated to regulate secretion of adipoctectin (45). Rarrus/chemerin, with a role in adaptive and innate immunity, is produced by adipocytes to regulate adipogenesis and adipocyte metabolism (9, 25). Thus additional examination of these microarray data, with the complete data set provided as supplementary information, is likely to yield further insights into important factors in adipogenesis.

Of the novel genes identified in our microarray comparisons, one caught our particular attention because it increased ~300-fold during brown adipogenesis, thus making it the 10th most upregulated gene on the array, and its protein sequence suggested it encoded a novel small secreted factor. This gene was designated as expressed sequence tag (EST) 1100001G20Rik on the microarray. BLAST searches determined that this EST was the murine homolog of the rat Wdnm1-like gene. The single publication to date on Wdnm1-like describes its identification during a serial analysis study of gene expression (SAGE), with enrichment of Wdnm1-like transcript reported in the stem cell-rich limbal region vs. the central corneal epithelium in rats (1). These authors termed this new gene Wdnm1-like on the basis of what they regarded as similarity in protein sequence to a second largely uncharacterized rat gene, Wdnm1 (1). Wdnm1, named for West-meath DMB-8 non-metastatic cDNA clone, also termed Exp1, was initially identified as a transcript whose expression was decreased in a nonmetastatic clone of the mammary adenocarcinoma cell line DMB-8 vs. expression in a metastatic clone (19). As described below, Wdnm1-like and Wdnm1 are members of the whey acidic protein/4-disulfide core (WAP/4-DSC) protein family.

We obtained and sequenced verified a cDNA I.M.A.G.E clone for murine Wdnm1-like and as shown in Fig. 1A, the transcript is comprised of 434 bases and encodes a protein of 63 amino acids with a calculated molecular mass of 6,792 Da. Start and stop codons are present at nucleotides 88 and 279, respectively. As depicted in Fig. 1B, a COOH-terminal region of murine Wdnm1-like has a limited degree of similarity with the WAP/4-DSC motif (6), ProDom database designation PD0026912 (53). The WAP/4-DSC motif consensus sequence consists of 8 cysteines (numbered 1–8) in the order of C1 (Xa), C2 (Xa), C3 (Xb), C4 (Xa), C5, C6 (Xa,3), C7 (Xa,4), and C8, where X is any amino acid and n represents any number of residues (54). Multiple members of this protein family have been identified in humans. As shown in Fig. 1B, Wdnm1-like has conservation of six of the eight cysteines comprising the WAP/4-DSC motif, as well as conservation of other residues found in this motif. The remaining region of the Wdnm1-like protein sequence lacks obvious homologies with other proteins.

The murine Wdnm1-like gene spans 5.7 kb and consists of three exons (Fig. 1C) with the first intron separating the WAP/4-DSC type motif region of the Wdnm1-like from the unique NH2-terminal half of the protein. Although nearly all genes encoding WAP/4-DSC proteins are clustered on murine chromosome 2 corresponding to human 20q12–20q13.1 (6), Wdnm1-like is present on murine chromosome 11. It is located in a region between a gene cluster for five chemokines including Ccl4 and that for the hypothetical protein Heatr6.

Interestingly, an ~45-kb region of murine chromosome 11 includes genes for Wdnm1-like, Wdnm1/Exp1 and for a third novel WAP/4-DSC protein with similarity to Wdnm1, designated NP001075426. This region is depicted in Fig. 1D. Wdnm1 transcript is induced upon mammary gland involution and is occasionally used as a molecular marker in studies of mammary gland biology (5). Although Wdnm1 has been postulated as a tumor suppressor gene (58), its biology has not been well studied and its function is unknown. No information is available regarding expression of the gene for NP001075426. The sequence for these three related proteins is shown in Fig. 1E. As this alignment illustrates, Wdnm1-like has limited sequence homology (~30% identity) with either Wdnm1/Exp1 or NP001075426, whereas a high degree sequence homology (~70% identity) is evident between Wdnm1/Exp1 and NP001075426. As previously stated, Wdnm1-like lacks two of the eight cysteines that comprise a single WAP/4-DSC motif (shown by upward arrows in Fig. 1E), whereas both Wdnm1/Exp1 (18) and NP001075426 have all eight (bolded).

