Fat-specific protein 27 modulates nuclear factor of activated T cells 5 and the cellular response to stress

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Abstract Fat-specific protein 27 (FSP27), a member of the cell death-inducing DNA fragmentation factor α-like effector (Cide) family, is highly expressed in adipose tissues and is a lipid droplet (LD)-associated protein that induces the accumulation of LDs. Using a yeast two-hybrid system to examine potential interactions of FSP27 with other proteins, a direct interaction with the N-terminal region of nuclear factor of activated T cells 5 (NFAT5) was identified. NFAT5 is a transcription factor that induces osmoprotective and inflammatory genes after its translocation to the nucleus. The interaction between FSP27 and NFAT5 was confirmed by bimolecular fluorescence complementation and communoprecipitation. Using immunocytochemistry, NFAT5 is detected in the cytoplasm and in the nucleus under isotonic conditions; however, overexpression of FSP27 inhibited the hypertonic-induced nuclear translocation of NFAT5. Consistent with the suppression of NFAT5 nuclear translocation, in cells transfected with a reporter construct containing the NFAT5 response element from the monocyte chemotactic protein 1 (MCP1) promoter, FSP27 overexpression repressed hypertonic-induced luciferase activity and the expression of NFAT5 target genes. Knockdown of FSP27 in differentiated 3T3-L1 adipocytes increased the NFAT5-mediated rise in MCP1. These results suggest that FSP27 not only modulates LD homeostasis but also modulates the response to osmotic stress via a physical interaction with NFAT5 at the LD surface.—Ueno, M., W.-J. Shen, S. Patel, A. S. Greenberg, S. Azhar, and F. B. Kraemer. Fat-specific protein 27 modulates nuclear factor of activated T cells 5 and the cellular response to stress. J. Lipid Res. 2013. 54: 734–743.

Supplementary key words adipose • inflammation • lipid droplet • nuclear translocation • protein-protein interaction

Lipid droplets (LDs) are organelles found in many types of eukaryotic cells and are composed of a core of neutral lipids, such as triacylglycerol (TAG), covered by a monolayer of phospholipids, free cholesterol, and specific proteins (1). Proteomic studies have identified LD-associated proteins involved in lipid metabolism and transport, intracellular trafficking, signaling, and cytoskeletal organization (1). The major LD-associated proteins belong to the perilipin-adipophilin-TIP47 (PAT) family and are characterized by sequence similarity (2).

Fat-specific protein 27 (FSP27), also known as Cidec for the human homolog, was discovered to be up-regulated during adipogenesis and is highly expressed in white adipose tissue (WAT) and brown adipose tissue (BAT) in mice (3–6) but only in WAT in humans (7). The LD localization of FSP27 and perilipin A (Plin1) in 3T3-L1 adipocytes is similar, and they share some conserved regions at the amino acid level (8), but there is no similarity within the PAT domains, which are regions highly conserved in PAT family proteins and which are not present in FSP27. Overexpression of FSP27 induces the accumulation of LDs and enhances LD size not only in adipocytes but also in many nonlipogenic cells (9, 10). Conversely, knockdown of FSP27 decreases LD size and increases LD number in 3T3-L1 adipocytes (6, 11). Thus, FSP27, like PAT proteins, appears to have an important role in the regulation of LDs but does not appear to function as effectively as Plin1 as a barrier to LD breakdown by cytosolic lipases. Two groups independently reported the phenotypes of FSP27-deficient

Abbreviations: BAT, brown adipose tissue; BiFC, bimolecular fluorescence complementation; Cide, cell death-inducing DNA fragmentation factor α-like effector; FSP27, fat-specific protein 27; HDAC, histone deacetylase 4; HSL, hormone-sensitive lipase; LD, lipid droplet; MCP1, monocyte chemoattractant factor 1; NF-κB, nuclear factor κB; ORE, osmotic response element; PAT, perilipin-adipophilin-TIP47; Plin1, perilipin A; TAG, triacylglycerol; TonEBP, tonicity element binding protein; WAT, white adipose tissue.

