Neuron-restrictive silencer factor functions to suppress Sp1-mediated transactivation of human secretin receptor gene

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Abbreviations: ChIP, chromatin immunoprecipitation; hSCTR, human secretin receptor; NRSF, neuron-restrictive silencer factor; NRSE, neuron-restrictive silencer element; PANC-1, human pancreatic ductile carcinoma; shRNA, short hairpin RNA; TSA, Trihostatin A

Abstract

In the present study, a functional neuron restrictive silencer element (NRSE) was initially identified in the 5' flanking region (-83 to -67, relative to ATG) of human secretin receptor (hSCTR) gene by promoter assays coupled with scanning mutation analyses. The interaction of neuron restrictive silencer factor (NRSF) with this motif was later indicated via gel mobility shift and ChIP assays. The silencing activity of NRSF was confirmed by over-expression and also by shRNA knock-down of endogenous NRSF. These studies showed an inverse relationship between the expression levels of NRSF and hSCTR in the cells. As *hSCTR* gene was previously shown to be controlled by two GC-boxes which are regulated by the ratio of Sp1 to Sp3, in the present study, the functional interactions of NRSF and Sp proteins to regulate *hSCTR* gene was investigated. By co-immunoprecipitation assays, we found that NRSF could be co-precipitated with Sp1 as well as Sp3 in PANC-1 cells. Interestingly, co-expressions of these factors showed that NRSF could suppress Sp1-mediated, but not Sp3-mediated, transactivation of *hSCTR*. Taken together, we propose here that the down-regulatory effects of NRSF on *hSCTR* gene expression are mediated via its suppression on Sp1-mediated transactivation.

Keywords: secretin receptor, neuron restrictive silencer factor, Sp-protein, transcriptional regulation.

1. Introduction

Secretin (SCT), a brain-gut peptide belonging to the secretin/vasoactive intestinal peptide/glucagon peptide family, functions to stimulate secretion of bicarbonate, electrolytes and water from pancreatic ductal epithelial cells [1], while its effects on other gastrointestinal tissues including intestine [2], stomach [3] and liver [4] were also suggested. Recently, the neuroactive functions of secretin were studied. It was suggested that in the cerebellum, secretin acts as a retrograde messenger to facilitate GABA release from the presynaptic basket cells, either directly or indirectly via an unknown glutamate source, resulting in potentiating evoked inhibitory postsynaptic currents (IPSCs) in Purkinje cells [5,6]. Recent evidences have suggested that SCT is potentially a neurohypophysial factor [7]. SCT and its receptor (SCTR) overlap with the functions of angiotensin II (ANGII), and more importantly, are needed in mediating the central actions of ANGII-induced responses [8].

The activities of secretin are mediated via a class II G protein-coupled receptor, secretin receptor (SCTR). Using human pancreatic ductal carcinoma (PANC-1) and bovine pancreatic ductal (BPD-1) cells as models, we have previously identified a 106-bp core promoter element (-263 to -158, relative to the ATG start codon) in the 5' flanking region of the human secretin receptor (hSCTR) gene [9]. This core promoter is controlled by the competitive binding of specificity protein 1 and 3 (Sp1 and Sp3) with two functional GC boxes (-240 to –226 and –203 to –194). In addition, the methylation status of CpG dinucleotides in the CpG island which overlaps with the core promoter was also found to be a critical factor to mediate the cell-specific expression of the *hSCTR* [10].

To understand further the spatial and temporal expression of *hSCTR*, in this report, we sought to investigate the functions of a putative neuron restrictive silencer

element (NRSE) located downstream (-83 to -67, relative to ATG) of the *hSCTR* core promoter. NRSE, also known as repressor element-1 (RE-1) with a consensus sequence "NTYAGMRCCNNRGMSAG" [11], was initially identified to regulate a number of neuron-specific genes by repressing their expressions in non-neural tissues [12]. Recently, NRSE is regarded a common repressor element as it can suppress an increasing number of non-neuronal genes. A genome-wide search indicated the presence of about 1,800 putative NRSE sites in both human and mouse genomes [11]. The protein factor that interacts with NRSE is neuron-restrictive silencer factor (NRSF), which is a member of the zinc-finger GLi-Krüppel family [13]. There are three domains in NRSF: a Krüppel-type zinc-finger for binding NRSE, an N-terminal repressor domain for interacting with SIN3 transcription regulator **(**Sin3), and a C-terminal repressor domain for recruiting the corepressor element 1 silencing transcription factor (CoREST). Both Sin3 and CoREST interact with histone deacetylase containing complex to deacetylate core histone proteins, and as a consequence, they work together to silence target genes by forming condensed chromatin structures and mediates developmental stage-specific gene expression [14,15,16]. An *in silico* analysis indicates that all members of the secretin receptor family contain at least one putative NRSE-like motif in their 5' flanking regions [17]. With the knowledge that most of NRSF-regulated genes also possess GC-boxes in their promoter regions [18,19], for this reason, the functional relationships between Sp-protein(s) and NRSF in controlling *hSCTR* expression was investigated. Findings reported here not only provide crucial information regarding the cell-specific expression of *hSCTR*, but also are applicable to the understanding of other GC-box/NRSF co-regulated genes, including several members of the secretin receptor family.

2. Materials and Methods

2.1 Cell culture

All cell lines were purchased from American Type Culture Collection (ATCC). PANC1-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) with 10% FBS. PC12 cells were cultured in DMEM with 10% horse serum and 5% fetal bovine serum (FBS). All cells were cultured at 37°C with 5% CO_2 in a medium supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin (Invitrogen).

