Identification of regulatory elements in the human adipose most abundant gene transcript-1 (*apM-1*) promoter: role of SP1/SP3 and TNF- α as regulatory pathways

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Abstract

Aims/hypothesis. The human adipocyte-specific *apM-1* (adipose most abundant gene transcript-1) gene encodes for a secretory protein of the adipose tissue that seems to play a role in the pathogenesis of obesity-related insulin resistance and its expression is inhibited by TNF- α . Our aim was to characterize the tissue-specific regulation of the recently cloned *apM-1* promoter and the mechanisms of TNF- α -induced down-regulation of the *apM-1* gene.

Methods. We characterised the *apM-1* gene by electrophoretic mobility shift assays (EMSA) and luciferase reporter gene assays (LRA).

Results. Although several putative binding sites for transcription factors known to be involved in adipogenesis such as C/EBP and PPAR γ are present in the promoter, we could not detect any binding of these nuclear proteins from differentiated adipocytes. However, a proximal SP1 binding site specifically binds both, recombinant SP1 protein and SP1 derived from adipocyte nuclear extracts. Since the expression of SP1 during adipocyte differentiation has not yet been

analysed, we could show by using EMSA, that binding activity of SP1 is increased during adipocyte differentiation. The stimulatory activity of SP1 was confirmed in LRA by cotransfection experiments in S2 Schneider cells lacking endogenous SP factors. An inhibitory activity of SP3 on the stimulatory effect of SP1 could be confirmed in LRA by contransfection experiments in adipocytes. Nuclear extracts from adipocytes incubated with TNF- α showed a reduced binding activity of SP1.

Conclusion/interpretation. SP1 is expressed and its binding activity is enhanced during adipocyte differentiation. SP1 has stimulatory effects, SP3 has inhibitory effects on *apM-1* promoter activity, mediated by a proximal SP1 binding site. The mechanism of TNF- α -induced inhibition of *apM-1* gene expression is, at least in part, due to a decrease of transcriptional SP1 binding activity caused by TNF- α and thus provides a new mechanism of TNF- α -dependent signalling. [Diabetologia (2002) 45:1425–1433]

Keywords *apM-1*, adipocyte, promoter, SP1, SP3, TNF- α , transcription, differentiation, diabetes, obesity.

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Abbreviations: *apM-1*, Adipose most abundant gene transcript-1; SP1/3, stimulatory protein 1/3; PPAR, peroxisome proliferator activated receptor; C/EBP, CCAAT-enhancer binding protein; LRA, luciferase reporter gene assay; EMSA, electrophoretic mobility shift assay.

The adipose tissue has been considered as a special type of connective tissue, that stores energy in the form of triglycerides and releases energy in the form of non-esterified fatty acids whenever needed. However, increasing evidence suggests that the adipose tissue produces and secretes several highly active molecules. During the last 5 years it has become widely accepted that adipocytes do function as endocrine cells by secreting a variety of highly active molecules. These so-called adipocytokines [1] are secreted from the adipose tissue into the blood stream.

In the murine system, two studies [2, 3] independently discovered an adipocyte-specific mRNA, which is induced during late adipocyte differentiation. The cDNA by [2] was named *AdipoQ*, the protein by [3] was described as Acrp30 (adipocyte complement related protein of 30 000 M_r). Others [4, 5] have described the human homologue of this adipocyte-specific mRNA. Because of its abundance in adipose tissue, it was named *apM-1* (adipose most abundant gene transcript-1). *apM-1* is composed of 244 amino acids containing a short noncollagenous N-terminal segment followed by a collagen-like sequence with 22 perfect Gly-X-Pro motifs. The globular domain of this protein and the "bouquet of flowers" structure of the oligomerisation complex shows striking homology to the complement component C1q [6]. Key amino acid conservations [6], β -strand topology [6], exon/intron structure [7], intron localizations [6, 7], sequence homologies [7] within the promoter region, the 3'-untranslated region and intronic regions, similarity of trimer interfaces [6], similarity of the 3-dimensional structure [6] both between C1q-*apM-1* (bouquet of flowers structure) and TNF- α -*apM-1* (globular head domain, β strand topology) suggest, that the TNF-ligands and the C1q complement proteins arose by divergence from a primordial recognition molecule of the ancient innate immune system [6].