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mice (5, 6), which display lean phenotypes, accompanied by smaller LDs in WAT and larger LDs in BAT, resulting in enhancement of insulin sensitivity and resistance to diet-induced obesity. However, a truncated mutation of Cidec was found in an individual with partial lipodystrophy and insulin-resistant diabetes (12), perhaps suggesting a different response in mice and humans.

FSP27 can form a complex with its family member Cidec (9), but the interaction of FSP27 with other cellular proteins has not been reported. In the current study, we sought to identify proteins that interact with FSP27 using a yeast two-hybrid system. Our results revealed that FSP27 directly interacts with the N-terminal region of tonicity response element binding protein (TonEBP, also known as nuclear factor of activated T cells 5 [NFAT5]). The interaction between FSP27 and NFAT5 was confirmed by bimolecular fluorescence complementation (BiFC) and coimmunoprecipitation. Moreover, we showed that FSP27 inhibits the normal translocation of NFAT5 from the cytoplasm to the nucleus under hypertonic conditions and attenuates its transcriptional activity. Thus, FSP27 appears not only to have an important role in LD metabolism but also to modulate cellular signaling in response to stress.

**MATERIALS AND METHODS**

**Yeast two-hybrid system**

Yeast two-hybrid screening was performed as previously described (13). Full-length rat FSP27 cDNA was cloned into the SalI/NcoI site of the pAS1-CYH2 yeast two-hybrid vector (14) to yield an FSP27-GAL4 DNA-binding domain vector that was used as bait for the screening of a rat adipocyte library.

**Construction of plasmids**

The vectors pBiFC-VN173 and pBiFC-VC155 were kindly provided by Dr. Chang-Deng Hu from Purdue University (15). Full-length mouse FSP27 cDNA was cloned into the EcoRI/Xhol site of the pBiFC-VC155. Rat NFAT5 (residues 66–274) was cloned into the pBiFC-VN173. The reporter constructs containing the putative NFAT5 element enhancer reporter gene pGL3-Wt for the luciferase assay was kindly provided by Dr. Ryoji.

**Cell culture**

HEK293 cells were plated onto 48-well plates at 2 × 10^4 cells/well and transfected with expression plasmids. Transfections were performed with Lipofectamine™ 2000 (Invitrogen) following the manufacturer’s protocol.

**RT-PCR analysis**

Cells were homogenized in TRIzol® Reagent (Invitrogen), and total RNA was extracted and purified following the manufacturer’s protocol. Total RNA was reverse-transcribed by SuperScript® II Reverse Transcriptase (Invitrogen) with random primers. Real-time PCR was performed in triplicate with an R2Real-Time™ SYBER Green/Rox PCR master mix (Qiagen, Hilden, Germany) and specific primer pairs (Table 1). The relative mass of specific RNA was calculated by the comparative cycle of threshold detection method according to the manufacturer’s instructions. Acid ribosomal phosphoprotein P0 (36B4) was used as an internal control.

**Immunoprecipitation**

Cells were homogenized in TNE buffer (10 mM Tris-HCl [pH 7.4], 8% sucrose, 1 mM EDTA, and 0.1 mM with 10 µg/ml leupeptin. For nuclear extracts, cells were lysed in NE-PER extraction reagent (Thermo Scientific, Rockford, IL) according to the manufacturer’s protocol. Protein concentrations were determined by Bradford protein assays. Approximately 20 µg proteins were resolved by 4–20% gradient SDS-PAGE (Lanza, Walkersville, MD) and blotted onto nitrocellulose membranes (Whatman, Dassel, Germany). Membranes were incubated with primary Ab at the following dilutions: anti-HA (1:5,000, 631207; Clontech Laboratories, Mountain View, CA), anti-Flag (1:5,000, F3165; Sigma-Aldrich Corp., St. Louis, MO), anti-NFAT5 (12,000, ab3446; Abcam, San Francisco, CA), anti-β-actin (1:10,000, sc-47778; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-histone deacetylase 4 (HDAC) (1:500, sc-5246; Santa Cruz Biotechnology, Inc.) at 4°C overnight and then with the appropriate secondary Ab conjugated to fluorescent dye (goat anti-mouse IgG-IR dye 800cw, goat anti-rabbit IgG-IR dye 680cw, or donkey anti-goat IgG-IR dye 800cw; Li-Cor Biosciences, Lincoln, NE) at room temperature for 1 h and were then detected on an Odyssey Infrared Fluorescent Imaging System.
RESULTS

