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Binding and Functional Characteristics of Two E-Box Motifs Within the S100A6 (Calcyclin) Gene Promoter

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Abstract S100A6 (calcyclin) is a small calcium-binding protein of the S100 family often associated with cancer and metastasis. We have previously shown that the E-box sequence at position −283/−278 of the S100A6 gene promoter interacts with USF transcription factor and contributes to promoter transcriptional activity. We now present evidence that a second E-box motif at position −593/−588 of the promoter also binds USF and that the USF1/USF2 heterodimer is the prevailing dimeric form of the transcription factor bound. Using the chromatin immunoprecipitation assay (ChIP), we show that USF is bound in vivo to the E-box regulatory element(s). Depletion of the endogenous USF pool by means of a decoy oligonucleotide evokes a severe inhibition of S100A6 gene promoter activity. Furthermore, we show that S100A6 gene promoter activity can be stimulated by palmitate and that mutation of the −283/−278 E-box sequence completely blocks this stimulation. J. Cell. Biochem. 97: 1017–1024, 2006.

Key words: S100A6; calcyclin; USF; promoter; chromatin immunoprecipitation; decoy; palmitate; E-box

S100A6 (calcyclin) is one of the homologous, low-molecular weight calcium-binding proteins of the S100 family whose expression has been functionally linked with cell cycle progression [Calabretta et al., 1986], cytoskeleton rearrangement [Breen and Tang, 2003], exocytosis [Timmons et al., 1993], and other cellular processes. Many different endogenous and exogenous stimuli including the action of gastrin [Kucharczak et al., 2001], vasopressin [Courtois-Coutry et al., 2002], phorbol esters [Gong et al., 1992], or cadmium ions [Leśniak et al., 2005] have been reported to induce an increase in S100A6 mRNA and/or protein level. An elevated level of S100A6 protein is also a common signature of many cancer cells and is often regarded as a marker of malignances [Hsieh et al., 2003]. Neither the reason for nor the molecular mechanism behind the increased expression in normal and cancer cells has been established and, in fact, experimental data concerning regulation of the S100A6 gene transcription are very limited. NFκB binding at position −460/−451 of the S100A6 gene promoter [Joo et al., 2003] points to the role of inflammatory processes in activation of this gene while the presence and functional characteristics of an antioxidant response element [Leśniak et al., 2005] suggest the role of oxidative stress. We have previously found that transcriptional activity of the S100A6 gene promoter is stimulated by the binding of upstream stimulatory factor (USF) to the E-box sequence located at position −283/−278 [Leśniak et al., 2000]. This transcription factor, represented by 43 kDa USF1 and 44 kDa USF2 proteins [Viollet et al., 1996], is ubiquitous and interacts with regulatory elements of many genes. It is now well documented that USF is acting as a mediator of glucose response in transcriptional regulation of genes engaged in glucose and fatty acid metabolism [Girard et al., 1997]. Evidence is also accumulating that USF can mediate cell response to ultraviolet light [Galibert et al., 2001]. The S100A6 gene promoter harbors two

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canonical E-box sequences and in this work we have studied their binding characteristics, in vivo occupancy, and the extent to which they contribute to the promoter activity. Since recent studies using microarray methodology indicated huge upregulation of calcyclin mRNA in palmitate- or oleate-treated cells [Busch et al., 2002], we have also checked whether calcyclin gene promoter is responsive to palmitate and examined the role of E-box sequence in this process.

MATERIALS AND METHODS

Cell Culture, Treatment, and Transfection

Hep-2 (human epithelial) cells were maintained in DMEM with 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded in 24-well plates and transfected after 24 h in DMEM without FCS or antibiotics using Lipofectamine Reagent (Gibco) for 6 h. HEK293 (human embryonic kidney) cells were maintained as above and transfected for 6 h by the calcium phosphate precipitate method. After transfection cells were grown in a complete medium for 12 h. Subsequently, transfected cells were treated with 10 μM SB203580 for 4 h prior to luciferase assay. As for cells treated with PA, after the initial 12 h post-transfection, they were incubated with 0.2 mM palmitate/0.48% BSA or 0.48% BSA for 48 h prior to luciferase assay. BSA and palmitate/BSA solutions were prepared as described [Busch et al., 2002]. Cells were subsequently lysed and luciferase activities were measured on a TD-20/20 luminometer (Turner designs) using Dual-Luciferase Reporter Assay System (Promega).