Epidemiological data available for *apM-1* report a negative correlation between *apM-1* plasma concentrations and the BMI both in men and women and in mice [3, 8, 9]. There is also a negative correlation with the occurrence of Type II (non-insulin-dependent) diabetes mellitus [10, 11, 12, 13]. Moreover, patients with Type II diabetes and coronary heart disease have lower *apM-1* plasma concentrations than diabetic patients without coronary heart disease [11]. Additionally, some *apM-1* gene mutations are correlated with increased concentrations of LDL-cholesterol [14].

Two recent publications [15, 16] showed that *apM-1* restores insulin sensitivity in obesity/lipoatrophic diabetes and improves hepatic insulin action. Thus, *apM-1* is a new and promising candidate tool in developing drugs for the treatment of Type II diabetes.

The receptor(s) for *apM-1* are currently not known and only few data on the regulation of *apM-1* exist [17, 18, 19, 20, 21]. Recently, we cloned 2.7 kb of the promoter region of the human *apM-1* gene and mapped the core promoter region within 1.3 kb of the proximal upstream sequence using luciferase reporter gene assays of 3T3-L1 adipocytes.

The aim of our study was therefore (i) to further characterize the human *apM-1* promoter in the context of adipocyte differentiation, (ii) to analyse several putative binding sites both for adipocyte-specific transcription factors (C/EBP and PPAR family) and for zinc finger proteins of the SP family of transcription factors, and (iii) to test the hypothesis of whether the well described inhibition of *apM-1* gene expression by

TNF- α [17, 18] might be due to an interaction with the pathway of SP1-induced gene transcription and whether SP1 protein is expressed and differentially regulated in adipocytes.

Materials and methods

Cloning of the human *apM-1* promoter. 2.7 kb of the human *apM-1* promoter region were cloned by 5'-genome walking, sequenced and analysed for putative transcription factor binding sites, as shown previously [7, 22].

Adipocyte cell culture. 3T3-L1-preadipocytes were cultured at a 10% CO₂ atmosphere at 37°C in DMEM-medium (Biowithaker, Verviers, Belgium) supplemented with 10% newborn calf serum (Sigma Biosciences, Germany) and penicillin and streptomycin (GIBCO BRL, Berlin, Germany). At confluence, cells were differentiated into adipocytes by treating them with DMEM/F12/glutamate-medium supplemented with 0.5 mmol/l 3-isobutylmethyl-xanthine, 10⁻⁷ mol/l corticosterone, 10⁻⁶ mol/l insulin, 200 μ mol/l ascorbat, 2 μ g/ml transferrin, 1 μ mol/l biotin, 17 μ mol/l panthothenate and 300 mg/l Pedersen-fetuin [23, 24] for 5 days. Thereafter, the cells were exposed to DMEM/F12/glutamate-medium with insulin 10⁻⁹ mol/l until they reached the fully differentiated phenotype [25, 26, 27, 28, 29, 30], that was controlled by light-microscopy for the existence of a more rounded cell shape and the typical appearance of extensive lipid droplet accumulation.

Preparation of nuclear extracts. 3T3-L1-Adipocytes at confluence, 48 h, 72 h, 96 h, 7 days and 9 days of differentiation were harvested by centrifugation, washed once with ice-cold PBS and washed twice with wash buffer composed of 10 mmol/l HEPES (pH 7.9), 1.5 mmol/l MgCl₂, 10 mmol/l KCl, 0.5 mmol/l dithiothreitol (DTT), and 0.5 mmol/l phenylmethylsulfonyl fluoride (PMSF). After resuspension in 1 ml ice-cold wash buffer, cells were centrifuged 1 min at 6000 rpm. Hypotonic buffer containing 0.1% NP40 was added to lyse the cell pellet. After 5 min of incubation on ice, nuclei were pelleted by centrifugation for 15 min (15000 rpm) at 4°C. Nuclei were resuspended in lysis buffer containing 20 mmol/l HEPES (pH 7.9), 0.42 mol/l NaCl, 1.5 mmol/l MgCl₂, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, 0.5 mmol/l PMSF, and 10% v/v glycerol, and incubated at 4°C for 15 min with gentle vortexing. Subsequently, the nuclear debris was pelleted by centrifugation at 4°C for 15 min and the supernatant was diluted 1:6 with storage buffer composed of 20 mmol/l HEPES (pH 7.9), 0.05 mol/l KCl, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, 0.5 mmol/l PMSF, and 20% v/v glycerol. The extracts were aliquoted and stored at -80°C. Protein concentration was measured using the Bradford method [31].

