Identification of a functional 3′,5′-cyclic adenosine monophosphate response element within the second promoter of the mouse somatostatin receptor type 2 gene

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Abstract Important physiological actions of somatostatin are mediated by the somatostatin receptor type 2. Its transcription is regulated by three tissue specific, alternative promoters. It is known that the mRNA of the somatostatin receptor type 2 gene is induced by cAMP, but little is known about the mechanisms underlying this regulation. We have identified and characterized a cAMP responsive element located at nucleotide −162 on the second promoter of the gene consisting of the classical palindromic octameric sequence 5′-TGACGTCA-3′. Using transient expression of reporter gene deletion constructs in NG108-15 cells the necessity of the intact element for forskolin-induced reporter gene activity was demonstrated. The first and the third promoter are not responsive to forskolin, nor did any promoter respond to the phorbol ester PMA. Electrophoretic mobility shift assays in combination with competition experiments suggest the interaction of the promoter element with the cAMP responsive element binding protein.

Key words: Somatostatin receptor 2; cAMP responsive element; Transcription regulation

1. Introduction

Somatostatin was initially isolated from ovine hypothalamic extracts as an inhibitor of growth hormone release from cultured pituitary cells [1]. Later, it became evident that somatostatin exerts diverse biological functions in many other tissues, for example the inhibition of insulin and glucagon release from pancreas [2], paracrine inhibition of gastric acid secretion [3] and neurotransmission and neuromodulation in the central nervous system [4].

The cellular actions of somatostatin are mediated by five distinct types of G protein coupled somatostatin receptors (sst1–5) [5–12]. Sst2 is alternatively spliced into two different receptor isoforms, a long (sst2A), and a short (sst2B) variant [13]. The structure of the mouse sst2 gene was recently analyzed by our group: the gene contains two previously unrecognized exons, separated by large introns. The first three exons are preceded by individual promoters, which are involved in tissue specific expression of the sst2 gene. The second promoter in front of exon 2 seems to be the main promoter, since it is used in tissues in which somatostatin has important functions such as brain, pituitary and pancreas. In addition, it contains a putative cAMP responsive response element (CRE) with the consensus sequence 5′-TGACGTCA-3′ [14]. A previous study has shown that the expression of sst2 mRNA can be induced by the second messenger cAMP [15]. Classically, elevation of intracellular cAMP levels leads to an activation of protein kinase A (PKA). The activated catalytic subunit of PKA enters the cell nucleus, where the cAMP response element binding protein (CREB) is activated by phosphorylation. Binding of phosphorylated CREB to a CRE within a promoter results in enhanced transcription of the gene (for review see [16]). In this report we demonstrate the functionality of the putative CRE of the sst2 promoter using transient transfection experiments and electrophoretic mobility shift assays (EMSA).

2. Materials and methods

2.1. Promoter constructs

For reporter gene constructs the chloramphenicol acetyltransferase (CAT) gene containing reporter plasmid pBLCAT2 [17] was used. Promoter sequences were inserted into the vector by simultaneously replacing the thymidine kinase promoter of pBLCAT2 with sst2 gene promoter sequences. Three series of sst-CAT reporter gene constructs were cloned with the following sst2 sequences: (i) a 672-bp *Pst*I/*Stu*I fragment including the first promoter and 247 bp of exon1 (P1-425), (ii) a 1318-bp *Sac*I/*Xho*I insert containing the promoter 5′ of exon2 and 181 bp transcribed sequences (P2-1137), and (iii) a 640-bp *Sac*I fragment including the transcription start site within exon3 (P3-572). Deletion constructs were made by cutting and religating sst-CAT-plasmids with appropriate enzymes or with the method described by Lin et al. [18]. The CRE deletion P2-1137Δ−606/−41 was obtained by deletion of a 561-bp *Sac*II fragment and religation of the vector. For P2-1137ΔCRE, P2-1137 was cut with *Aat*II, a restriction enzyme which recognizes the central 6 bp of the CRE, then the cohesive ends were removed by T4 DNA polymerase and finally the plasmid was religated, turning the original 5′-TGACGTCA-3′ into 5′-TGCA-3′.

2.2. Cell culture

NG108-15 cells were cultured at 37°C with 10% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and HAT (0.1 mM hypoxanthine, 10 μM aminopterin, 17 μM thymidine).