Wdnm1-like encodes a novel 6.7-kDa secreted protein. To begin studies on the Wdnm1-like protein, we assessed the size of the Wdnm1-like primary translation product with coupled in vitro transcription and translation using the Wdnm1-like-HA construct as template; the HA tag adds an additional ~1 kDa to the protein mass. Western blot analysis in Fig. 2A shows that lysate programmed with this template resulted in a major protein species migrating at ~8 kDa, in good agreement with the mass predicted from the primary amino acid sequence of Wdnm1-like. Kyte-Doolittle hydrophobicity analysis indicated that the NH2 terminus of Wdnm1-like was enriched in hydrophobic residues, consistent with the presence of a signal sequence (Fig. 2B). To determine whether Wdnm1-like protein was a secreted factor, the Wdnm1-like-HA construct or empty vector control was transiently transfected into 293T cells and media or cell lysates harvested at 24 h posttransfection. The Western blot in Fig. 2C, wherein a similar percentage of the total volume collected of cell lysate or medium was analyzed, indicates that the majority of Wdnm1-like protein is in the culture medium. The fact that the molecular mass found for the Wdnm1-like expressed in mammalian cells appears the same as that of the Wdnm1-like primary translation product (first two lanes of Fig. 2A) indicates that the protein does not undergo the types of posttranslational modifications, such as glycosylation, that would be predicted to result in measurable alterations in protein mass.

Wdnm1-like transcript expression is restricted to adipose tissue and liver. To garner further information on the in vivo tissue expression pattern of Wdnm1-like, we used real-time PCR analysis of multiple murine tissues. Figure 3A indicates that the Wdnm1-like transcript is highly restricted in expression to WAT and liver; it is present in these tissues at levels several hundred times higher than in the other tissues examined. We next compared Wdnm1-like transcript level in BAT and three distinct WAT depots, subcutaneous (SC), epididymal (EP) and retroperitoneal (RP) (Fig. 3B, left), and determined that it is expressed in SC WAT at levels ~500 times higher than that detected in BAT. It is also more than 100-fold higher in EP and RP WAT relative to BAT. In this regard, Wdnm1-like expression is similar to that for resistin (Fig. 3B, middle), in contrast to stearoyl CoA desaturase 1 (SCD1) and adipocyte...
fatty acid-binding protein (aFABP), wherein more uniform expression across the adipose depots are noted (Fig. 3B, right).

To determine which cellular fraction of adipose tissue expresses Wdnm1-like transcript, murine WAT was separated into the nonadipocyte SVF and the adipocyte-containing fraction. Real-time PCR analysis indicates that Wdnm1-like transcript expression is highly enriched in the adipocyte fraction (Fig. 3C, left), similar to the adipocyte marker transcript SCD1 (Fig. 3C, middle) and in contrast to the SVF enrichment of collagen 1A1 (Fig. 3C, right). We also compared the levels of Wdnm1-like transcript in wild-type mice vs. the ob/ob genetic model of murine obesity. Figure 3D shows a 1.5-fold increased transcript expression in the EP WAT and a 3.3-fold increase in BAT of ob/ob mice. In liver, ob/ob levels of Wdnm1-like transcript are decreased to 60% of that found for wild-type animals.

Fig. 1. Characteristics of Wdnm1-like protein sequence. A: sequence of the 434-bp Wdnm1-like transcript and the encoded 63-amino acid protein (bolded) are shown. The italicized region of the protein sequence corresponds to the region of whey acidic protein/four-disulfide core (WAP/4-DSC) homology. The underlined G in the protein sequence indicates the position of the first intron.

B: alignment of the WAP/4-DSC type region of the Wdnm1-like protein with a consensus WAP/4-DSC sequence (ProDom PD026912).

C: intron-exon arrangement of the murine Wdnm1-like gene. Open boxes represent the untranslated region, and solid boxes represent the translated region of the transcript. Thin dashed lines indicate introns.

D: location of the murine gene for Wdnm1-like and 2 additional WAP/4-DSC type genes on murine chromosome 11.