Identification of NFAT5 as a partner of FSP27

To identify protein(s) that interact with FSP27, we used the yeast two-hybrid system to screen a rat adipocyte expression library for proteins that would interact with full-length rat FSP27 used as bait. Ten positive clones were identified out of the $5 \times 10^6$ colonies screened, and sequencing results revealed that one of the clones contained an in-frame fragment of TonEBP/NFAT5 (amino acid residues 157–269). This domain includes the nuclear localization signaling motif, which is highly conserved among rat, mouse, and human (>99% at the amino acid level) (Fig. 1).

NFAT5 is a widely expressed transcription factor that is a major inducer of osmoprotective gene products in mammalian cells (19, 20). To identify tissues where FSP27 and NFAT5 are coexpressed and could potentially interact, we examined their relative mRNA expression in various tissues. FSP27 was detected in all WAT and BAT (Fig. 2A) and in heart and muscle in the mouse. NFAT5 mRNA was expressed in all tissues we tested, including WAT and BAT, with relatively similar levels of expression (Fig. 2B). The expression of FSP27 (Fig. 2C) and NFAT5 (Fig. 2D) increased during differentiation in 3T3-L1 adipocytes, but FSP27 was much more highly expressed after differentiation.

To confirm the interaction of FSP27 with NFAT5, we used a BiFC assay to visualize the complexes in living cells. We constructed plasmids that express the genes of interest (NFAT5 66-274 and full-length FSP27) as fusion proteins and visualized using an Odyssey® Imaging System.

BiFC analysis by fluorescence microscopy

HEK293 cells were cotransfected with a combination of pBiFC-VN173, pBiFC-NFAT5-VN, pBiFC-VC155, and pBiFC-FSP27-VC. Cells were incubated with 120 µM oleate and palmitate or 1% BSA as a control for 24 h and observed by fluorescence microscopy (Carl Zeiss AG, Germany).

Immunofluorescence staining

HEK293 cells were transfected with pBiFC-FSP27-VC or pBiFC-VC155 as a control. Cells were incubated with 120 µM oleate and palmitate or 1% BSA as a control for 24 h, and osmotic stress was induced by 100 mM NaCl for 12 h. Cells were fixed in 4% paraformaldehyde in PBS (pH 7.4) at 4°C for 1 h, washed with PBS with 0.2% Triton-X100 at 25°C for 30 min, and blocked with 3% BSA in PBS. Anti-NFAT5 Ab and anti-HA Ab (1:500, 2367S; Cell Signaling, Danvers, MA) were added to the cells at 4°C overnight and visualized by incubation with Alexa Fluor 488 or 568-conjugated secondary Ab (1:500, A11034 and A11031; Invitrogen).

Luciferase assay

HEK293 cells were cotransfected with expression plasmids, firefly luciferase reporter construct, and pRL (Renilla luciferase)-SV40 reporter vector (Promega Corp., Madison, WI) as a control of transfection. Cells were incubated with 120 µM oleate and palmitate or 1% BSA as a control for 24 h, and osmotic stress was induced by 100 mM NaCl for 12 h. Cells were then analyzed by the Dual-Luciferase® Reporter Assay (Promega) following the manufacturer’s protocol.

Knockdown of FSP27

A recombinant AAV2 expressing shRNA targeting FSP27 was constructed by the Neuroscience Gene Vector and Virus Core, Stanford Institute of Neuro-innovation and Translational Neuroscience, Stanford University. The nucleotide sequences for the shRNA against FSP27 and a scrambled control were as follows: FSP27, AAAAGGAAGGTTCGCAAAGGCATCATTCGTGATGCC, scrambled reverse, AAAAGCGCGCTTTGTAGGATTCGTTCGCGAATCCTACAAAGCGCGC (3). For in vitro infection of AAV2-shRNA, fully differentiated 3T3-L1 adipocytes were incubated with 1.0 × 10^11 AAV2-shRNA for 24 h, and cells were harvested 48 h later.