Plasmids

pGL2 vectors containing luciferase gene and the −1371/+134 and −361/+134 fragments of the human S100A6 gene promoter were described earlier [van Groningen et al., 1995]. Plasmid bearing the −361/+134 promoter fragment with the E-box sequence at position −283/−278 (E-box1) mutated to CAGCTT was obtained as described [Leśniak et al., 2000]. Mutation in E-box2 (CACGTG to CAGCTT) within the −1371/+134 promoter fragment was introduced according to the QuikChange Site-Directed Mutagenesis method (Stratagene) using the following sense and antisense oligonucleotides: 5′-GGGCTGGGCTCCCAGCTTCATGCACATAACAC-3 and 5′-GTTAATGTGCATGAAGCTGGGAGCCCAGCCCTG-5′ overlapping the E-box2 sequence at position −593/−588 of the S100A6 gene promoter (mutated E-box sequences are underlined). PCR program consisted of an initial incubation for 1 min at 94°C followed by 30 s at 95°C, 1 min at 55°C, and 12 min at 68°C. Two 25 μl samples containing the non-mutated plasmid template and either the sense or antisense primer were cycled four times and then combined and processed for another 18 cycles. pEGFP and pEGFP-USF1 plasmids were used in USF1-overexpression experiments. The pRL-SV40 vector was used to assess transfection efficiency. In the decoy experiments a 22 bp double stranded oligonucleotide 5′-GCCGAGTCACGTGTCACGAAGA-3′ corresponding to the −290/−268 region of the S100A6 gene promoter encompassing E-box1 (underlined) or the same oligonucleotide but with mutation in the E-box were transfected together with the appropriate reporter plasmids.

Electrophoretic Mobility Shift Assay (EMSA)

Preparation of nuclear extracts from Hep-2 cells, labeling of the DNA probe, sample incubation, and electrophoresis were performed as previously described [Leśniak et al., 2000]. The sequence of the double stranded oligonucleotide probe 5′-CTGGGCTCCCACGTGCATGCAC-3′ corresponded to the −602/−581 fragment of the S100A6 gene promoter. E-box2 is underlined. Antibodies used for the supershift assay were from Santa Cruz Biotechnology (USF1, sc-229X; USF2, sc-862X).

Chromatin Immunoprecipitation Assay (ChIP)

The assay, including all buffers used, was performed essentially according to the Upstate Biotechnology protocol available at www.upstatebiotech.com/support/protocols/chip. Eighty percent confluent Hep-2 cells were crosslinked by addition of 37% formaldehyde (1% final concentration) directly to the medium and incubated 20 min at 37°C. Cells were then scraped in PBS, washed by centrifugation, resuspended in 0.2 ml ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8,0) supplemented with protease inhibitor cocktail (Sigma) and incubated 10 min on ice. The sample was then sonicated 6 × 15 s at 30% duty cycle using Branson Sonifier 250 and centrifuged for 10 min at 15,000*g*. As judged by agarose gel electrophoresis this treatment resulted in

reducing DNA length to 200–400 bp fragments. Protein concentration was measured in the supernatant and the content was diluted with the lysis buffer to achieve 0.2 mg/ml final protein concentration. This solution (0.2 ml) was further diluted to 1.8 ml with DNA dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl) and 50 μl was preserved as the input fraction. Protein A agarose slurry (80 μl) was added to the remaining sample and the content was incubated at 4°C for 45 min with rotating. After pelleting agarose the supernatant was divided into two equal portions. Anti-USF1 antibody (3 μl) was added to one of them and the samples were incubated overnight at 4°C with rotating. After that 100 μl agarose slurry was added to each sample and they were incubated for another 2 h. The agarose pellet was then washed by centrifugation in low- and high-salt buffer, lithium-containing buffer, and twice in TE, exactly as described in the protocol. Agarose bound proteins were eluted upon vortexing in two 250 μl portions of the elution buffer for 30 min in RT. Twenty microliters of the input fraction were also mixed with 0.5 ml elution buffer and all samples were incubated overnight at 65°C to reverse the crosslinks, followed by 2 h incubation with 20 μg of proteinase K at 50°C. After that 10 μg of yeast RNA was added as a carrier, the samples were extracted with two 0.5 ml portions of phenol-chloroform and DNA was ethanol-precipitated from the aqueous phase. This DNA served as a template for the subsequent PCR with primers designed to encompass E-box1: 5′-CTGGCT-CGAGGGTCACAT-3′ (−368) and 5′-GGGCT-GTTCCGGGTTAGGA-3′ (−167) and E-box2: 5′-CTGTAGGAAGGGCATGTC-3′ (−796) and 5′-TAGGGGGAGTAGCCAGTG-3′ (−449) where numbers in brackets refer to the 5′ position of a given primer in relation to the transcription start site [Ferrari et al., 1986]. Both reactions were amplified 30 times using the following PCR program: 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C.