E: alignment of protein sequence for the 3 related WAP/4-DSC type proteins present in a cluster on murine chromosome 11. The canonical cysteines of the WAP/4-DSC motif are shown in bold typeface; those not found in Wdnm1-like are indicated with upward arrows.
Given the close chromosomal location of Wdnm1-like with Wdnm1/Expi and NP001075426, and their sequence similarities, we also determined the expression of these two transcripts in the same murine tissue panel used in Fig. 3A. We found that Wdnm1 was highly enriched in salivary gland, present at levels 1,101,112,000-fold higher than in any other of the tissues examined.

Fig. 2. Wdnm1-like encodes a novel 6.7-kDa secreted protein. A: Western blot analysis of in vitro translation products of HA-tagged Wdnm1-like expression construct or empty vector (EV). A sample (10 µl or 2 µl) of a 1:10 dilution of in vitro translation products was subjected to Western blot analysis performed with anti-HA antibody. Positive control (+) is medium from Wdnm1-like-transfected HT1080 cells. Molecular mass markers in kDa are shown at right. B: hydrophobicity analysis of Wdnm1-like protein sequence. Wdnm1-like protein sequence was subjected to Kyte-Doolittle hydrophobicity analysis using DS Gene 2.1 software with a window size setting of 10. The hydrophobicity score is shown on the y-axis and the amino acid number on the x-axis. C: Western blot analysis of Wdnm1-like protein expression in cells and culture medium. 293T cells were transfected with an HA-tagged Wdnm1-like expression construct or EV, and a portion of the cell lysate or conditioned medium (as described in MATERIALS AND METHODS) was analyzed by Western blot analysis using an anti-HA antibody (Ab). Coomassie blue gel staining (for medium) or membrane reprobed with anti-cyclophilin A antibody (for cell lysate) are shown as loading controls. Arrows in A and C indicate signal for Wdnm1-like protein. Lanes comprising the single panels shown in A or C were generated from the same Western blot exposure; however, some lanes have been removed and/or rearranged for clarity and/or economy of presentation. Minor adjustments to brightness and/or contrast were utilized for better visualization.

Fig. 3. Adipose tissue is a primary site of Wdnm1-like expression in vivo. A: real-time PCR assessment of Wdnm1-like transcript level in a panel of murine tissues. Transcript level in muscle tissue was set to a value of 1. *P < 0.001 for white adipose tissue (WAT) or liver vs. the other tissue samples. Sal. gland, salivary gland. B: expression of Wdnm1-like and selected adipocyte marker transcripts in adipose tissue depots. Brown adipose tissue (BAT) (B), subcutaneous (SC) (S), epididymal (E) and retroperitoneal (R) WAT cDNA were used for real-time PCR analysis for transcript level of Wdnm1-like (left), for resistin (Retn, middle) or stearoyl-CoA desaturase 1 (SCD1) and adipose fatty acid-binding protein (aFABP, right). The transcript level in BAT was set to a value of 1. *P < 0.001 for Wdnm1-like transcript level in S, E, and R compared with the B depot. C: cellular fractionation of WAT. cDNA derived from the cells of the stromal vascular fraction (SVF) or adipocyte fraction (AF) of SC WAT was used for real-time PCR analysis of Wdnm1-like (left). Transcript levels of SCD1 (middle) and collagen 1A1 (Col1A1, right) are shown to validate effective fractionation of WAT. The transcript level in SVF was set as a value of 1 for the left and middle panels and for AF for the right panel. *P < 0.001 for AF vs. SVF. D: real-time PCR analysis of Wdnm1-like transcript in wild-type C57BL/6 mice (WT) and ob/ob mice. The value in the respective WT depot was set to 1. *P < 0.002 for ob/ob vs. wild-type. S, E, and B are as defined in B.
(data not shown). Wdhn1 is often used as a molecular marker in studies of the mammary gland; we do not know at this time the relative expression level in mammary vs. salivary gland for Wdhn1 transcript. The level for transcript for NP001075426 was nearly undetectable in the tissues examined, precluding an accurate assessment of differential expression (data not shown).