Electrophoretic mobility shift assays. 10 μ g of nuclear extracts were incubated with 2 μ g poly (dI-dC) in a volume of 20 μ l binding buffer containing 50 mmol/l HEPES (pH 7.9), 6 mmol/l MgCl₂, 50 mmol/l KCl, 5 mmol/l DTT, 100 μ g/ml BSA, and 0.01% NP40. [³²P] end-labelled synthetic oligonucleotides (30 000 cpm) were added and the reaction mixture incubated for 20 min at room temperature. In competition experiments nuclear extracts were preincubated for 10 min with a 50-fold molar excess of non-labelled competitor oligonucleotide. Supershift assays were carried out with antibodies purchased from Santa Cruz Biotechnology. The DNA-protein complexes were analysed on a 8% polyacrylamide gel at 80 V using 0.25 $\times$ TBE as electrophoresis buffer. Gels were dried

and exposed to Kodak X-Omat AR films overnight at -80°C . The following oligonucleotides annealed to their respective complementary oligonucleotides were used (Table 1): SP1 consensus oligonucleotide, wild type promoter fragment $-28/-80$ (prom $-28/-80$) carrying the proximal SP-1 binding site, mutated fragment $-28/-80$ (prom-mut $-28/-80$) carrying a mutated SP1 binding site. Additional putative binding sites were tested in electrophoretic mobility shift assays using the following oligonucleotides: *PPAR* γ binding site at $-593/-622$, *C/EBP* binding sites at $-157/-204$, at $-222/-262$, at $-434/-382$, at $-753/-782$, at $-883/-912$, *AP-1* binding sites at $-28/-80$, at $-109/-156$, at $-420/-466$, at $-483/-512$, *USF-1* binding site (E-box) at $-167/-204$, and *YY1* at $-683/-712$.

Transient transfection of adipocytes and luciferase reporter gene assays. To study promoter activity, a genomic DNA fragment derived from the 5'-proximal region of the *apM-1* gene, fragment $-256/-41$ carrying the proximal putative SP1 binding site was isolated and cloned in the luciferase expression vector pGL3-enhancer (Promega, Madison, Wis., USA). It was excised with BamH-I and SacI from the pUC18 cloning vector and recloned into the pGL-3 enhancer vector digested with SacI and Bgl-II (with BamH-I and Bgl-II leaving compatible ends). The PCR products were purified by Qiaex, digested with Sac-I and Bgl-II and cloned into the luciferase vector that had also been cut with SacI and BglII. The fragments had a common 3'-terminus located at nucleotide -41 referring to the ATG codon in the first exon. All chimeric plasmids were sequenced (ALFExpress, Pharmacia) before transfection.

After the cells have grown at a density of 80 to 90%, cells were transiently transfected with 10 μg plasmid DNA using the Superfect reagent based on activated dendrimer technology (Qiagen, Hilden, Germany) as described by the manufacturer. After 3 h of incubation, cells were differentiated as described above. Luciferase activity was measured 48 h after the start of differentiation. In each experiment, cells were transfected with pGL-3 enhancer and pGL-3 control plasmids that served as negative and positive controls, respectively. The pGL-3 enhancer vector has no eukaryotic promoter upstream of the luciferase gene. The pGL-3 control vector contains the SV40 early promoter driving the expression of luciferase mRNA transcripts. To standardise the transfection efficiency, 7.5 μg of *pSV β -galactosidase* plasmid (Promega) were always cotransfected. Moreover, the amount of measured relative light units was normalized to total protein content of each cell lysate and represents the means and standard error of the mean (mean $\pm$ SD) to three separate experiments, each done in triplicate.