Apoptosis

For evaluation of apoptosis, HEK293 cells in 48-well plates were transfected with pBiFC-VC155 or pBiFC-FSP27-VC. Cells were incubated with 120 µM oleate and palmitate or 1% BSA as a control for 24 h to promote lipid accumulation, and osmotic stress was induced by 100 mM NaCl or 200 mM D-glucose for 12 h. After that, apoptotic cells were stained using an annexin-V-FLUOS staining kit (#81 858 777 001; Roche, Mannheim, Germany) following the manufacturer’s protocol.

TABLE 1. Primer sequences

| Primer   | Forward                  | Reverse                  |
|----------|--------------------------|--------------------------|
| FSP27 (mouse) | TACTCGCTTTCTTCTAGGCCTGG    | AACTGTCGCACTAGCTGGGTTG    |
| NFAT5 (human/mouse) | AGTCAGACAAGCGGCTGGTTG    | GGTGGTAAGAAGACTGCAAG    |
| MCP1 (mouse) | GTCCCTGCTGCTGCTGAGT    | GCTCTCCAGCTACTCATTG    |
| MCP1 (human) | CTTCCAGCTGAAAGGTTTC    | TCTGCAACTGATCTTCCATTG    |
| 36B4 (mouse) | GTCCAGGCAAGGAGA    | CCGGAGTGAGCCAGCAAG    |
| 36B4 (human) | TGCTTGTGACGGACAAAAGCAGG    | GCTTTGGAACCAAAAGGCAACAT    |

San Diego, CA) and visualized using an Odyssey® Imaging System.