Differentiation-dependent upregulation of Wdhn1-like in white adipogenesis. The above data indicates that Wdhn1-like is highly enriched in adipocytes of WAT. We next conducted Northern blot analysis to examine the expression of Wdhn1-like during a daily time course of in vitro adipogenesis of 3T3-L1 preadipocytes to white adipocytes. The 3T3-L1 cell line is a highly characterized and frequently utilized cell culture model of adipogenic conversion, wherein fibroblastic 3T3-L1 preadipocytes convert to mature lipid-laden adipocytes when induced with a 2-day treatment with an adipogenic induction cocktail comprised of FBS, Dex, and MIX. The Northern blot analysis in Fig. 4A shows that the Wdhn1-like transcript is first detected very early in adipogenesis, by 1 day postonset of the differentiation program. Its level increased until day 4 and was sustained through day 6. Upregulation of Wdhn1-like transcript is detected earlier than that for two markers of terminal adipocyte differentiation, SCD1 and aFABP, whose expression is first detected at day 3 and is not apparent before the removal of adipogenic differentiation agents. We also utilized real-time PCR to quantitate Wdhn1-like transcript expression in preadipocytes vs. adipocytes for 3T3-L1 cells and in a second model of in vitro white adipogenesis recently developed in this laboratory, ScAP-23. ScAP-23 is an immortalized preadipocyte cell line derived from preadipocytes present in murine WAT. 3T3-L1 adipocyte conversion is accompanied by an ~17,000-fold increase in Wdhn1-like transcript level and ScAP-23 adipogenesis by an ~6,000-fold increase in Wdhn1-like expression (Fig. 4B). Figure 4C indicates that Wdhn1-like is also upregulated during in vitro differentiation of primary murine cultures of the preadipocyte-containing stromal-vascular cell population of adipose tissue (left). Figure 4C, right, confirms effective adipose conversion of the primary cultures with increased transcript levels for the adipocyte markers aFABP and SCD1.

Given that the Northern blot analysis in Fig. 4A indicated upregulation of Wdhn1-like transcript by day 1 postinduction of differentiation, we conducted a second time-course study focusing on the first 2 days of adipogenesis. Figure 4D illustrates that increased levels of Wdhn1-like transcript occur within 6 h of treatment with Dex and MIX and that this reaches 78-fold by 12 h and ~2,300-fold by 24 h. This dramatic early upregulation is in contrast to the relatively minor changes noted for FABP and SCD1 transcripts. On the other hand, increased levels of transcript of PPARγ2 are found within 6 h of treatment with Dex and MIX.
postinduction, and levels continue to increase through 48 h. That this was not discernable in the Northern blot in Fig. 4A is perhaps due the fact that the Northern analysis detects both the γ1 and γ2 forms of PPAR. It remains to be determined whether the coincident increase in PPARγ2 and Wdnm1-like transcript in early adipogenesis indicates possible PPARγ2-mediated transcriptional regulation of Wdnm1-like.

Regulation of adipocyte Wdnm1-like transcript expression. To investigate signals that might regulate Wdnm1-like transcript level in adipocytes, we first individually tested the effects of the components of the standard adipogenic cocktail on expression of Wdnm1-like transcript. We find that only Dex and MIX in combination result in increased Wdnm1-like transcript levels (Fig. 5A). This is first evident at day 1 and is readily apparent at days 2 and 5. The finding that neither individual component of the adipogenic differentiation cocktail leads to detectable levels of Wdnm1-like transcript further serves to tie expression of this gene to the early events of adipogenesis.

We next treated 3T3-L1 adipocytes with TNF-α, a cytokine that impacts adipocyte transcriptional downregulation of the key adipocyte transcription factors PPARγ (64) and C/EBP-α (31, 59). We find that TNF-α treatment of 3T3-L1 adipocytes increases the level of Wdnm1-like transcript by 2.4-fold (P < 0.001) (Fig. 5B). Whereas neither the PI 3-kinase inhibitor wortmannin nor the mTOR inhibitor rapamycin had an inhib-
itory effect, pretreatment with the p38 MAP kinase inhibitor SB203580 blocked TNF-α-induced upregulation of the Wdnm1-like transcript (Fig. 5B). This is consistent with several recent reports that the effects of TNF-α on cell types such as osteoblasts, monocytes, and human umbilical vascular endothelial cells can be mediated via p38 MAP kinase and with the limited reports on the role of p38 MAP kinase in the effects of TNF-α on adipocyte gene expression (43, 61). The expression pattern of Wdnm1-like in murine tissues, with restriction to WAT and liver, and its upregulation by TNF-α are somewhat reminiscent of that for several acute phase reactants that are secreted products of adipocytes, such as haptoglobin and serum amyloid A3 (23, 55, 60). We also addressed whether LPS, a proinflammatory agent that acts in macrophage activation could regulate levels of Wdnm1-like transcript. Figure 5C shows that a 4-h treatment of RAW264.7 murine macrophages results in a robust upregulation of Wdnm1-like transcript, suggestive of a possible role for Wdnm1-like in the inflammatory response.