For luciferase assay, transfected cells were harvested 48 h after differentiation and lysed in 1 ml of reporter lysis buffer (Promega). Aliquots of 20 μl of lysates were mixed with 100 μl luciferase assay reagent containing luciferyl-CoA. The luciferase activity was measured in a Lumat LB9501 (Berthold, Munich, Germany).

The transcription factor expression plasmids for SP1 (pPacSP1) and SP3 driven by the actin promoter were a gift from Dr. R. Tijan (University of California, Berkeley, USA) and from Dr. L. Lania (Naples, Italy).

Transient transfection of drosophila-S2-schneider-cells. Schneider cells were cultured at $22-24^{\circ}\text{C}$ in the absence of CO_2 . They were cultured in Complete DES Expression Medium with L-glutamine (Invitrogen, Groningen, Netherlands) containing 10% heat-inactivated foetal bovine serum. After the cells have grown at a density of 70 to 80%, cells were transiently transfected with 19 μg plasmid DNA using the CaCl_2 method as described by the manufacturer (Invitrogen). This mixture was removed after 24 h, and cells were grown for ad-

Fig. 1. Electrophoretic mobility shift assay. The expression of transcription factor SP1 during adipocyte differentiation is investigated by binding of nuclear extracts from 3T3-L1-adipocytes to a SP1 consensus oligonucleotide during different stages of differentiation. lane 1: free probe (radiolabelled SP1 consensus oligonucleotide without nuclear extract), lane 2: nuclear extract from preadipocytes, lane 3: nuclear extract from preadipocytes preincubated with 50-fold molar excess of unlabelled probe as specific competitor, lane 4: nuclear extract from 48-h cultured adipocytes, lane 5: nuclear extract from 4 days cultured adipocytes, lane 6: nuclear extract from 7 days cultured adipocytes, lane 7: nuclear extracts from 9 days cultured adipocytes

ditional 24 h. All experiments and procedures of our investigation were conducted according to the guidelines expressed in the "Declaration of Helsinki" (revised in 1996).

Results

Expression of transcription factor SP1 during adipocyte differentiation. Transcriptional binding activity of SP1 protein was investigated by electrophoretic mobility shift assay using a radiolabelled SP1 consensus oligonucleotide and nuclear extracts from preadipocytes and from adipocytes during different stages (48 h, 4 days, 7 days, 9 days) of differentiation (Fig. 1). Incubation of the radiolabelled SP1 consensus oligonucleotide with nuclear extracts from 3T3-L1-preadipocytes produces a weak, but reproducible band shift (Fig. 1, lane 2). The specificity of binding was shown by competition with unlabelled oligonucleotide, which eliminates this complex completely (Fig. 1, lane 3). SP1 is stepwise and strongly induced during adipocyte differentiation (Fig. 1, lanes 4–7), increasing from 48 h of differentiation to a maximum at day 9 of late adipocyte differentiation.

These results indicate that transcription factor SP1 is expressed in adipocytes and its DNA-binding activity is induced during differentiation from preadipocytes to adipocytes with a maximum in late adipocyte differentiation.

Fig. 2. Electrophoretic mobility shift assay. The binding of purified recombinant SP1 factor and nuclear extracts from fully differentiated adipocytes to the proximal promoter fragment *prom* -28/-80 carrying a putative SP1 binding site is investigated. lane 1: free probe (radiolabelled promoter fragment), lane 2: recombinant SP1 factor, lane 3-5: recombinant SP1 preincubated with 1, 2 or 3 µl of unlabelled probe as specific competitor, lane 6: recombinant SP1 preincubated with SP1 antibody (supershift complex), lane 7: nuclear extracts from fully differentiated adipocytes, lanes 8-9: nuclear extracts from fully differentiated adipocytes preincubated with 1 and 2 µl of unlabelled probe as specific competitor, lane 10: nuclear extracts from fully differentiated adipocytes preincubated with SP1 antibody (supershift complex). Shifted complexes are marked with brackets. The asterisks (*) denote non-specific interaction bands, which were not consistently seen with different nuclear extract preparations

Characterisation of the proximal SP1 binding site within the human apM-1 promoter. Since we found the existence of transcriptionally active SP1 protein in adipocytes and its increase during late adipocyte differentiation (as does apM-1), it was our aim to investigate, whether recombinant purified SP1 factor and SP1 factor in nuclear extracts from mature adipocytes bind specifically to the putative binding site within the proximal apM-1 promoter.