2.3. Transfection and CAT assay

For transfections 1×10^6 cells were plated on 5-cm tissue culture dishes and allowed to grow for 4–6 h. Then medium was replaced and cells were transfected with 8 μg plasmid DNA using a calcium phosphate method as described in Chen and Okayama [19]. After 16–18 h medium was renewed again and cells were allowed to grow for 48 h before harvesting. Harvesting and CAT ELISA were performed according to the protocol of the CAT ELISA kit from Boehringer Mannheim.

2.4. Nuclear protein extraction

NG108-15 cells were harvested in cold phosphate buffered saline (PBS) and collected in a 50-ml plastic tube. The cells were pelleted and washed once in cold PBS. The volume of the pellet (PCV) was estimated and the cells were resuspended in 5× PCV buffer A (10 mM HEPES-KOH, pH 7.6, 10 mM KCl, 0.15 mM spermine/HCl, 0.5 mM spermidine, 1 mM EDTA, 0.5 mM DTT). During an incubation of at

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least 10 min on ice, the cells were checked under microscope to ensure swelling of the cells. The cells were centrifuged at 800×*g* and 0°C for 5 min and resuspended in 2× PCV of buffer A. The cells were broken in a Dounce homogenizer by several strokes with a tight pestle, which was also monitored under microscope. When 90% of the cells were broken, one-tenth volume of buffer A-iso (10 mM HEPES-KOH, pH 7.6, 100 mM KCl, 0.15 mM spermine/HCl, 0.5 mM spermidine, 1 mM EDTA, 0.5 mM DTT) was added, mixed gently and then pelleted with 1100×*g* and 0°C for 10 min. The pelleted crude nuclei were resuspended in 2.5 ml/10^9 used cells nuclear lysis buffer (10 mM HEPES-KOH, pH 7.6, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM DTT). The suspension was put in an ice-water bath, and one-tenth volume of 4 M $(NH_4)_2SO_4$ was added dropwise and stirred for 30 min. After centrifugation at 90 000×*g* and 0°C for 40 min, the supernatant was recovered, put on a magnetic stirrer and in ice-water bath, and 0.3 g/ml of solid $(NH_4)_2SO_4$ was added slowly. After another centrifugation (90 000×*g*, 0°C, 25 min) the pellet was carefully resuspended in one-tenth PCV of dialysis buffer (25 mM HEPES-KOH, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT). The nuclear extract was dialyzed twice against 200 ml dialysis buffer at 4°C (average pore diameter, 24 Å), frozen in aliquots in liquid nitrogen, and stored at −80°C.

2.5. Electrophoretic mobility shift assay

Synthetic oligonucleotides (MWG Biotech, Ebersberg, Germany) carrying putative transcription factor binding sites were labeled with [γ-^{32}P]ATP (Amersham, Braunschweig, Germany) according to standard methods [20]. For each reaction 5000 cpm labeled probe DNA was incubated with 2 μl NG108-15 cell nuclear extract for 30 min at room temperature in a 20-μl reaction mixture containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol and 1 μg poly(dI-dC) DNA. Some reactions also contained various amounts of competitor DNA. Samples were loaded onto 6% polyacrylamide gels and separated electrophoretically. The gels were dried and exposed to Kodak X-Omat AR films.

2.6. Statistical analysis

For statistical evaluation Student's *t*-tests were performed. Stars (*) indicate significantly different values ($P < 0.05$).

3. Results and discussion

Recently, the complete 5′ regulatory region of the sst2 gene has been characterized, identifying three promoters as shown in Fig. 1A [14]. The three promoters (P1–3) were assayed in transiently transfected NG108-15 cells. This was done (i) to test the putative CRE on the second promoter and (ii) to exclude the possibility that other promoter elements mediate a cAMP response, as was shown for AP-1 [21,22] and AP-2 [23].

First, basal CAT activities of reporter gene constructs of P1–P3 were compared to the reporter gene activity under control of the herpes simplex virus thymidine kinase promoter (pBLCAT2), which was defined as 100% (Fig. 1B). For the P1-425 construct an activity of 109%, for P2-1137 161% and for P3-572 118% was observed. Then CAT activities for all constructs were measured after stimulation with forskolin (1 μM). No significantly altered CAT activities could be detected for constructs carrying sequences of the first and third promoter (P1-425 and P3-572). Testing the putative CRE bearing second promoter P2-1137, a 2.2-fold significant induction of CAT activity was observed after forskolin administration, indicating the involvement of the putative CRE in cAMP induced transcription. For further characterization of this effect, a series of 5′ deletions of the second promoter (P2-181, P2-85)