Ectopic expression of Wdnm1-like leads to increased levels of active MMP-2. Several proteins of the WAP/4-DSC family, for example elafin and secretory leukocyte proteinase inhibitor (SLPI) (21), have demonstrated roles as proteinase inhibitors (6) with wide ranging impact on physiology. Given that Wdnm1-like possesses only a single WAP/4-DSC type motif, wherein only six of the eight cysteines are present, it appears to be only distantly related to other WAP/4-DSC proteins. We nonetheless hypothesized that perhaps it might have a similar proteinase inhibition function. An important class of extracellular proteases that function in adipocyte differentiation and adipose tissue are the MMPs (7, 8, 13, 15, 17, 20, 36). MMPs play key roles in governing cell-matrix interactions by their ability to degrade ECM components. This, in turn, has multiple cellular consequences, such as for example, the release of growth factors from the ECM (27). We therefore hypothesized that Wdnm1-like might affect MMP activity. To test this, we utilized HT1080 human fibrosarcoma cells, which are commonly used in MMP functional studies because they produce readily detectable levels of the gelatinases MMP-2 and MMP-9. The Wdnm1-like-HA expression construct or an empty vector control plasmid was transiently transfected into HT1080 cells, and media were collected for analysis at 48 h posttransfection. Figure 6A demonstrates effective ectopic expression of Wdnm1-like transcript by real-time PCR (left) and by Western blot analysis of culture media for Wdnm1-like protein with the use of anti-HA antibody (right). Figure 6B, bottom, shows the result of gelatin zymography analysis of duplicate cultures of HT1080 cells transfected with either empty vector control or the Wdnm1-like-HA expression construct. These data indicate that expression of Wdnm1-like leads to increased levels of the intermediate and active forms of MMP-2 and a reduction in the level of pro-MMP-2. No alterations in MMP-9 activities were noted (Fig. 6B, top). We used real-time PCR to assess transcript level for MMP-2 and MT1-MMP (also known as MMP-14); the latter is a key regulator of MMP-2 activation via its action to remove the MMP-2 propeptide. Although a degree of variation is found across the experimental replicates, these data indicate no appreciable alteration in the level of neither MMP-2 nor MT1-MMP/MMP-14 transcript as a result of Wdnm1-like expression (Fig. 6C). This suggests that Wdnm1-like does not cause elevated MMP-2 activity via enhanced expression of the MMP-2 or MT1-MMP/MMP-14 gene but rather by a presently undefined novel mechanism.

DISCUSSION

The MMP systems are key for matrix remodeling that accompanies many developmental and physiological processes, including adipogenesis and obesity (4, 34, 60). Adipose tissue growth occurs by both hyperplasia and hypertrophy, and these events involve remodeling of the cellular and extracellular environment. The robust upregulation of Wdnm1-like in early adipogenesis is consistent with Wdnm1-like having a regulatory role in the process. Our functional studies of Wdnm1-like indicate that one way in which this novel adipokine may exert effects in a paracrine or autocrine manner in adipose tissue/adipocytes is via an impact on MMP-2 activity.