To test this hypothesis, we did electrophoretic mobility shift assays with a radiolabelled proximal promoter fragment *prom* -28/-80 carrying a putative SP1 binding site (Fig. 2). Incubation of the radiolabelled promoter fragment *prom* -28/-80 with recombinant SP1 factor produces a strong band shift (Fig. 2, lane 2), that can be competed using unlabelled probe (Fig. 2, lanes 3-5). Coincubation of the radiolabelled promoter fragment with SP1 factor and SP1 antibody produces a specific supershift band (Fig. 2, lane 6).

These results indicate, that SP1 binds specifically to the proximal SP1 binding site within the *apM-1* promoter.

We repeated the same experiments using nuclear extracts from fully differentiated adipocytes instead of recombinant SP1 factor. Incubation of the radiolabelled promoter fragment with nuclear extracts from fully differentiated adipocytes produces a band shift

Fig. 3. Electrophoretic mobility shift assay. Mutation of the SP1 recognition element within the proximal promoter fragment *prom* -28/-80. The binding of SP1 factor in nuclear extracts from preadipocytes (lanes 2-4 and 9-11) and 9 days cultured adipocytes (lanes 5-7 and 12-14) to the wildtype (lanes 1-7) and a mutated promoter fragment (lanes 8-14) is investigated. lane 1: free probe (radiolabelled promoter fragment), lane 2: nuclear extracts (NE) from preadipocytes, lane 3: nuclear extracts preincubated with 50-molar excess of unlabelled probe as specific competitor, lane 4: nuclear extract coincubated with SP1 antibody, lane 5: NE adipocytes (9 days), lane 6: NE adipocytes (9 days) and unlabelled probe as specific competitor, lane 7: NE adipocytes (9 days) coincubated with SP1 antibody, lane 8: free probe (mutated SP1 binding site), lane 9: NE from preadipocytes, lane 10: NE preincubated with 50-molar excess of unlabelled probe as specific competitor, lane 11: NE coincubated with SP1 antibody, lane 12: NE adipocytes (9 days), lane 13: NE adipocytes (9 days) and unlabelled probe as specific competitor, lane 14: NE adipocytes (9 days) coincubated with SP1 antibody. Shifted complexes are marked with brackets. The asterisk (*) denotes non-specific interaction bands, which were not consistently seen with different nuclear extract preparations

(Fig. 2, line 7), that can be competed using unlabelled probe (Fig. 2, lanes 8-9). Again, coincubation of the radiolabelled promoter fragment with nuclear extracts from fully differentiated adipocytes and SP1 antibody produces a specific supershift band (Fig. 2, lane 10).

These results show that recombinant SP1 factor as well as SP1 factor in nuclear extracts from adipocytes bind specifically to the proximal SP1 binding site within the *apM-1* promoter.

Mutation of the SP1 binding site (Fig. 3) within the proximal promoter fragment abolishes SP1 binding from nuclear extracts of preadipocytes (Fig. 3, lanes 9-11) and adipocytes (Fig. 3, lines 12-14). The SP1 band shift of preadipocytes (Fig. 3, lane 2) and of adipocytes (Fig. 3, line 5) is completely abolished using a mutated SP1 binding site (Fig. 3, lines 9 and 12), providing additional evidence for specific binding of SP1 to the *apM-1* promoter site.

Exclusion of co-binding of SP1/SP3 to the proximal SP1 binding site. Incubation of the radiolabelled proximal promoter fragment *prom* -28/-80 with nuclear

extracts from adipocytes produces a band shift (Fig. 4, lane 2), that can be competed by unlabelled probe (Fig. 4, lane 3) and that produces a supershift band using SP1 antibody (Fig. 4, lane 4), whereas no supershift band could be observed using SP3 antibody alone (Fig. 4, lane 5). Again, coinubation of nuclear extracts with both the SP1 antibody and the SP3

antibody produces the former supershift band (Fig. 4, lane 6). These results show, that SP1 and SP3 are not co-bound on the same DNA molecule, since the anti-SP3 antibody did not affect the formation of the SP1-specific complex. However, that does not exclude the possibility, that SP1 and SP3 compete for the binding to this promoter region.