2.2 Plasmid construction

Cloning of the *hSCTR* promoter was described previously [9]. The plasmid p263/158 (–263 to –158 relative to the ATG start codon) was generated by exonuclease III/S1 nuclease digestion (Amersham Pharmacia, Arlington Heights, IL) followed by cloning into the *Mlu* I/*Xho* I sites of the pGL-2 basic vector (Promega Corp, Madison, WI). The region -263/-46 was obtained by PCR amplification of human genomic DNA and then subcloned into the *Mlu* I/*Xho* I sites of the pGL2 basic vector (Stratagene, La Jolla, CA). Expression vectors, Sp1/CMV. Sp3/CMV and Sp4/CMV were kindly provided by Prof. C. Paya (Mayo Clinic, Rochester, US). The NRSF expression vector, REEX1/CMV, and the dominant negative expression vector, p73/CMV, were gifts from Dr. Mandel (State University of New York) [20]. The potential shRNA target sites of human NRSF was determined using the Ambion design program (Ambion, CA), and the sequences were BLAST-confirmed for specificity. Sense and antisense oligos were annealed in an annealing buffer (100 mM potassium acetate, 30 mM HEPES (pH 7.4) and 2 mM magnesium acetate) at 90°C for 3 min, followed by incubation at 37°C for 1 h. The annealed DNA was cloned into

the *Apa* I/*Hind* III sites of pSilencer 1.0-U6 shRNA expression vector (Ambion) for constructing shNRSF vector. As a negative control, siControl (Ambion) with a sequence that shares no significant identity to any known gene sequence was used.

2.3 PCR-linker Scanning Mutagenesis

Site-directed mutagenesis was carried out by a three-step PCR method (Nelson & Long 1989), using mutagenic primers MA, MB, MC, MD, ME, M1, M2 or DM and universal primers GL2-MP, GL1 and MP (Table 1). The region –263/-158 was initially subcloned into *Mlu* I/*Xho* I sites of pGL2, and this construct was used as a template for all PCR mutagenesis. Mutations introduced were subsequently confirmed by DNA sequencing.

2.4 Transient transfection assay

PANC-1 and PC12 cells were plated at a density 2.5 x 10^5 cells / 35 mm well (six-well plate, Costar). After 2 days incubation, promoter-luciferase construct and β-gal control vector were cotransfected into cells using the Genejuice reagent (Novagen, Darmstadt) according to the manufacturer's protocol. Cells were harvested 48 h after transfection and cell extracts were assayed for luciferase and β-galactosidase activities as described previously [21]. For *in vitro* functional studies, 2 days after plating, promoter constructs and various amounts of the expression vector (Sp1/CMV, Sp3/CMV, Sp4/CMV, REEX1, and p73) and/or shRNA vector in a total of 3.5 μg DNA (adjusted by pBluescript KS^+) were cotransfected into cells by Genejuice reagent. The transfected cells were incubated for two more days before luciferase and β-galactosidase activities were determined.

2.5 Co-immunoprecipitation and gel mobility shift assay

PANC-1 nuclear extract was prepared as described before [22], and was incubated with 2 μg of the appropriate antibodies (NRSF, Sp1, Sp3 and Sp4, Santa Cruz Biotechnology, CA) for 2 h at 4^{o}C. Protein G-agarose beads (20 μl) were used to pull-down the Ab-Ag complex. Western blotting was performed essentially according to a protocol described earlier [23]. The presence of NRSF in the nuclear extract was visualized by the ECL system (Amersham Biosciences, Buckinghamshire, UK). For gel mobility shift assays, double-stranded oligonucleotide probes (DNA sequences listed in Table 1) were end-labeled with [γ-^{32}P] by the Ready-To-Go T4-polynucleotide kinase labeling kit (Amersham Biosciences). Gel mobility shift assays were carried out at room temperature for 20 min in a 20 μl reaction mixture containing 10 mM Tris (pH 7.5), 50 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 4% glycerol, 2 μg poly(dI:dC) (Amersham Biosciences) and 1 pmole probe. Free and bounded probes were separated by electrophoresis in a 5% polyacrylamide gel. For competition assays, a graded concentration of unlabeled DNA was added with the labeled probe. In the supershift assay, antibody against NRSF (sc-15118X) or AP-2 (sc-8975X) (Santa Cruz Biotechnology) was included in the reaction mix.

2.6 Chromatin immunoprecipitation (ChIP) assay

The ChIP assays were performed essentially according to Baek *et al.* [24]. PANC-1 cells were cross-linked with 1% formaldehyde for 15 min at room temperature. Cells were harvested by centrifugation and resuspended in the lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml Aprotinin and 1.5 μg/ml Pepstatin A). After sonication in

Sonifier 450 (Branson, Danbury, CT), 10 μg antibody and 20 μl Protein A/G plus agarose were added to precipitate the DNA-protein complex. Precipitated DNA-protein complex was washed in the ChIP buffer (0.1% SDS, 1% Triton X-100, 0.1% Na deoxycholate, 140 mM NaCl , 1 mM PMSF, 1 μg/ml Aprotinin and 1.5 μg/ml Pepstatin A) and eluted in the elution buffer (1% SDS and 0.1 M $NaHCO_3$). The mixture was incubated at 65°C for 4 h to reverse the formaldehyde cross-linking. Proteins were digested by proteinase K (200 μg/ml) at 45°C for 2 h and followed by phenol/$CHCl_3$ extraction. The extracted DNA was used for PCR using the primers showed in Table 1. For real-time PCR analysis, the extracted DNA was amplified by the SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA) and the signal was detected by the iCycler iQ detection system (Bio-Rad, Hercules, CA). The percentage recovery was normalized by the Ct value from the input control.