Adipose tissue in vivo and adipocytes from in vivo and in vitro sources produce a number of MMPs (7, 8, 13, 15, 17, 20, 36). Although a comprehensive assessment of expression and the effects of all members of the MMP family has not been done in adipogenesis/adipose tissue, the level of either activity, protein, or transcript for a number of MMPs has been demonstrated to be altered during adipogenesis (7, 8, 13, 15, 17, 20, 36). Although it is generally regarded that the obese state is accompanied by an overall shift to increased matrix degradation (13, 37, 40), knockout of individual MMPs has also been found to promote a diet-induced obesity phenotype (38, 44). For example, MMP-11/stromelysin-null (38) and MMP-19-null (19) mice are prone to increased levels of nutritionally induced obesity with effects on adipogenesis and fat cell hypertrophy, respectively. Studies in vitro with broad spectrum and specific MMP inhibitors as well as blocking antibodies have demonstrated that blocking MMP activities inhibits adipogenesis (7, 8, 13, 14, 17, 40). In regard to the gelatinases MMP-2 and MMP-9, their expression has been reported to increase during adipogenesis, and they have been shown to be important for adipogenic conversion in vitro (7, 8, 13). In vivo studies show that MMP inhibitors diminish adipose tissue accumulation (20, 36). However, no obvious alterations in adipocyte cellularity were observed for mice null for MMP-2, MMP-3, or MMP-9, suggesting that in vitro observations may not hold in the in vivo setting. However, to our knowledge, the response of such animals to dietary obesity or other manipulation was not examined (15). In regard to the function we describe for Wdnm1-like in the activation of MMP-2, MT1-MMP/MMP-14 has a major role in the activation of MMP-2 and has been demonstrated to be key to adipose tissue development in vivo. Mice null for this membrane-anchored collagenase were observed to have poorly developed adipose tissue (15). Thus Wdnm1-like, with its early upregulation in adipogenesis, may be important for initial ECM remodeling events that would favor adipogenesis; given its sustained expression in adipocytes, it may also be important in adipocyte-ECM interactions.

The global transcriptional response of 3T3-L1 adipocytes and adipose tissue to TNF-α treatment was studied by Lodish and coworkers (49, 50) who reported suppression of many adipocyte genes and the activation of expression of many preadipocyte genes. Given this, one might make the assumption that TNF-α would likewise decrease expression of
Wdnm1-like transcript in 3T3-L1 adipocytes; in contrast, we observed upregulation. A common theme that emerged from the Lodish report was the central role of TNF-α-mediated gene regulation in the promotion of insulin resistance via downregulation of adipocyte genes that are key for the storage or uptake of free fatty acids or glucose (49, 50). Many of these encode proteins that function in the promotion or attenuation of lipolysis and lipogenesis, respectively (49, 50). Thus, one would not necessarily expect a diminution of adipocyte Wdnm1-like transcript expression upon TNF-α treatment because there is no evidence that Wdnm1-like has a role in insulin responsiveness or energy metabolism. Moreover, although a large percentage of adipocyte and preadipocyte genes are coordinately decreased or increased, respectively, upon TNF-α treatment of adipocytes, this is clearly not a hard and fast rule. Expression of transcript for the preadipocyte marker gene Pref-1 is not upregulated in TNF-α-treated 3T3-L1 adipocytes (64), and transcript for adipocyte-expressed haptoglobin increases upon TNF-α treatment of 3T3-L1 adipocytes (49). We have yet to define the transcriptional underpinnings of the TNF-α upregulation of Wdnm1-like. However, in their study, Lodish and coworkers (49) report that NF-kB activation is obligatory for the suppression of adipocyte-specific and for the activation of preadipocyte genes by TNF-α (49). It is possible that TNF-α upregulation of Wdnm1-like in adipocytes does not fall under the domain of this transcription factor. The transcriptional decrease of adipocyte genes by TNF-α has also been ascribed to its ability to decrease transcript levels for the key adipogenic transcription factor PPAR-γ (64). Although our studies have not directly addressed whether Wdnm1-like transcription is controlled by PPAR-γ, we report herein that upregulation of Wdnm1-like transcript is a very early event in adipogenesis. As such, it is possible that PPAR-γ may not be a key player in the transcriptional signals underlying adipocyte-specific expression of Wdnm1-like. Of interest, in regard to the function we report herein for Wdnm1-like, it is the observation by Lodish and coworkers (49) that a subset of the TNF-α-mediated gene expression during involution of mammary gland (Review). Int J Mol Med 2: 39–44, 1998.


ACKNOWLEDGMENTS

We thank Dr. Ji Young Kim for generously providing RNA samples.

GRANTS

This work was supported by NIDDK/NIH grant 5R21DK064992 to C. M. Smas.