Fig. 4. Electrophoretic mobility shift assay. Interaction of SP1 and SP3 on binding of nuclear extracts from adipocytes to the proximal promoter fragment *prom* -28/-80 is investigated. lane 1: free probe (radiolabelled promoter fragment), lane 2: nuclear extract from adipocytes, lane 3: nuclear extracts preincubated with 50-molar excess of unlabelled probe as specific competitor, nuclear extract coincubated with SP1 antibody, lane 5: nuclear extract coincubated with SP3 antibody, lane 6: nuclear extract coincubated with SP1 antibody and SP3 antibody. Shifted complexes are marked with *brackets*. The bands marked with a *cross* are specific low molecular weight complexes that have not been identified

Characterisation of putative binding sites for adipocyte-specific transcription factors and several additional binding sites within the apM-1 promoter. The following putative binding sites were tested in electrophoretic mobility shift assays and did not produce any specific supershift bands: *C/EBP* (-898/-892), *C/EBP* (-244/-233), *C/EBP* (-199/-185), *C/EBP* (-776/-768), *C/EBP* (-372/-359), *PPAR* (-605/-610), *AP-1* (-127/-116), *AP-1* (-46/-34), *AP-1* (-60/-450), *AP-1* (-506/-498), *YY1* (-706/-696), *USF* (E-box) (-176/-171).

Functional assessment of the SP1-stimulated promoter activity in S2-Schneider cells using SP1-cotransfection experiments. Since S2-Schneider cells lack a constitutive SP1 expression, we investigated the promoter activity of fragment -256/-41 carrying the proximal SP1 binding site in these cells after transient transfection with luciferase reporter gene constructs (Fig. 5). As expected, in the absence of SP1, promoter activity of this construct was low (24.6±1.5% for fragment -256/-41 compared with the control vector). Co-transfection of a SP1 expression plasmid resulted in about a tenfold induction of promoter activity (fragment -256/-41: 294.4±85.3%). These data indicate, that the apM-1 promoter constructs are not activated in a non-adipocytic environment and in the absence of

Fig. 5. Promoter activity of human apM-1 luciferase gene chimeras transiently transfected in S2-Schneider cells. The localization of the tested promoter fragment with or without coexpression plasmids are shown on the left. Numbers indicate the relative positions with respect to the ATG start codon. The first

exon is marked by a *solid box*. The SP1 binding sites are represented by an *open box*. Data are normalized to total protein content. Relative promoter activity equal to 100% corresponds to the luciferase activity of the SV 40 control vector

Fig. 6. Promoter activity of human *apM-1* luciferase gene chimeras transiently transfected in 3T3-L1 adipocytes. The localization of the tested promoter fragment with or without coexpression plasmids are shown on the left. Numbers indicate the relative positions with respect to the ATG start codon. The first exon is marked by a *solid box*. The SP1 binding sites are represented by an *open box*. Data are normalized to total protein content. Relative promoter activity equal to 100% corresponds to the luciferase activity of the SV40 control vector

constitutive SP1 expression. The presence of SP1, induced by coexpression, is required for induction of promoter activity by activating the proximal SP1 binding site.

Functional assessment of the SP1-stimulated promoter activity in 3T3-L1 adipocytes using SP1 / SP3-cotransfection experiments. Transient transfection of adipocytes (Fig. 6) with promoter constructs carrying the proximal (fragment -256/-41) SP1 binding site induces a basal promoter activity (fragment -256/-41: $12.9 \pm 3.5\%$) compared with the control vector. Cotransfection of fragment -256/-41 with SP1 expression plasmid resulted in an increase of promoter activity from $12.9 \pm 3.5\%$ to $26.8 \pm 6.4\%$ ($p=0.012$), whereas coexpression of SP3 alone has no effects. The increase in promoter activity observed by cotransfection of fragment -256/-41 with SP1 ($26.8 \pm 6.4\%$) can be reduced back to control levels by simultaneous cotransfection of SP3 ($16.2 \pm 4.5\%$). Thus, SP3 inhibits the promoter activity induced by SP1 at the proximal SP1 binding site possibly by competing with SP1 for its binding site.