2.7 Real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis

Total RNAs from cells were isolated using the TriPure reagent (Roche Molecular Biochemicals, Switzerland) and reverse-transcribed (5 μg) by an oligo-dT primer and Superscript III (Invitrogen). One-tenth of the first strand cDNAs was used for real-time PCR analysis, in which secretin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels were measured by an Assays on Demand System (ID: Hs00161610_m1; Applied Biosystems) or Pre-developed Taq-man probe with the Taqman Universal Master Mix (Applied Biosystems) using the iCycler iQ detection system (Bio-Rad). Relative expression levels of transcripts were calculated by the $2^{-\Delta\Delta Ct}$ method [25] using GAPDH as the endogenous control.

2.8 Data analysis

Data from the transfection assays were shown as the means ± SEM of triplicate assays in at least three independent experiments. Data from quantitative PCR were shown as the means of ± SEM of duplicate assays in at least three independent experiments. All data were analyzed by one-way ANOVA and followed by Dunnett's test using the computer software PRISM (version 3.0, GraphPad Software Inc., San Diego, CA).

3. Results

3.1 Identification of a functional NRSE in the 5' flanking region of the hSCTR gene

In human and mouse *SCTR* genes, putative NRSE sites at -83/-67 and -314/-298, respectively, relative to the ATG codon were identified (Figure 1A). To initially investigate whether this motif is functional in *hSCTR*, several DNA fragments with different 3' regions (ranged from -158 to -1) were linked to the luciferase reporter gene for transient promoter assays using the human pancreatic PANC-1 cell as a model. Constructs with the core promoter and the putative NRSE (p-263/-45, p-263/-1) exhibited promoter activities significantly lower than that of the control (core promoter construct p-263/-158) (Figure 1B). To locate the repressor/silencer element, scanning mutants (MA to ME Figure 1C) were constructed, and among them, changes of NRSE sequences led to either complete or partial recovery in promoter function (MD: 101%; ME: 79% vs control p-263/-158). The importance of NRSE was then

confirmed by mutating the core nucleotides [11] of the motif (Figure 1D). In this study, mutation of either 1 or 2 core nucleotides resulted in partial but significant recovery of promoter activities (M1: 49% recovery, M2: 71%, and DM: 56%). Taken together, the conserved NRSE is a functional motif that down regulates the *hSCTR* gene expression.

3.2 In vitro and in vivo interaction of NRSF with NRSE in the hSCTR gene

To identify the transcription factor(s) that interacts with the NRSE, initially, gel mobility shift assays were performed. Using an NRSE-I oligo, two specific DNA-protein complexes were observed in PANC-1 nuclear extract (complexes A and B, Figure 2A, lanes 2 to 4). These complexes were specific as only cold NRSE-I or NRSE consensus (rM4RE1) [11,26,27], but not non-specific oligo, could abrogate their formation (Figure 2B). Consistently, as shown in supershift assays, addition of NRSF antibody reduced the intensities of these complexes (Figure 2C, lane 3) while the addition of an un-related AP2 antibody or non-specific bovine serum albumin (BSA) was ineffective in supershifting these complexes (Figure 2C, lanes 4 and 5).

To show the *in vivo* binding of NRSF to NRSE, ChIP assays (from -263 to -45) were performed (Figure 2D). In this study, we observed no PCR signals from the negative controls (anti-rabbit IgG, lane 3; No immunoprecipitation, lane 5; and PCR

negative, lane 6), indicating that there were neither non-specific precipitations nor PCR contamination. Positive PCR signals were detected only when NRSF antibody was used (lane 4, sample; lane 2, positive control). Moreover, by amplifying the exon 3 to intron 4 sequence of hSCTR as the control, there are no positive signal was observed in all antibody precipitation, including the NRSF antibody. This further confirmed the specificity of the ChIP assay. Similar results were also observed in ChIP-real time PCR assay (Figure 2E). NRSF immunoprecipitation shows strongest signal (with 34% recovery) when compare to IgG and No IP control (<5% recovery). In summary, gel mobility shift assays and ChIP assays showed the *in vitro* and *in vivo* interaction of NRSF with the *hSCTR* promoter.

3.3 Transcriptional down-regulation of hSCTR gene by NRSF

To investigate the *in vivo* roles of NRSF to repress *hSCTR* expression, an NRSF expression vector (REEX1) [20] was cotransfected with the NRSE-containing promoter construct p-263/-45 into PC12 cells. We used PC12 cells as these cells do not express endogenous NRSF [28].

As shown in Figure 3A, over-expression of NRSF caused a significant and dose-dependent decrease in *hSCTR* promoter activity in PC12 cells. The *hSCTR* promoter activity was decreased 53% when 2.0 μg of REEX1 was transfected into

PC12 cells. In the *hSCTR* expressing PANC-1 cells, over-expression of NRSF also led to a significant drop in *hSCTR* transcript levels (Figure 3B). By real-time RT-PCR, a 64% decrease of *hSCTR* mRNA was observed when 2.0 μg NRSF vector was transfected into the cells. These data therefore confirmed that an increased expression of NRSF could down-regulate *hSCTR* promoter, leading to reduced *in vivo* transcript levels of *hSCTR*.