Statistical Analysis

The results of luciferase assays are presented as means ± SE of at least three experiments. Differences in mean values were tested by Student's *t*-test. The level of statistical significance was set at $P < 0.05$.

RESULTS

Binding of USF to the E-Box Sequence Located at Position −593/−588 of the S100A6 (Calcyclin) Gene Promoter

We have previously shown that the transcription factor USF binds to the E-box sequence located at position −283/−278 of the human S100A6 gene promoter and stimulates its activity [Leśniak et al., 2000]. Since the promoter harbors another E-box sequence (E-box2) more distal from the transcription start site, that is, at position −593/−588, we checked whether this site is also recognized by USF. As shown in Figure 1, lane 2, a 22 bp double-stranded oligonucleotide corresponding to the −602/−581 promoter fragment binds a protein complex in EMSA. Addition of the anti-USF1 or anti-USF2 antibody resulted in a supershift of this complex indicating that USF is the transcription factor involved in the binding (Fig. 1, lanes 3–5). Since each antibody supershifted a major part of the complex it is justified to conclude that the prevailing form of USF bound to E-box2 is the USF1/USF2 heterodimer. Thus, USF appears to interact with at least two binding sites within the S100A6 gene promoter.

In Vivo Binding of USF to the E-Box Motifs in the S100A6 Gene Promoter

EMSA is an in vitro method employed to study the potential of a transcription factor to bind a defined DNA sequence comprised in a short, double stranded oligonucleotide. However, even when positive, this assay leaves open the question of whether the protein–DNA interaction indeed persists in vivo. We have performed chromatin immunoprecipitation assay to check the in vivo binding of USF to two E-box sequences in the S100A6 gene promoter. Figure 2 shows positive PCR reactions encompassing E-box1 and E-box2, respectively, in the input fraction (positive control) and in the sample incubated with anti-USF1 antibody, but not in the control sample without antibody. This result indicates that DNA fragments containing E-box motifs are effectively co-immunoprecipitated by the anti-USF1 antibody and demonstrates that USF is bound in vivo to the S100A6 gene promoter in Hep-2 cells. As the two E-boxes are only about 300 bp apart it is difficult to unambiguously prove if USF binds to only one or both of these motifs. However, taking into account that the average length of