Fig. 7. Electrophoretic mobility shift assay. The binding of nuclear extracts (NE) from adipocytes during different stages of differentiation treated with 0.2 nmol/l or 2 nmol/l TNF- α for 24 h to the proximal SP1 binding site *prom* -28/-80 is investigated. lane 1: free probe (radiolabelled promoter fragment *prom* -28/-80), lane 2: NE pre-adipocytes, lane 3: NE pre-adipocytes + 0.2 nmol/l TNF- α , lane 4: NE pre-adipocytes + 2 nmol/l TNF- α , lane 5: NE adipocytes (7 days), lane 6: NE adipocytes (7 days) + 2 nmol/l TNF- α , lane 7: NE adipocytes (7 days) + 0.2 nmol/l TNF- α , lane 8: NE adipocytes (48 h), lane 9: NE adipocytes (48 h) + 0.2 nmol/l TNF- α , lane 10: NE adipocytes (48 h) + 2.0 nmol/l TNF- α . Shifted complexes are marked with brackets

TNF- α treatment of adipocytes reduces binding of SP1 to the proximal SP1 binding site. Since TNF- α has been reported to downregulate *apM-1* expression, we were interested to see whether this effect has an impact on SP1 binding to the promoter.

Treatment of preadipocytes (Fig. 7, lines 2–4) and adipocytes differentiated for 7 days (Fig. 7, lines 5–7), 48 h (Fig. 7, lines 8–10) with different amounts of TNF- α (0.2 nmol/l and 2 nmol/l) leads to an inhibition of SP1 binding to the proximal promoter fragment *prom* -28/-80.

Discussion

Since *apM-1* represents an adipocyte-specific gene, we first tested the binding of nuclear extracts from adipocytes during different stages of differentiation to several putative binding sites for adipocyte-specific transcription factors such as C/EBP and PPAR γ . However, no specific supershift bands could be observed in our EMSA's concerning several C/EBP and PPAR binding sites. Accordingly, *apM-1* seems to be regulated differently from other adipocyte-specific genes. Recently, a study [18] found that ligands for PPAR γ increase the expression of *apM-1*. Since we could not verify any specific interactions between putative PPAR binding sites and adipocytic nuclear proteins, this induction might be caused by secondary effects involving PPAR-inducible genes and not by specific activation of PPAR response elements in the proximal *apM-1* promoter. Our data on the putative C/EBP binding sites in the *apM-1* promoter sequence do not support the hypothesis [20], suggesting a regulation by a proximal C/EBP binding site. In those experiments, no supershift bands and no functional data (luciferase reporter gene assays) concerning the C/EBP sites were presented [20].

TNF- α is known to be a negative regulator of several adipocyte-specific genes as *C/EBP* genes, PPAR γ , Glut-4, fatty acid binding protein, adipsin, lipoprotein lipase, acetyl-CoA-carboxylase, glycerophosphate dehydrogenase [34, 35, 36, 37] and of adipocyte differentiation in general [35, 36]. TNF- α has been shown to inhibit *apM-1*-mRNA expression [17, 18] by influencing *apM-1* promoter activity [18] via largely unknown mechanisms. It is our hypothesis, that the TNF- α -induced inhibition of the transcriptional binding activity of SP1 is the mechanism responsible for the inhibitory effect of TNF- α on *apM-1* gene expression. This was confirmed due to the fact that (i) SP1 has stimulatory effects on *apM-1* promoter activity and that (ii) SP1 binding to the *apM-1* promoter can be reduced by preincubation of adipocytes with TNF- α .

This mechanism of regulation seems to be very useful. Since TNF- α generally inhibits adipocyte differentiation, the specific downregulation of a candidate gene by TNF- α without disturbing the process of differentiation itself, must be caused by mechanisms not affecting the common adipogenic pathways (for example C/EBP, PPAR γ). Due to our data, SP1 represents a potential mediator of these non-adipogenic regulatory pathways.