To better reflect the *in vivo* actions of NRSF, we sought to knock-down endogenous NRSF by a vector-based shRNA approach. The knock-down effect of the shRNA (shNRSF) on NRSF expression level was confirmed by Western blotting (Figure 3E). As shown in the figure, the increase of shNRSF vector in the transfection can significantly reduce the protein level of NRSF in the transfected PANC-1 cells. By cotransfecting shNRSF with the promoter constructs, a significant augmentation of luciferase activity was observed only in the NRSE-containing promoter (p-263/-45: 2.7-fold against control with no shNRSF, Figure 3C), but not in the core promoter (p-253/-158). Finally, we found also that the shNRSF vector could up-regulate endogenous *hSCTR* expression (Figure 3D: 3.8-fold increase in *hSCTR* transcript level). In summary, changes in NRSF concentrations in PANC-1 cells could oppositely regulate *hSCTR* expression and this effect is mediated by the NRSE cis-acting motif located at the 5' region of the *hSCTR* gene.

3.4 NRSF-mediated transcriptional down-regulation is Sp1 dependent

As it has previously been shown that *hSCTR* is controlled by the Sp1/Sp3 ratio [10] and the repressor effects of NRSF is mediated via inhibition of Sp1 [18], we therefore investigated the potential interactions of NRSF and Sp-proteins in regulating *hSCTR* promoter in PANC-1 cells. To show the physical contact of Sp-proteins with NRSF on *hSCTR* promoter, we performed co-immunoprecipitation assays. As shown in Figure 4A, NRSF co-immunoprecipitated with Sp1 and Sp3 antibody and provided strong signals in the Western blot. Consistently, the NRSF-immunoprecipitated proteins contained also Sp1 and Sp3 as shown in the Western blots (Figure 4B, C). These data showed the endogenous interactions of NRSF with Sp1 and Sp3 in PANC-1 cells, although we cannot prove that these interactions are *hSCTR*-promoter specific. The next series of experiments were then designed to test the *in vivo* functional interactions of Sp1 and Sp3 with NRSF. By over-expressing Sp1, Sp3 or Sp4 with p-263/-45 (Figure 5A), we found that, Sp4 consistently had no effect, while both Sp1 and Sp3 could activate the NRSE-containing promoter. More interestingly, the activities of Sp1 and Sp3 in the NRSE containing promoter were different from our previous [10] and also present (Figure 5D) studies with the core promoter (-263 to -158). When the core promoter was used, Sp1 was a stronger activator comparing

with Sp3 (Figure 5D, Sp1: 2.5-fold vs Sp3: 1.2-fold, respectively). However, when the NRSE-containing promoter was employed, Sp3 was a stronger activator on promoter activity (Fig 5B, Sp3: 3.7-fold vs Sp1: 2.2-fold). These data suggest differential functions of Sp1 and Sp3 in controlling *hSCTR* expression in the presence or absence of NRSF.

To investigate the mechanisms underlining the differential activities Sp1 and Sp3, we co-expressed Sp1 or Sp3 with NRSF or a dominant negative NRSF p73 [20] (Figure 5B). NRSF over-expression significantly reduced the Sp1-mediated activation (from 219% to 60.3% against p-263/-45), whereas over-expression of the dominant negative NRSF p73 had no effect (Fig 5B). On the contrary, the Sp3-mediated transactivation was not significantly affected by NRSF or p73 (Fig 5B: lanes 7-9). To confirm these observations, shNRSF was used to knockdown the endogenous NRSF in PANC-1 cells in normal and Sp-protein over-expressed conditions. In the absence and presence of Sp1, silencing of endogenous NRSF could up-regulate *hSCTR* promoter activity (Fig 5C: lanes 1-6), while shNRSF seemed to exhibit no effect in Sp3-over-expressed cells (Fig 5C: lanes 7-9). As a control, over-expressing or silencing of endogenous NRSF had little effects on the core-promoter without the NRSE (Figure 5D). Taken together, our data suggested that the differential functions of Sp1 and Sp3 is a result of the specific inhibition of NRSF on

Sp1-mediated transactivation in the *hSCTR* promoter. It was previous reported that NRSF could recruit histone deacetylases (HDAC) to repress gene expression through chromatin remodeling. In this study, when a HDAC inhibitor (Trihostatin A, TSA) was employed, *hSCTR* promoter (-263/-45) activity was significantly increased in Sp1- (1.8-fold increase), but not Sp3-transfected cells (Figure 6). In the control experiment, when the NRSE motif was removed from the promoter (DM), the TSA treatment did not show significant effects on the promoter activities after either Sp1 or Sp3 oeverexpression. Our present data, taken together, therefore suggest that NRSF, via its interactions with Sp1 and HDAC, functions to repress *hSCTR* gene.

4. Discussion

4.1 NRSF and cell-specificity of secretin receptor

Secretin receptor was previously considered to be localized to classical sites such as the pancreatic and biliary ductal cells. Now it has been demonstrated that secretin receptors are expressed in specific cell types in various parts of the brain [5,29,30] as well as peripheral tissues [31.32,33]. The spatial and temporal regulation of this receptor is the key to understanding secretin's physiology, but remains largely unknown. The present study provides new information regarding the transcriptional mechanisms controlling *SCTR* gene involving NRSF. NRSE was originally found in

the 5' flanking region of the gene encoding the voltage-gated sodium type II channel [34]. NRSF silences NRSE-containing genes in a cell-specific manner and it is expressed in many cells except mature neurons and pancreatic β-cells [28]. NRSF are reported to regulated many G-protein coupled receptors, such as GPR10 [35], metabotropic glutamate receptor 1 [36], mu opioid receptor [37, 38], and AMPA glutamate receptor subunit GluR2 [39].