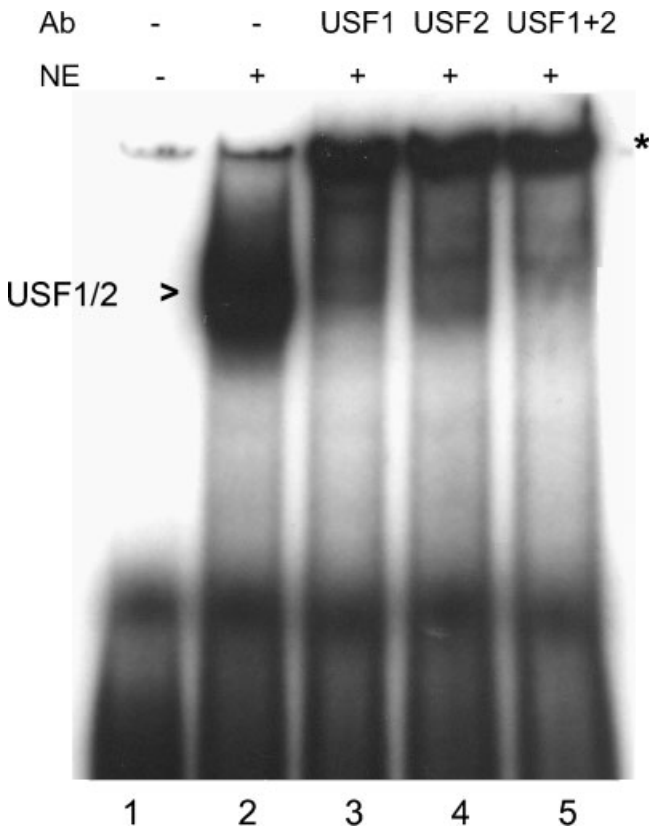

Fig. 1. Analysis of protein binding to the −602/−581 fragment of the S100A6 gene promoter. Nuclear extracts (NE), 10 μg, obtained from Hep-2 cells were incubated with radioactive double-stranded oligonucleotide corresponding to the −602/−581 fragment of the promoter. Antibodies (Ab), 1 μl, were incubated with the samples for 30 min at RT prior to the addition of the radioactive probe. Samples were run on a 5% non-denaturating polyacrylamide gel, the gel was dried and exposed at −70 for 20 h. No extract (lane 1), control sample (lane 2), addition of the antibodies against USF1 (lane 3), USF2 (lane 4), both USF1 and USF2 (lane 5). The complex formed by USF1/2 heterodimer is indicated by an open arrowhead. The position of supershifted bands is indicated by asterisk.

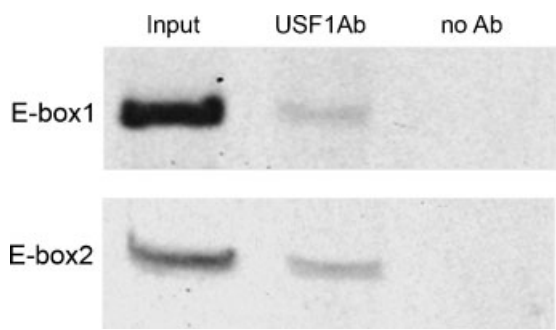

Fig. 2. Analysis of USF binding in vivo to the S100A6 gene promoter. Chromatin immunoprecipitation assay and PCR reactions were performed as described in Materials and Methods. Upper panel: 200 bp PCR product encompassing E-box1 at position −283/−278 of the calcyclin gene promoter. Lower panel: 345 bp PCR product encompassing E-box2 at position −593/−588 of the calcyclin gene promoter. Two microliters of the input fraction and 5 μl of the sample incubated with anti-USF1 antibody (USF1Ab) and of the control sample without antibody (no Ab) were used as template for PCR.

sonicated DNA is 200–400 bp the scarcity of longer DNA fragments that could serve as templates for both PCR reactions strongly supports the latter possibility. Thus, we have established that USF interacts in vivo with E-box motif(s) in the calcyclin gene promoter and because of this may be considered as a relevant physiological factor involved in the regulation of its transcriptional activity.

Effects of E-Box2 Mutation, Competition for USF Binding and Inhibition of USF1 Phosphorylation on S100A6 Gene Promoter Activity

To check whether the binding of USF to E-box2 contributes to the S100A6 promoter activity we have introduced a 3 base mutation in the E-box2 sequence which results in a complete loss of USF binding [Leśniak et al., 2000]. Hep-2 cells were subsequently transfected with either wild-type −1371/+134 promoter construct or its mutated counterpart. As