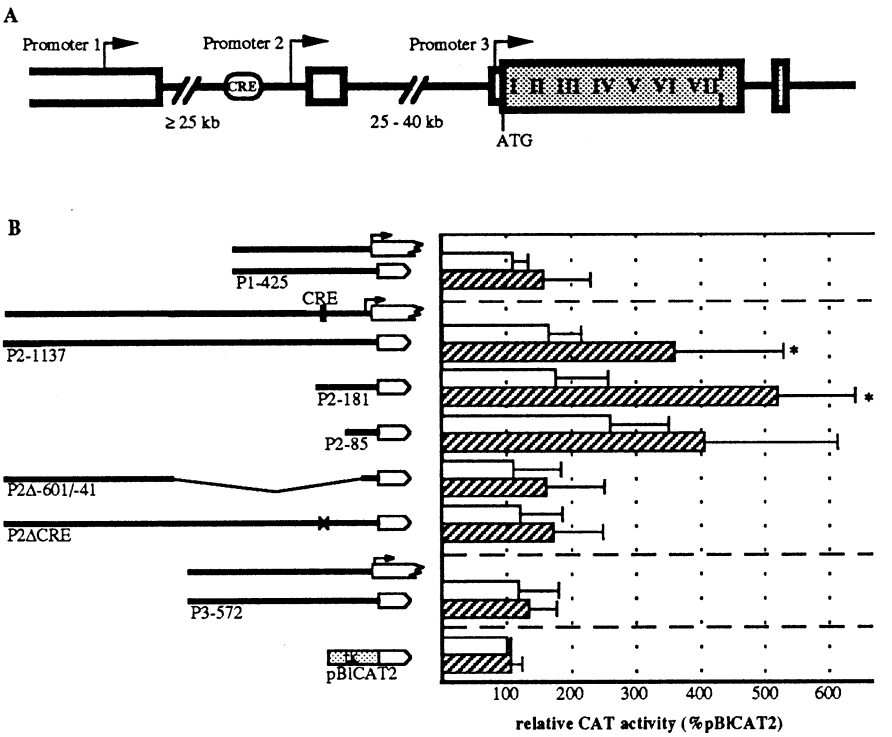

Fig. 1. A: Genomic organization of the sst2 gene. Exons are shown as boxes, translated parts of the exons are shaded, transmembrane domains I–VII are indicated. Lines represent intronic sequences. Three transcription initiation sites are indicated as arrows. A putative CRE within the second promoter is depicted. B: Functional analysis of the sst2 promoter. NG108-15 cells were transiently transfected with sst2-CAT reporter gene constructs designated after the respective promoter (P1, P2, P3). Numbering of the reporter genes is according to the transcription initiation site (arrows) of the respective promoter. The CRE within the second promoter is indicated (filled box). Basal CAT activities (open bars) and activities after forskolin stimulation (1 μM, dashed bars) are expressed relative to that of pBLCAT2 which contains the herpes simplex thymidine kinase (tk) promoter. Data are shown as the mean ± S.E.M. of at least three independent experiments performed in duplicate.