In the present study, we demonstrated the repressor effects of NRSF by binding onto the NRSE motif (-83 to -67, *hSCTR* gene), hence, we proposed here that this mechanism could be responsible for the suppression of *SCTR* gene in *SCTR*-expressing and non-SCTR expressing cells. In this study, we have used the *hSCTR*-expressing PANC-1 cell [9,10] that also expresses NRSF (Western blotting, data not shown). As previous studies [13,20,40], a threshold level of NRSF is required for complete silencing of transcription. As shown here, over−expression of NRSF could still reduce *hSCTR*, suggesting that the endogenous NRSF level in PANC-1 cells is not sufficient to completely silence the *hSCTR* gene. In summary, in NRSF-expressing PANC-1 cell, the endogenous NRSF level is therefore a key factor in controlling *SCTR* expression.

Other than the well known gastrointestinal functions of SCT, our recent data also suggested that SCT is potentially a neurohypophysial factor [7] and it is an important

central regulators in osmoregulation [8] and appetite control [41]. We showed that the SCTR is expressed in hypothalamus, including the arcuate nucleus (Arc), supraoptic nucleus (SON) and paraventricular nucleus (PVN) [8, 41]. It is interested to note that the expression of NRSF is lower in brain, hypothalamus and pancreatic islets [42]. The contrary expression pattern between SCTR and NRSF further suggests the role of NRSF in down-regulating the SCTR. It has been shown that the expression of corticotropin-releasing hormone can be reprogramed by the NRSF expression in stress-responsive hypothalamic neurons [43]. Recently, we found that the expression of SCT in hypothalamus was up-regulated by hypertonicity and angiotensin II stimulation [44]. Therefore, the expression of hSCTR may be regulated by similar stimulation through the NRSF in hypothalamus and in other tissues.

4.2 Mechanism of NRSF's repressor activity on secretin receptor gene

Recent studies have suggested that the transcriptional repression mediated by NRSF is Sp1-dependent [18]. In our previous works, we showed that Sp1 is a predominant activator, whereas Sp3, itself a weak activator, inhibits *hSCTR* expression by competing with Sp1 for the same binding sites [10]. As Sp1/Sp3 ratio varies in different cells and also at different stages of the cell cycle [45,46], our data indicate the importance of the stochastic ratio of Sp1 and Sp3 in the control of *hSCTR*

gene expression. In the present study, we provided evidence that NRSF could specifically down-regulate Sp1-mediated transactivation. Our data clearly suggested that NRSF interacts with both Sp1 and Sp3 (Figure 4). Hence, the specificity of NRSF on Sp1 is not likely due to differential interactions. Actually, in a TATA-less promoter such as the *hSCTR* promoter, Sp1 facilitates binding of transcription factor IID (TFIID) to recruit factors for forming the preinitiation transcriptional complex [47,48,49]. It is possible that interactions of NRSF with Sp1 could block the binding of Sp1 with other transcriptional-associated proteins and finally prevent the formation of the preinitiation complex. Alternatively, it has been reported that HDAC could interact with Sp1 to form a silencing complex [50]. Since NRSF recruits HDAC via both the C- and N- termini through Sin3 or CoREST, NRSF could enhance formatting of the silencing complex for histone deacylation or gene silencing [18]. Meanwhile, treatment of an HDAC inhibitor, TSA, relieves the down-regulation of NRSF to Sp1 [40], suggesting that HDAC is key to the silencer activity of NRSF.

Our data also indicated a specific role of Sp1, but not Sp3, in linking NRSF with HDAC in changing the local chromatin structure for transcriptional down regulation. This observation is consistent with previous reports showing that a direct interaction of Sp3 and HDAC could not be detected in the two-hybrid system [51]. The difference between the interactions of Sp1 and Sp3 with HDAC may be the key to

understand the differential actions of these Sp-proteins on *SCTR* gene, as well as the secretin receptor super-family expression.

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Figure Legends

Figure 1. A putative NRSE represses the *hSCTR* promoter activity. (A) Sequence comparison of NRSE motifs in human and mouse *SCTR* with NRSE consensus sequence. Conserved nucleotides were highlighted in bold letters. Nucleotides which are necessary for NRSF functions are underlined. Position numbers are relative to the ATG start codon of *SCTR*. (B) The *hSCTR* core promoter with different 3' extension were inserted into the pGL-2 basic vector. Luciferase activities are normalized by β-galactosidase expression and are shown as the fold increases in relative promoter activities compared with the empty vector, pBasic (pGL-2 basic). (C) Scanning mutation analysis of the region (-113/-57). Upper panel: DNA sequences of the region (-113/-57), and the underlined sequences are the sites of the scanning mutations (MA–ME). The sequences in the open box represent the putative NRSE site. Lower panel: A family of 6-bp block mutants were constructed and used for transient promoter assays. The boxes with a cross inside are the mutated sites. (D) Mutation Analysis of the NRSE (-83/-67) site. Two mutants with 4-bp block replacement (M1-M2) and the double mutants (DM) were constructed and used for transient promoter assays. The DNA sequences of NRSE (-83/-67) were shown and the underlined sequences are the sites of mutations. Luciferase activities are normalized by β-galactosidase activity and are shown as the percentage change in relative promoter activities compared with that in core promoter (-263/-158). Values reported in the figure represent the means ± SEM of three independent experiments, each in triplicate. *, ($P < 0.001$ and **, $P < 0.05$).