The regulation of transcriptional binding activity of SP1 by TNF- α has not been demonstrated extensively. However, a report [38] showed that TNF- α -induced downregulation of murine hepatic growth hormone receptor expression is mediated by inhibition of SP1 and SP3 binding to the growth hormone receptor promoter. Our data are in strong agreement with these data and support this new mechanism of TNF- α -dependent signalling.

SP1 represents a DNA-binding protein which interacts with a variety of gene promoters containing GC box elements. SP1 has contiguous zinc finger motifs and requires zinc for sequence-specific binding to DNA [39]. SP1 has been shown to activate transcription of a large number of regulated and constitutively expressed genes. This SP1 mediated transcription is independent of whether the promoter comprises a TATAA box or not [32]. SP1 and SP3 contain a similar DNA-binding domain with three zinc fingers and two glutamine- and serine/threonine-rich trans-activation domains [33, 40]. SP3 comprises an additional inhibitory domain, leading to either activation or repression depending on the promoter and cellular context [40, 41, 42, 43, 44, 45, 46]. In this context, we reported, that the *apM-1* promoter lacks a classical TATAA box, but contains a classical CCAAT box and has several transcription start sites [22], thus belonging to the group of class II promoters.

To date, no data are available concerning SP1 expression or regulation in adipocytes. Our data show that transcriptional binding activity of SP1 is present at very low levels in preadipocytes but increases dramatically during adipocyte differentiation with a maximum level in late adipocyte differentiation. *apM-1* represents a late marker of adipocyte differentiation and both the amount of *apM-1* and the transcriptional binding activity of SP1 parallels along adipocyte differentiation.

As shown by our EMSA experiments using recombinant SP1 factor and nuclear extracts from adipocytes, SP1 factor binds putative SP1 binding sites within the proximal *apM-1* promoter. The data obtained by our LRA experiments show that SP1 functions in driving *apM-1* promoter activity. In adipocytes expressing SP1 protein, basal *apM-1* promoter activity can be observed. In adipocytes, overexpression of SP1 activates basal *apM-1* promoter activity. In cells lacking constitutive SP1 expression (S2-Schneider cells), we could not show any basal promoter activity. However, coexpression of SP1 in S2-Schneider cells using a SP1 expression plasmid induces *apM-1* promoter activity. Similar data were obtained concerning the regulation of the promoter of the *PAI-1* (plasminogen activator inhibitor-1) gene [47], which is also expressed in adipocytes. In insect cells devoid of SP1, coexpression of SP1 with a *PAI-1* promoter construct led to an induction of promoter activity [47].

An interaction of SP1 and SP3 has been observed [40, 47, 48]. SP3 has inhibitory partial effects on those

effects mediated by SP1 [40, 41, 42]. The data obtained by our LRA's show that SP3 has inhibitory effects on *apM-1* promoter activity due to the fact that simultaneous coexpression of SP3 with SP1 reduces promoter activity induced by expression of SP1 alone. These data concerning SP1 and SP3 interactions are in agreement with previous reports [40, 41, 42, 47, 48]. SP3 alone is not able to activate SP1-responsive promoters [48] and it fails to activate reporter constructs in cells lacking endogenous SP factors [40, 47]. However, SP3 is able to repress SP1-mediated activation, due to a competition with SP1 for their common binding sites [40]. The mechanism of a competition between SP1 and SP3 for binding to the promoter site is supported by our data from EMSA, which showed, that application of a SP3 antibody alone could not produce a similar supershift band that was obtained by an antibody against SP1. In addition, simultaneous application of antibodies against SP1 and SP3 does not further influence the SP1 supershift band. Accordingly, a simultaneous binding of a putative SP1/SP3 co-complex can be excluded.

In conclusion, SP1 is expressed and its binding activity is enhanced during adipocyte differentiation. In this context, SP1 has stimulatory effects, SP3 exhibits inhibitory effects on *apM-1* promoter activity, mediated by a proximal SP1 binding site. The mechanism of TNF- α -induced inhibition of *apM-1* gene expression seems to be due to a decrease of transcriptional SP1 binding activity caused by TNF- α and thus could provide a new mechanism of TNF- α -dependent signaling. In addition, our data show the existence of a non-adipogenic regulatory pathway composed of SP1/SP3.

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