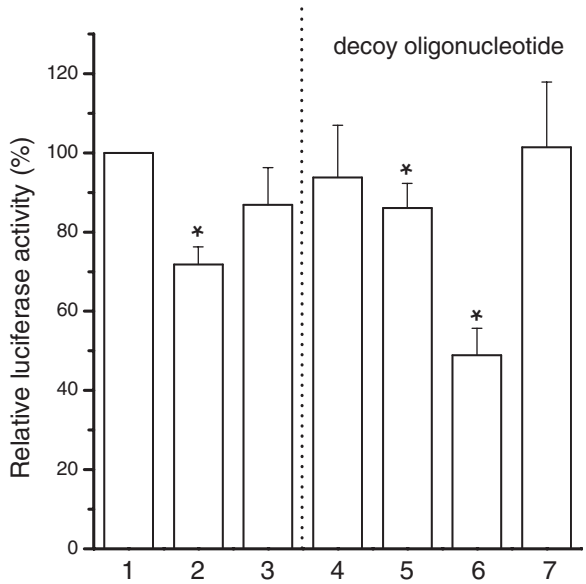

Fig. 3. Effect of E-box2 mutation, p38 inhibition, and of the USF decoy oligonucleotide on the S100A6 gene promoter activity. Hep-2 cells were transfected with a reporter luciferase vector containing the −1371/+134 fragment of the S100A6 gene promoter (1) or with a vector containing the same promoter fragment but with mutation in E-box2 (2). To assess the effect of p38 kinase inhibition cells transfected with the wild-type vector were incubated with 10 μM SB203580 for 4 h (3). For the decoy experiment Hep-2 cells were transfected with the wild-type vector and with a decoy oligonucleotide containing either wild-type (4, 5, 6) or mutated E-box sequence (7). The decoy oligonucleotide concentration used was: 0.055 μM (4), 0.11 μM (5) and 0.22 μM (6). The mutated decoy oligonucleotide was used at 0.22 μM concentration (7). Luciferase assay was performed as described in Materials and Methods. All data are mean ± SE of at least three independent experiments. $^*P < 0.05$, relative to control (1).

can be seen in Figure 3 this mutation resulted in a 29% inhibition of the S100A6 gene promoter activity confirming that the binding of USF to this sequence has a significant effect on transcription. To study the overall impact of USF on the calcyclin gene transcription we employed the decoy method to see how depletion of the nuclear USF pool by an exogenously introduced oligonucleotide containing E-box sequence would affect its promoter activity. To ensure that the oligonucleotides can effectively enter cells a series of preliminary experiments was performed using an oligonucleotide labeled with FITC on its 5′ end. The optimal transfection conditions were selected by monitoring the intracellular fluorescence (not shown). The effect of the decoy oligonucleotide was concentration dependent and was most pronounced at 220 nM concentration, which resulted in 50% inhibition of S100A6 gene promoter activity (Fig. 3). The same oligonucleotide, but with mutation in the E-box, was practically without effect (Fig. 3). These data on the manipulation of the endogenous pool of USF clearly show, in accordance with the results presented in previous sections on in vitro and in vivo binding, that a large fraction of basal promoter activity could be attributed to the binding of USF. Since it was shown that phosphorylation of USF1 by p38 kinase enhanced its transactivating properties [Galibert et al., 2001] we also checked to what extent this modification would affect S100A6 gene promoter activity. For this we have treated Hep-2 cells for 4 h with 10 μM SB 203580, a p38 kinase inhibitor, before the luciferase assay. As can be seen in Figure 3, incubation with SB 203580 had only a slight effect on promoter activity suggesting that, at least in non-stimulated cells, USF1 phosphorylation does not significantly contribute to the transactivating properties of USF and, in consequence, to the S100A6 gene transcription efficiency.

Involvement of the E-Box Sequence in the Stimulation of the S100A6 Gene Promoter Activity by Palmitate

It has been shown by means of microarray and RT-PCR methods that calcyclin gene expression was several times higher in MN16 cells treated with oleate or palmitate [Busch et al., 2002]. Thus we checked whether the effect of palmitate could be reproduced in transfection studies using S100A6 gene promoter constructs, which would indicate a transcriptional regulation of the gene by fatty acids. For these studies we have employed palmitate and HEK293 cells, which were shown to actively take up palmitate [Qanbar and Bouvier, 2004] and could tolerate palmitate treatment better than Hep-2 cells. As shown in Figure 4, 48 h incubation with 0.2 mM palmitate almost doubled calcyclin gene promoter activity. The effect was also seen, although it was less pronounced, with the shorter −361/+134 promoter construct containing only one E-box sequence. To establish whether this sequence is involved in the observed stimulation we compared luciferase activity in cells transfected with the wild-type and mutated −361/+134 construct. Mutation within the E-box sequence totally abolished the effect of palmitate indicating that it is indispensable for the activation to occur. In fact, palmitate slightly inhibited the