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FIG. 1. Schematic representation of mammalian NFAT5 proteins. A: NFAT5 proteins share an N-terminal region that includes a nuclear localization signaling (NLS) motif, a DNA binding domain (DBD), and a C-terminal region including a transactivation domain. The NLS motif (RRK419-212 and RRK420-212) is highly conserved and plays an important role in translocation.
with the N-terminal (VN173) or C-terminal (VC155) of Venus for the BiFC assay. If FSP27 and NFAT5 interact with each other, the interaction should bring VN173 and VC155 into close proximity and reconstitute an intact Venus molecule, which can be detected by direct visualization with fluorescence imaging (21). To confirm the interaction between the fusion proteins, we transiently expressed the proteins in HEK293 cells that had been loaded with fatty acids (FAs) to promote LD formation. Because HSL and Plin1 have previously been reported to interact (22), we used HSL-VN and Plin-VC as a positive control for the BiFC assay. Consistent with a physical interaction between HSL and Plin1, the BiFC assay showed a fluorescent signal that appeared localized on the LDs (Fig. 3A, B). In contrast, HSL-VN and FSP27-VC did not demonstrate any fluorescent signal (Fig. 2C, D). Coexpression of NFAT5-VN and FSP27-VC yielded a fluorescent signal that appeared to surround the LDs that had been stained with bodipy (Fig. 3E–H). Thus, FSP27 and NFAT5 appear to physically and specifically interact by genetic screening and BiFC. As an additional means of documenting the interaction between FSP27 and NFAT5, Flag-NFAT5 and HA-FSP27 were expressed in HEK293 cells, immunoprecipitated with anti-HA antibody, and immunoblotted with anti-Flag antibody. Fig. 4A documents the expression of the Flag-tagged constructs. NFAT5 coimmunoprecipitated with FSP27 (Fig. 4B), but another cytosolic protein, Flag-HSL, expressed as a control, did not coimmunoprecipitate with FSP27; however, Flag-HSL was coimmunoprecipitated when HA-Plin1 was coexpressed, consistent with our previous observations (22). To document the interaction of endogenously expressed FSP27 and NFAT5, proteins extracted from the fat cake obtained from isolated normal mouse adipose cells were immunoprecipitated with anti-NFAT5 antibody and then immunoblotted with anti-FSP27 antibody (Fig. 4C). FSP27 was highly expressed in the fat cake (lane 1) and coimmunoprecipitated with NFAT5 (lanes 3 and 5) but not when nonimmune IgG (lanes 2 and 4) was used for the immunoprecipitation, thus substantiating the physical
in HEK293 cells in the presence or absence of overexpressed FSP27. HEK293 cells were transiently transfected with FSP27-VC or VC155 as a control for transfection, incubated with fatty acids (FAs) to induce LDs, and then cultured in hypertonic medium with an additional 100 mM NaCl. Under basal conditions, VC155 is ubiquitously expressed throughout the cell (Fig. 5B), and endogenous NFAT5 is observed throughout the cytoplasm and nucleus (Fig. 5D), with the merged image of DAPI (Fig. 5C) and NFAT5 shown in Fig. 5E. After hypertonic stress, VC155 continues to be found throughout the cytoplasm and nucleus (Fig. 5G), whereas NFAT5 is now localized almost exclusively to the nucleus (Fig. 5I), with the merged image shown in Fig. 5J. Under basal conditions, FSP27-VC is found throughout the cytoplasm (Fig. 5L), and endogenous NFAT5 is again observed throughout the cytoplasm and nucleus (Fig. 5N, O). However, after hypertonic stress, in cells that express FSP27-VC (Fig. 5Q), NFAT5 is not exclusively nuclear but remains localized to the cytoplasm and nucleus (Fig. 5S, T). As another means to evaluate the effects on the nuclear trafficking of NFAT5, HEK293 cells were transiently transfected with FSP27-VC or VC155 as a control for transfection, incubated with FAs to induce LDs, after cultured in isotonic (Control) or hypertonic medium (100 mM NaCl added), and then the cells were lysed, nuclei were isolated, and nuclear proteins were immunoblotted for NFAT5 (Fig. 6). The expression of total cellular NFAT5 was induced by osmotic stress, and this stimulation was not affected by overexpression of FSP27 (Fig. 6A). Cell fractionation experiments confirmed the immunocytochemistry findings, demonstrating that the amount of NFAT5 found in the nuclear fraction was increased markedly after osmotic stress, but the amount of nuclear NFAT5 was decreased 60% by overexpression of FSP27 under both basal and osmotic stress conditions (Fig. 6B). Thus, heterologous expression of FSP27 decreases the nuclear trafficking of NFAT5 normally observed after hypertonic stress.

FSP27 modulates the transcriptional activity of NFAT5

Because the expression of FSP27 attenuates the translocation of NFAT5 to the nucleus, we tested whether the transcriptional activity of NFAT5 was similarly inhibited in the presence of FSP27. Kojima et al. (16) identified the cis-acting regulatory enhancer elements within the monocyte chemoattractant protein 1 (MCP1) gene that contains the putative NFAT5 binding sequence and that is responsive to hypertonic stress. We used a reporter gene construct containing the putative NFAT5 element from the MCP1 promoter with the luciferase reporter gene and the basic plasmid as a control for promoter analysis. HEK293 cells were transiently transfected with VC155 or FSP27-VC along with reporter plasmids, incubated with or without FAs, and cultured in hypertonic medium with an additional 100 mM NaCl. Promoter activity was increased 2.4-fold by NaCl, 1.6-fold by FA loading, and 2.5-fold by FA loading along with NaCl (Fig. 7A). Thus, although FA loading increased reporter activity, NaCl-induced hypertonic stress was most potent. We also incubated cells with D-glucose to induce osmotic stress, which resulted in a
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conditions had no effect on apoptosis when VC155 was overexpressed as a control, but overexpression of FSP27 tended to increase apoptosis, and this was slightly augmented under hypertonic conditions such that differences compared with overexpression of VC155 were now statistically significant in the presence of hypertonic NaCl or glucose. FSP27 controls MCP1 expression in 3T3-L1 adipocytes