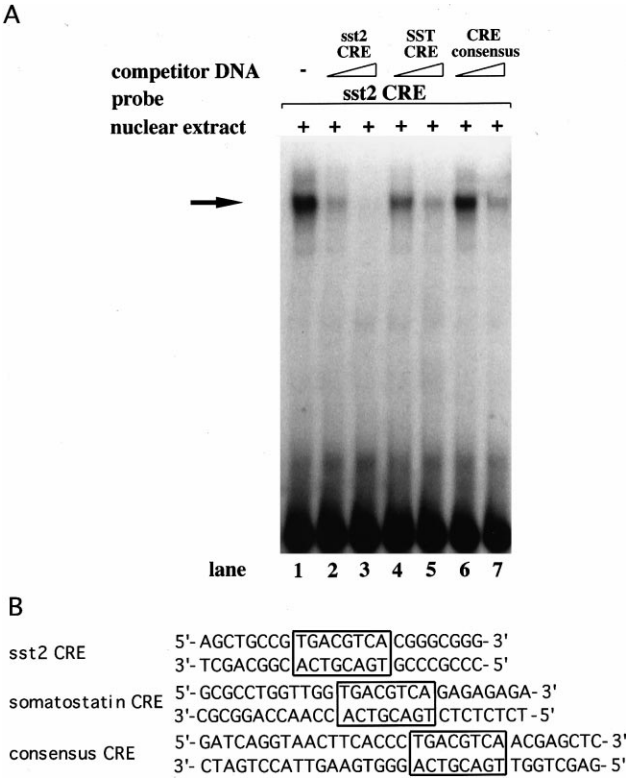

Fig. 2. A: Electrophoretic mobility shift assay with nuclear extract from NG108-15 cells targeting the cAMP responsive element binding protein. ^{32}P-labeled sst2 CRE oligonucleotides were incubated for 60 min with nuclear extract of NG108-15 cells (lanes 2–8). Competition experiments were performed by incubating with 5-fold or 25-fold molar excess of unlabeled oligonucleotides: sst2 CRE (lanes 3–4), somatostatin CRE (lanes 5–6) and consensus CRE (lanes 7–8). The arrow indicates the position of the specific complex. B: Sequence of double-stranded oligonucleotides used for EMSA. The sst2-CRE contains the putative cAMP responsive sequence of the sst2 gene, located at nt −162 to nt −155. The somatostatin-CRE was characterized by Montminy [24]. The consensus-CRE contains the classical CRE sequence with random flanking sequences. The solid box indicates the CREB recognition site.

was prepared. The P2-181 deletion, which still contains the CRE, exhibited a 3.0-fold induced CAT activity in response to forskolin. In contrast, after deletion of the CRE sequence (P2-85) the CAT activities remained on basal levels after stimulation, indicating that the putative CRE sequence is functional. To provide further evidence for involvement of the CRE, two additional deletions were cloned, which should show that no other sequences than the CRE could mediated the forskolin induced effect. In P2-1137Δ−606/−41 the CRE and adjacent regions were deleted, whereas in P2-1137ΔCRE only the CRE was destroyed by eliminating four nucleotides within the CRE sequence, leaving the rest of the promoter intact. Both constructs showed no altered CAT activities after stimulation. Taken together, these results clearly demonstrate that the CRE within the second promoter of the sst2 gene is the functional element, responsive for the forskolin induced transcriptional activation.

These results are consistent with data published by Patel et al. [15], who reported an induction of sst2 mRNA levels after forskolin stimulation of AtT-20 mouse pituitary cells. Interestingly, the authors showed in their experiments that only the shorter form of two detected mRNA transcripts (2.3 kb, 2.8 kb) was upregulated, speculating that this was the spliced variant sst2B. It is not possible to align the 2.3-kb transcript to a specific promoter or splice variant due to alternative promoter usage, 3′ alternative splicing and varying length of poly(A) tails. One band seen in Northern blot experiments may thus contain several mRNA species. However, these results are in good accordance with our findings that not all mRNA species of sst2 are induced by forskolin but only those deriving from the second promoter. Whereas sst2 mRNA is induced by forskolin, no induction can be detected after PMA stimulation either in Northern blots [15] or in our transfection experiments (data not shown).

Electrophoretic mobility shift assay was used to characterize interactions of nuclear proteins with the sst2-CRE (Fig. 2A). Incubation of the sst2 CRE with NG108-15 cell nuclear extract resulted in a typical 'band shift' (lane 1). The shifted complex was specifically competed by the unlabeled sst2 CRE oligonucleotide (lanes 2 and 3). A similar competition occurred when using an unlabeled somatostatin CRE oligonucleotide or an unlabeled oligonucleotide with a CRE consensus sequence (lanes 4–7; for nucleotide (nt) sequences see Fig. 2B). For the CRE within the somatostatin gene the binding of CREB was clearly demonstrated previously [24]. Considering the competition pattern, it must be assumed that the same nuclear protein binds to all used oligonucleotides, which is most likely CREB.

The cAMP/CREB signalling pathway is an important common mechanism used by many cells and tissues to regulate the expression of a variety of target genes. Interestingly, sst genes are upregulated in many tumor cells. Sst2 is especially highly expressed in meningiomas, neuroblastomas, pituitary adenomas, small cell lung carcinomas, lymphomas, and breast tumors [25,26]. The upregulation of sst genes might be considered as a kind of protective mechanism of the cell by rendering it susceptible to antiproliferative action of somatostatin. This property of stable analogs of somatostatin is used to treat a variety of tumors [27]. In this context one may imagine sst 'manipulation' (artificial up-regulation) [28] in tumor tissue which may be achieved for example via the cAMP/CREB pathway investigated here.

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