Figure 2. *In vitro* and *in vivo* interaction of NRSF with *hSCTR* promoter. (A) Gel mobility shift assays using PANC-1 nuclear extracts and NRSE-I oligo. Different

amounts of nuclear extract (ranged from 5 to 15 μg, lane 2 - 4) were incubated with the labeled NRSE-I probe. Arrows indicate specific DNA-protein complexes. (B) PANC-1 nuclear extract (15 μg) was pre-incubated with the competitor (NRSE-I or non-specific oligo; lanes 3 - 6) or NRSE consensus oligo (rM4RE1, lanes 7 - 8). (C) Supershift assays. PANC-1 nuclear extract (15 μg) was pre-incubated with an antibody (2 μg; lanes 3 - 4) specific for NRSF or AP2. BSA was used as a negative control. (D) ChIP assay shows *in vivo* binding of NRSF to *hSCTR* promoter. Chromatin from PANC-1 cells was crosslinked, and immunoprecipitated with anti-NRSF antibody (lanes 4), and used for PCR. Upper: A 194-bp fragment containing the NRSE region of the *hSCTR* promoter is observed. Immunoprecipitation without an antibody (No IP, lane 5) and with a non-specific antibody against rabbit IgG (IgG, lane 3) were carried out as negative controls. Lane 6, PCR negative control (without DNA). Input DNA from fragmented chromatin prior to immunoprecipitation was used as a positive control (lane 2). Lane 1, DNA size standards (100-bp DNA ladder; Invitrogen). Lower: PCR of a 381-bp fragment flanking the exon 3 to intron 4 of *hSCTR* was used as the control. Only the input DNA (lane 1) shows positive signal. No PCR product was found in all the immunoprecipitation samples and negative controls, including the NRSF (lane 4). (E) Real-time PCR analysis of NRSF ChIP assay. After immunoprecipitation, the hSCTR gene promoter was amplified by real-time PCR. N.D., not detectable.

Figure 3. Effects of NRSF on *hSCTR* expression. (A) Overexpression of NRSF in PC12 cells down-regulated the *hSCTR* promoter activity. p-263/-45 was cotransfected with various amounts of NRSF-expression vectors (REEX1) into NRSF-deficient PC12 cells. Total DNA amount was adjusted to 4 μg by pBluescript KS^+ DNA.

Luciferase activities are normalized by protein concentrations and are shown as the percentage increases in relative promoter activities compared with that of the control. Values reported represent the means ± SEM of at least three independent experiments, each in triplicate. * and ** indicate significant difference ($P < 0.001$, $P < 0.05$) from p-263/-45 without REEX1. (B) Overexpression of NRSF decreased *hSCTR* transcript levels in PANC-1 cells. REEX1 (1 or 2 μg) transfected into PANC-1 cells were used for real-time PCR. The *hSCTR* mRNA / GAPDH mRNA ratio was calculated by the $2^{-\Delta\Delta Ct}$ method. The mRNA level in the control (no REEX1) is defined as 1.0. Data represent the means ± SEM of three experiments performed in duplicates. **, $P < 0.05$ vs control. (C) Activity of a NRSE-containing promoter (p-263/-45) was up-regulated by cotransfection of shNRSF. Constructs (p-263/-158 or p263/-45) were cotransfected with either pSilencer empty vector or shNRSF construct (2 μg) into PANC-1 cells. Luciferase activities are normalized by β-galactosidase activity and are shown as the percentage changes in relative promoter activities compared with that in the core promoter (-263/-158). Values reported in the figure represent the means ± SEM of three independent experiments, each in triplicate. *, $P < 0.001$. (D) shNRSF vector increased endogenous *hSCTR* transcripts in PANC-1 cells. The *hSCTR* mRNA/GAPDH mRNA ratio was calculated by the $2^{-\Delta\Delta Ct}$ method. The mRNA level in the control (pSilencer) is defined as 1.0. Data represent the means ± SEM of three experiments performed in duplicates. *, $P < 0.001$ vs control. (E) Effects of silencing endogenous NRSF by shNRSF. shNRSF-1 (0.5, 1.0 and 2.0 μg), empty vector (pSilencer) and generic gene-targeting siControl were used as controls were transfected into PANC-1 cells. Left, Western blot analysis of NRSF protein in PANC-1 cells transfected with (1) pSilencer, (2-4) shNRSF (0.5 - 2.0 μg) and (5) siControl. Right, Coomassie blue staining of a SDS-PAGE run in parallel as a loading control.

Figure 4. NRSF interacts with Sp1 and Sp3. (A) Sp proteins were immunoprecipitated using 0.5 mg total cell lysate from NRSF-expressing PANC-1 cells. The proteins were separated by 8% SDS-PAGE and NRSF was detected by Western blotting using the NRSF antibody. (B and C) Total cell lysate (0.5 mg) was immunoprecipitated with NRSF antibody. Samples were separated by 10% SDS-PAGE and the presence of (B) Sp1 or (C) Sp3 was detected by the corresponding antibodies. Loading controls were done by Coomassie blue staining of SDS-PAGE (Data not shown).

Figure 5. Effects of Overexpressing Sp1, Sp3 and Sp4 on *hSCTR* Promoter (-263 to -64) in PANC-1 Cells. (A) p-263/-64 was cotransfected in the absence and in the presence of 0.5 or 1 μg of Sp1, Sp3 or Sp4 vector. (B) Effects of overexpressing Sp proteins in the presence of NRSF. p-263/-64 and NRSF or DN-NRSF were cotransfected with Sp1 or Sp3 vector. (C) Cotransfection of p-263/-64 with shNRSF and Sp1 or Sp3. (D) The core promoter (p-263/-158) was cotransfected with Sp1 or Sp3 vector in the presence of shNRSF or NRSF. Luciferase activities are normalized by protein concentrations and are shown as the percentage increases in relative promoter activities compared with that in the control (p-263/-158 or p-263/-45 alone). Values reported represent the means ± SEM of three independent experiments, each in triplicate. *, $P < 0.001$ vs p-263/-158 or p-263/-45.