Because the prior studies were conducted in a cell where FSP27 is not normally expressed, we next tested the functional interaction of FSP27 and NFAT5 in differentiated 3T3-L1 adipocytes under hypertonic conditions (Fig. 9A). FSP27 mRNA was decreased 50% by NaCl (100 mM added) and 60% by D-glucose (200 mM) incubation. NFAT5 mRNA was increased 3.1-fold by NaCl and 3.5-fold by 1.8-fold increase in reporter activity. Importantly, coexpression of FSP27 significantly decreased luciferase activity under each condition \((p<0.05)\) (Fig. 7A). Furthermore, RT-PCR showed that FSP27 overexpression inhibited endogenous MCP1, PAI-1, TNF-\(\alpha\), and IL-6 expression in HEK293 cells under hypertonic conditions (Fig. 7B). Thus, heterologous expression of FSP27 not only inhibits the nuclear trafficking of NFAT5 normally observed after hypertonic stress but also inhibits the transcriptional activity of NFAT5. Because FSP27 and its other cell death-inducing DNA fragmentation factor \(\alpha\)-like effector (Cide) family members are known to induce apoptosis (24), particularly in cells lacking LDs (25), we assessed the effects of overexpression of FSP27 on apoptosis in HEK293 cells under isotonic and hypertonic conditions (Fig. 8). Hypertonic conditions had no effect on apoptosis when VC155 was overexpressed as a control, but overexpression of FSP27 tended to increase apoptosis, and this was slightly augmented under hypertonic conditions such that differences compared with overexpression of VC155 were now statistically significant in the presence of hypertonic NaCl or glucose.

**FSP27 controls MCP1 expression in 3T3-L1 adipocytes**

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**Fig. 4.** Interaction of FSP27 and NFAT5 detected by immunoprecipitation. A and B: HEK293 cells were transfected with Flag-NFAT5-VN173 and HA-FSP27-VC155 (lane 1), Flag-HSL-VN173 and HA-FSP27-VC155 (lane 2), or Flag-HSL-VN173 and HA-Plin1-VC155 (lane 3) and loaded with lipid. A: Total cell extracts were immunoblotted with anti-Flag Ab. B: Cell extracts were immunoprecipitated with anti-HA Ab and then immunoblotted with anti-Flag Ab. NFAT5 coimmunoprecipitates with FSP27 (lane 1). HSL fails to coimmunoprecipitate with FSP27 (lane 2) but coimmunoprecipitates with Plin1 (lane 3). Results are representative of five independent experiments. C: Total proteins were extracted from the fat cake obtained from mouse adipose tissue and immunoblotted with anti-FSP27 Ab. Lane 1, protein extract; lanes 2 and 4, proteins immunoprecipitated with nonimmune Ab and then immunoblotted; lanes 3 and 5, proteins were immunoprecipitated with anti-NFAT5 Ab and then immunoblotted. Results are representative of two independent experiments, and each lane represents an independent immunoprecipitation.

**Fig. 5.** FSP27 overexpression inhibits nuclear translocation of NFAT5. HEK293 cells were transfected with HA-VC155 (A-J) or with HA-FSP27-VC155 (K-T) and incubated in isotonic (Control, A-E, K-O) or hypertonic (additional NaCl 100 mM, F-J, P-T) conditions for 6 h. Cells were then visualized by light (A, F, K, P) or fluorescent microscopy (B-E, G-J, L-O, Q-T), immunostained with anti-HA Ab to visualize expression of the transfected proteins (B, G, L, Q), stained with DAPI to visualize nuclei (C, H, M, R), and immunostained with anti-NFAT5 Ab to localize endogenous NFAT5. Merged images are displayed in E, J, O, T. Results are representative of 5 independent experiments.
DISCUSSION