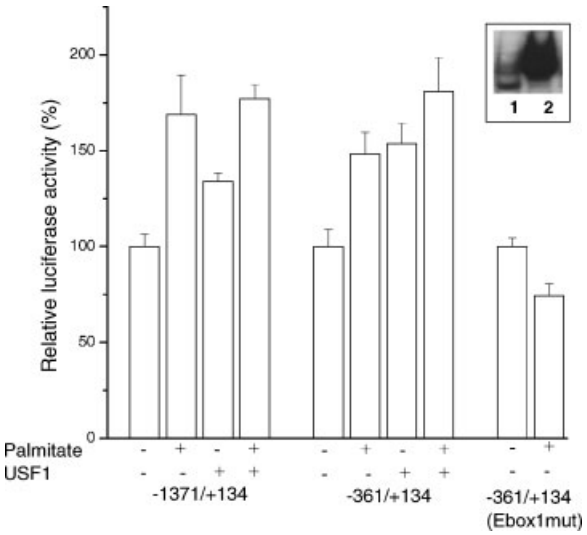

Fig. 4. Effect of palmitate on the S100A6 gene promoter activity. HEK293 cells were transfected with 0.8 μg of the appropriate reporter plasmid and with 0.33 μg of the pEGFP or pEGFP-USF1 plasmids and incubated for 48 h in the presence of BSA or BSA/ palmitate. Luciferase activity measurements were performed as described in Materials and Methods. All data are mean ± SE of at least three independent experiments. $P < 0.05$ for all ± USF and ± palmitate results except the palmitate effect on the USF-cotransfected −361/+134 reporter plasmid ($P < 0.25$). Luciferase activities of vectors bearing the −1371/+134 and the −361/+134 E-box1mut promoter fragments are 82.2 ± 5% and 59.7 ± 6%, respectively, of that obtained for the −361/+134 S100A6 gene promoter fragment. Inset: EMSA performed as in Figure 1 but using a labeled probe comprising E-box1; (1) NE from control HEK293 cells, (2) NE from HEK293 cells transfected with pEGFP-USF1 plasmid.

activity of the −361/+134 mutated promoter (Fig. 4). We have subsequently investigated whether the effect of palmitate can be further augmented by transiently transfecting cells with USF1. Transfection with USF1 increased basal promoter activity by about 33% in the case of the −1371/+134, and by about 50% in the case of the −361/+134 S100A6 gene promoter fragment (Fig. 4). However, although as judged by visual examination the transfection efficiency was close to 80% and the amount of USF1 bound to E-box1 in vitro increased dramatically (Fig. 4, inset), the effect exerted by palmitate was not higher than in cells transfected with the control pEGFP plasmid for both promoter constructs. Thus, overexpression of the USF1/USF1 homodimer did not boost the effect of palmitate.

DISCUSSION

In this work we have shown that the E-box sequence at position −593/−588 of the S100A6 gene promoter (E-box2), similarly to the sequence at position −283/−278 (E-box1) [Leśniak et al., 2000], is recognized by the transcription factor USF and that the prevailing form of USF bound to these E-boxes is the USF1/USF2 heterodimer. Thus, there are at least two potential USF binding sites, each represented by a canonical E-box sequence, CACGTG, in the S100A6 gene promoter. To ensure that the binding of USF observed in EMSA reflects an in vivo interaction occurring on the promoter we employed the chromatin immunoprecipitation assay. Our results show that in Hep-2 cells USF interacts in vivo with at least one, and most probably with both, of the E-box sequences within the S100A6 gene promoter. In accordance with this finding mutation in E-box2 resulted in a significant reduction in the promoter activity. This inhibition, 29% of control value, is comparable to that exerted by mutation of E-box1 in the context of the −361/+134 promoter both in HEK293 (Fig. 4) and in Hep-2 cells [Leśniak et al., 2000]. Moreover, the depletion of the endogenous USF pool which affects binding to both E-box sequences, using a "decoy" assay, led to 50% inhibition of transcription. It is thus fully justified to consider this transcription factor as an important in vivo mediator in the regulation of the S100A6 gene expression. Since decoy oligonucleotides are regarded as potential tools in anti-cancer therapies [Cho-Chung, 2003] our results offer a possible strategy to hamper increased calcyclin expression in cancer. We have also shown that phosphorylation of USF1 by the p38 kinase is not critical for its function in the regulation of the *S100A6* gene basal transcriptional activity.