Figure 6. Sp1 increased *hSCTR* promoter activity in TSA-treated cells. PANC-1 cells were transfected with p-263/-45 or p-263/-45(DM) in the presence of Sp1 or Sp3 vector. The cells were then treated with 100 nM TSA for 24 h. Relative luciferase activities were expressed in percentage changes against control (no TSA and no

Sp-protein). Values reported in the figure represent the means ± SEM of three independent experiments, each in triplicate. *, $P < 0.001$ vs control.

Name	Oligonucleotide (5′ to 3′)
Site-directed mutagenesis	
MA	GCAGCGGCCGGCATTCTACCGGGACCCTGC
MB	GGCCGGAGCCCGTTCAAATGCGCGGGGCGC
MC	CCGGGACCCTGCTATTTTCGCTGAGCTCCC
MD	CAGCTCGAGCGCTCGGGCTAGACGCGCCCCGCGCA
ME	CTCGGGGCGCAGATTATTGCCGGAGCCCG
M1	CAGCTCGAGCGCTCGGGAGCTCCTTCCCCCGCGCAGGGTCC
M2	CAGCTCGAGCGCTCGGGAGCTCAGCGCCCCCCGACTGGTCCCGGGCTC
DM	CAGCTCGAGCGCTCGGGAGCTCCTTCCCCCCCGACTGGTCCCGGGCTC
ChIP assay	
-45-R	TCGCTCGAGCGCTCGGGAGCTCAG
-238-F	CAGACGCGTCCCGCGCCAGTCCCTGCCG
hSR-exon3-F	ACCTTACGGGCTCTAGGAC
hSR-intron4-R	CCCCTGACCTTGTTCCGG
Gel mobility shift assay	
NRSE-I	ACCCTGCGCGGGGCGCTGAGCTC
Non-specific	GGCCACTAGAGGGAATTAAA
rM4RE1	GGCTTCAGCACCTCGGAGAGCTCC
shRNA construction	
NRSF-sh-top	TACAGTTATGGCCACCCAGTTCAAGAGACTGGGTGGCCATAACTGTATTTTTT
NRSF-sh-bottom	AATTAAAAAATACAGTTATGGCCACCCAGTCTCTTGAACTGGGTGGCCATAACTGTAGGCC
siControl-top	TTCTTCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTT
siControl-bottom	AATTAAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAAGGCC

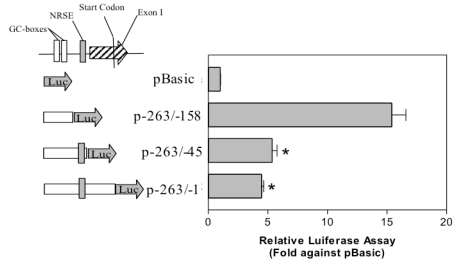
Table 1 Oligonucleotide sequences. Sequences listed are from 5’ to 3’. The underlined regions of oligonucleotides for site-directed mutagenesis indicate the positions that carry mutations.

Figure 1

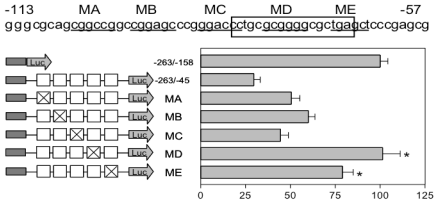
A

Consensus	NTYAGMRCCNNRGMSAG	% Match	Position
Human	CTCAGCGCCCCGCGCAG	88%	-83 to -67
Mouse	TTCAGAGAAGTGGGCAG	82%	-314 to -298