It is widely accepted that obesity and type 2 diabetes are characterized by evidence of a chronic, low-grade inflammatory process (26, 27), as manifested by elevated circulating levels of acute phase proteins (e.g., C-reactive protein) and cytokines (e.g., TNF-α, IL-1, and IL-6). In addition, adipose tissue in these settings has increased expression of chemokines, such as CC-chemokine ligand 2 (also known as MCP1), that can recruit macrophages. Inflammation in obesity is associated with an increased infiltration of macrophages, mast cells, and T cells into adipose tissue, with an associated increase in inflammatory mediators. Several mechanisms have been proposed to explain the inciting insult leading to inflammation, such as tissue hypoxia, cell death due to an enlargement of an adipose cell that exceeds the boundaries of its cell membrane, and cell stress due to lipotoxicity, ER stress, oxidative stress, or glucotoxicity. Each of these mechanisms for cell stress has been associated with the activation of several signaling pathways, such as inhibitor of κB kinase, which activates nuclear factor κB (NF-κB) and controls the transcription of a wide variety of inflammatory genes, and JNK, which controls the activation of several transcription factors. In addition, several other signaling pathways are activated by cell stress in diabetes and obesity (28), including PKR, ERK, PKC, and p38 MAPK.

NFAT5, also known as TonEBP and as osmotic response element (ORE) binding protein, is a member of the Rel family of transcription factors, which also includes NF-κB.
and NFAT1-4 (19, 20). In contrast to NFAT1-4, NFAT5 does not contain a calcineurin-sensitive domain and is not responsive to calcineurin inhibitors; however, it shares sequence homology with all other Rel family members in its DNA binding domain. All Rel family members, including NFAT5, function as homo- and heterodimers. NFAT5 is the largest Rel protein, containing almost 1,500 amino acids, and is the major transcription factor activated in response to osmotic stress, where it regulates an osmoprotective gene expression program via binding to OREs, which increases enzymes and transporters that elevate intracellular organic osmolytes and heat shock proteins. NFAT5 is ubiquitously expressed and functions not only in intracellular organic osmolytes and heat shock proteins, which increases enzymes and transporters that elevate intracellular organic osmolytes and heat shock proteins. NFAT5 is ubiquitously expressed and functions not only in the renal medulla but also in other tissues, where it regulates a variety of other genes not directly involved in osmoregulation, including inflammatory genes such as MCP1 (16) and TNF-α, among others (29). Moreover, NFAT5 has been recently shown to interact with NF-κB, thereby enhancing the transcriptional activity of NF-κB (30). NFAT5 is localized to the cytoplasm under hypotonic conditions and translocates to the nucleus after hypertonic stress. This nucleocytoplasmic trafficking is mediated via a nuclear localization signal regulated by a number of different kinases, including p38, Fyn, ATM, PKA, and CDK5 (19, 31), and via a nuclear export sequence (32).

LDs are intracellular organelles that store neutral lipids within cells, where the LD serves as a reservoir for energy stores and to protect the cell from lipotoxic effects of FAs via their incorporation into TAG within the LD (1). Formation of LDs in response to lipotoxic effects of FAs and TNF-α, observed after osmotic stress, is associated with ER stress (33), linking LDs with inflammation. Proteomic analyses of LDs from a variety of cells have revealed the existence of many LD-associated proteins. The most abundant of these proteins belong to the PAT family, with Plin1 being the predominant member in adipocytes. In addition to PAT proteins, the Cide (cell death-inducing DNA fragmentation factor α-like effector) family of proteins associates with and regulates LD physiology in addition to their role in apoptosis (25). The Cide family consists of three proteins, Cidea, Cideb, and Cidec (also called FSP27), and shares regions of homology with Plin that are outside the conserved PAT region. Cide proteins are predominantly expressed in adipose tissue and liver in mice, with Cidea primarily in BAT and FSP27 in WAT and Cideb in liver. Ectopic expression of Cide proteins promotes LD formation and reduces TAG hydrolysis. Moreover, knockdown of FSP27 decreases LD size and increases LD number in adipocytes (5, 6, 11), suggesting that FSP27 is involved in promoting the formation and maintenance of a unilocular LD within adipocytes, apparently by mediating the clustering and fusion of LDs and promoting lipid exchange (34, 35).

In the current work we identified a direct interaction of FSP27 with NFAT5 using a yeast two-hybrid screen. The interaction between FSP27 and NFAT5 was confirmed by BiFC and by communoprecipitation and appeared to occur on LDs where FSP27 is normally localized. We could not detect NFAT5 localization to LDs in the absence of FSP27 expression (data not shown). The interaction of FSP27 with NFAT5 seems to result in the sequestration of NFAT5 at the LD surface, thus attenuating the nuclear trafficking of NFAT5 normally observed after activation of signaling pathways induced by osmotic stress.