The use of microarray technology makes possible identification of a wide spectrum of genes whose expression is changed under specific experimental conditions. Recent data obtained by this technique showed S100A6 mRNA to be one of these most upregulated by incubation of MN16 cells, a model for pancreatic β-cells, in the presence of palmitate and oleate [Busch et al., 2002]. In previous studies, the presence of calcyclin in pancreatic β-cells was functionally linked to insulin secretion [Niki et al., 1997]. Since fatty acids increase the basal level of insulin secretion upregulation of the S100A6 protein level may be linked to this phenomenon. An increase in S100A6 gene expression was also noticed in studies on visceral and subcutaneous adipose tissue in obese people [Linder et al.,

2004]. It has been reported that proteins homologous to S100A6, namely the S100A8/A9 heterodimer and S100A7 are engaged in fatty acid binding [Siegenthaler et al., 1997; Hagens et al., 1999]. Thus, in non-β cells, upregulation of calcyclin expression may be linked to the elevated level of fatty acids. In this work, we show that stimulation of S100A6 expression by palmitate is due to transcriptional activation of the gene. Since data on the signaling properties of fatty acids are rather scarce, we extended our observations to gain more insight in the molecular mechanism involved in the stimulation of S100A6 gene promoter activity by palmitate. Studies on palmitate-induced expression of interleukin-6 in human myotubes pointed to the role of NF-κB transcription factor in cell response to palmitate on the basis of its increased binding to DNA, IκB phosphorylation, and rapid degradation [Weigert et al., 2004]. However, no effect of the mutation in NF-κB binding site on palmitate-induced IL-6 expression was studied. Work on the uncoupling protein-2 gene in β-cells which, similarly to the calcyclin gene, can be stimulated by both palmitate and oleate, implicated the sterol response element (SRE) and E-box sequences in a coordinated action in response to fatty acids [Medvedev et al., 2002]. Thus, two candidate regulatory elements, that is, E-box and NF-κB binding sequence [Joo et al., 2003] possibly engaged in the response to fatty acids are present in the S100A6 gene promoter. To differentiate the role of these regulatory elements in response to palmitate we compared activity of the −1371/+134 promoter fragment containing all the identified elements with that of the −361/+134 fragment containing only E-box1. We found that the −361/+134 fragment of the S100A6 gene promoter is also, although to a lesser extent, responsive to palmitate. Furthermore, mutation in E-box1 completely abolished the effect of palmitate suggesting that this element was indispensable for the observed activation at least within the context of the shorter promoter fragment. Since overexpression of USF1 did not have any effect on the stimulation exerted by palmitate it may be speculated that the USF1/USF1 homodimer may not have, even when overexpressed, sufficient transactivating properties compared to the USF1/USF2 heterodimer or USF2 homodimer. The latter one, rather than USF1, was shown to be indispensable for the response of L-type pyruvate kinase to glucose in USF2 −/− mice [Vallet et al., 1997]. Since the metabolic (and probably also the signaling) pathways of glucose and fatty acids are highly intertwined it is possible that USF2 is also a necessary mediator of fatty-acid effect. Alternatively, the E-box-USF interaction in the S100A6 gene promoter may indirectly contribute to the stimulatory effect of palmitate by facilitating protein–DNA interactions occurring elsewhere in the promoter. In this respect studies on the uncoupling protein-2, a protein implicated in insulin secretion, showed that E-box sequence played a supportive role in response of its gene promoter to oleate since, although USF overexpression was not stimulatory, E-box mutation abolished the stimulation [Medvedev et al., 2002]. The presence of the E-box sequence was also necessary but not sufficient to activate fatty acid synthase gene upon feeding [Latasa et al., 2003]. In both cases the sterol response element binding protein (SREBP), viewed as an intermediate in lipid signaling [Shimano, 2002], appeared to be indispensable. Although there is no consensus SRE in the −361/+134 promoter fragment, SREBP was earlier shown to be able to interact with E-box sequence [Kim et al., 1995] so its involvement in the stimulation of the S100A6 gene promoter activity cannot be excluded at this stage. Thus, we have shown that the S100A6 gene promoter activity is stimulated by palmitate and that the E-box sequence is indispensable for this process.

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