Consistent with the attenuation of nuclear trafficking of NFAT5, overexpression of FSP27 suppressed the transcriptional activity of a luciferase reporter construct containing the putative ORE from the MCP1 promoter and attenuated the increase in MCP1 mRNA expression as well as the expression of mRNA of other inflammatory genes, such as TNF-α, PAI-1, and IL-6, observed after osmotic stress induced by NaCl or glucose in HEK293 cells. FA loading of HEK293 cells also increased the activity of the ORE promoter reporter construct and MCP1 expression under isosmotic conditions. Compatible with the FA induction of MCP1 being mediated via NFAT5, this, too, was attenuated by overexpression of FSP27. This suggests that one of the mechanisms through which elevated FA levels and higher rates of lipolysis induce inflammation and the
recruitment of macrophages into adipose tissue (36, 37) might be mediated via NFAT5. Nonetheless, osmotic stress resulted in a maximal response of MCP1 transcription in the current studies that was not influenced by FA loading. Not surprisingly, the overexpression of FSP27 was associated with an increase in apoptosis, though this is minimized in the presence of LD induction by fatty acid loading (25); however, it seems unlikely that the small changes observed in apoptosis substantially contributed to the ability of FSP27 to inhibit the nuclear trafficking and repress the transcriptional activity of NFAT5 because overexpression of FSP27 had no effect on the induction of NFAT5 by osmotic stress. Moreover, our observations were not confined to HEK293 cells because expression of differentiated 3T3-L1 adipocytes to osmotic stress (either NaCl or glucose) resulted in an increased expression of NFAT5 and MCP1 mRNA. In these adipocytes where FSP27 is endogenously suppressed, whereas NFAT5 and MCP1 were endogenously expressed, osmotic stress resulted in the simultaneous suppression of FSP27, whereas NFAT5 and MCP1 were increased. This suggests that the decrease of FSP27 that occurs as a part of the response to osmotic stress removes the restraint on the movement of NFAT5 to the nucleus and magnifies the transcriptional function of NFAT5. The results of the knockdown of FSP27 in differentiated 3T3-L1 adipocytes are consistent with this interpretation because the removal of FSP27 resulted in an increase in MCP1 even in the absence of osmotic stress. In addition to removing the restraint of the movement of NFAT5 to the nucleus, the osmotic stress-induced suppression of FSP27 would be expected to increase lipolysis by alleviating the barrier function of FSP27 toward intracellular lipases.

Therefore, these experiments have documented the modulation of the inflammatory response to stress by an LD-associated protein (FSP27) and that this occurs through the interruption of the normal nuclear trafficking and, thus, activity of a transcription factor (NFAT5), which is important in the cellular response to stress. Interestingly, similar to the current observations with FSP27 and NFAT5, lipin, a protein important in TAG synthesis and LD formation and which is known to interact with several nuclear proteins (38), has been reported to interact with NFATc4 and to repress inflammatory genes (39).

Hyperglycemia results in significant osmotic stress, and postprandial hyperglycemia occurs in many patients even with apparently normal fasting glucose values (40). In addition, the DNA binding activity of NFAT5 is increased in diabetics with microvascular complications (41). Moreover, aldose reductase, a key enzyme in the polyol pathway that has been mechanistically linked with hyperglycemia-induced complications (42), is a target gene of NFAT5 (43). In addition to TNF-α and MCP1, which are direct NFAT5 target genes (16, 44), IL-1, IL-6, and IL-18 contain putative NFAT5 consensus sites in their promoter regions and can be regulated by NFAT5 under hypertonic conditions (20). Thus, the results of the current experiments provide a potential mechanistic link in dissecting the regulation of inflammation in obesity and diabetes, where reduced expression of LD-associated proteins Cidea, Cidec/FSP27, and Plin1 is associated with insulin resistance (8).

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