B



C



D

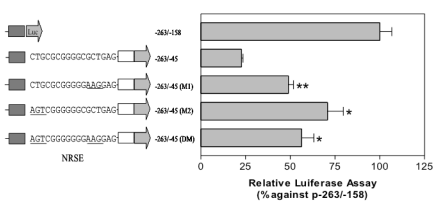


Figure 2

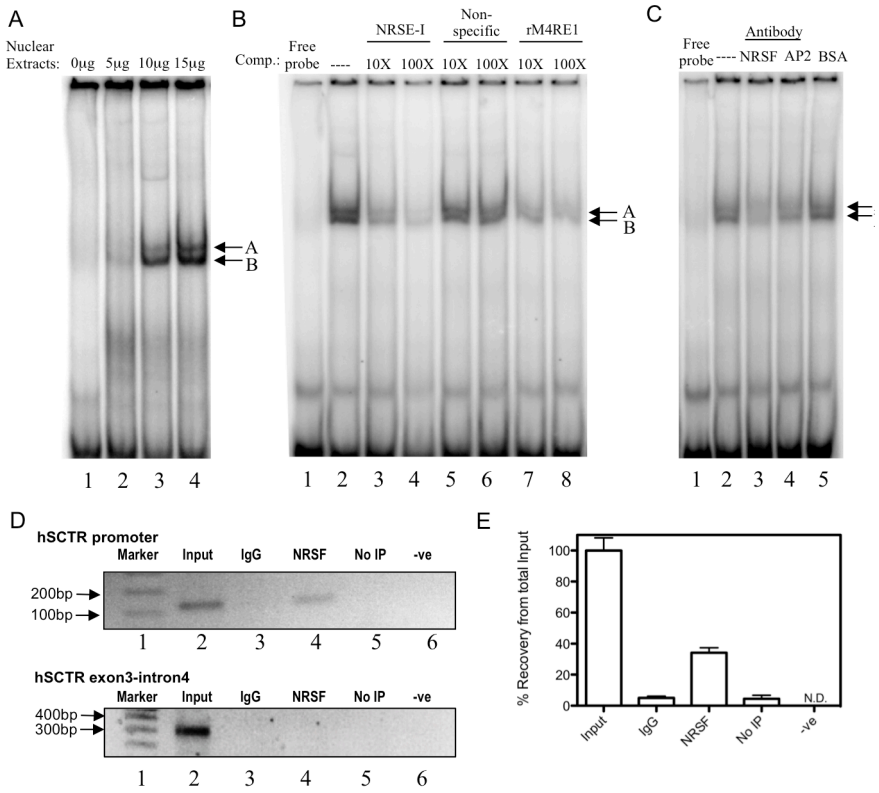

A
Nuclear Extracts: 0µg 5µg 10µg 15µg
A
B
1 2 3 4
B
Comp.: Free probe ---- NRSE-I 10X 100X Non-specific 10X 100X rM4RE1 10X 100X
A
B
1 2 3 4 5 6 7 8
C
Free probe ---- Antibody NRSF AP2 BSA
1 2 3 4 5
D
hSCTR promoter
Marker Input IgG NRSF No IP -ve
200bp
100bp
1 2 3 4 5 6
hSCTR exon3-intron4
Marker Input IgG NRSF No IP -ve
400bp
300bp
1 2 3 4 5 6
E
% Recovery from total Input
100
80
60
40
20
0
N.D.
Input
IgG
NRSF
No IP
-ve

Figure 3

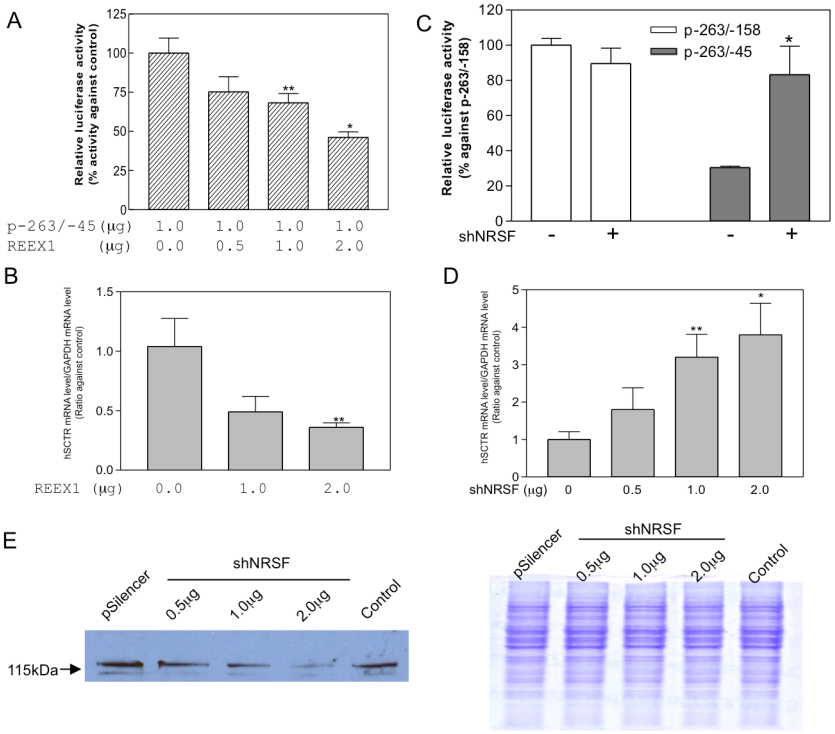

A
Relative luciferase activity (% activity against control)
125
100
75
50
25
0
**
*
p-263/-45 (μg) 1.0 1.0 1.0 1.0
REEX1 (μg) 0.0 0.5 1.0 2.0
C
Relative luciferase activity (% against p-263/-158)
120
100
80
60
40
20
0
p-263/-158
p-263/-45
*
shNRSF - + - +
B
hSCTR mRNA level/GAPDH mRNA level (Ratio against control)
1.5
1.0
0.5
0.0
**
REEX1 (μg) 0.0 1.0 2.0
D
hSCTR mRNA level/GAPDH mRNA level (Ratio against control)
5
4
3
2
1
0
**
*
shNRSF (μg) 0 0.5 1.0 2.0
E
shNRSF
pSilencer
0.5μg
1.0μg
2.0μg
Control
115kDa
shNRSF
pSilencer
0.5μg
1.0μg
2.0μg
Control

Figure 4

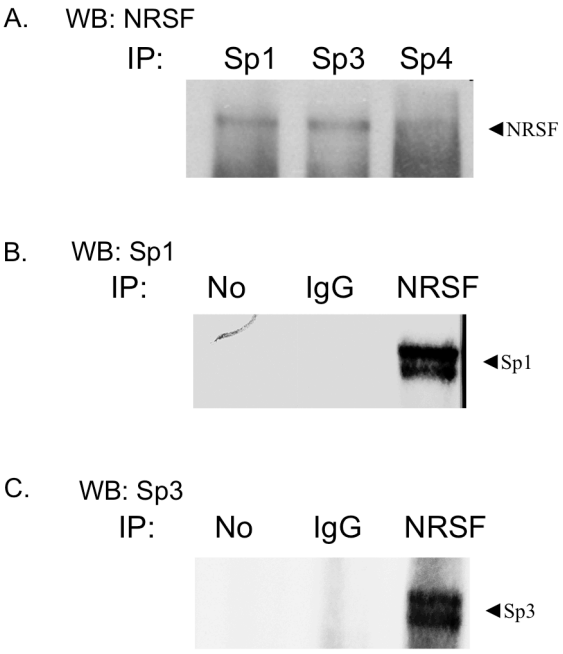

A.
WB: NRSF
IP:
Sp1
Sp3
Sp4
◄NRSF
B.
WB: Sp1
IP:
No
IgG
NRSF
◄Sp1
C.
WB: Sp3
IP:
No
IgG
NRSF
◄Sp3

Figure 5

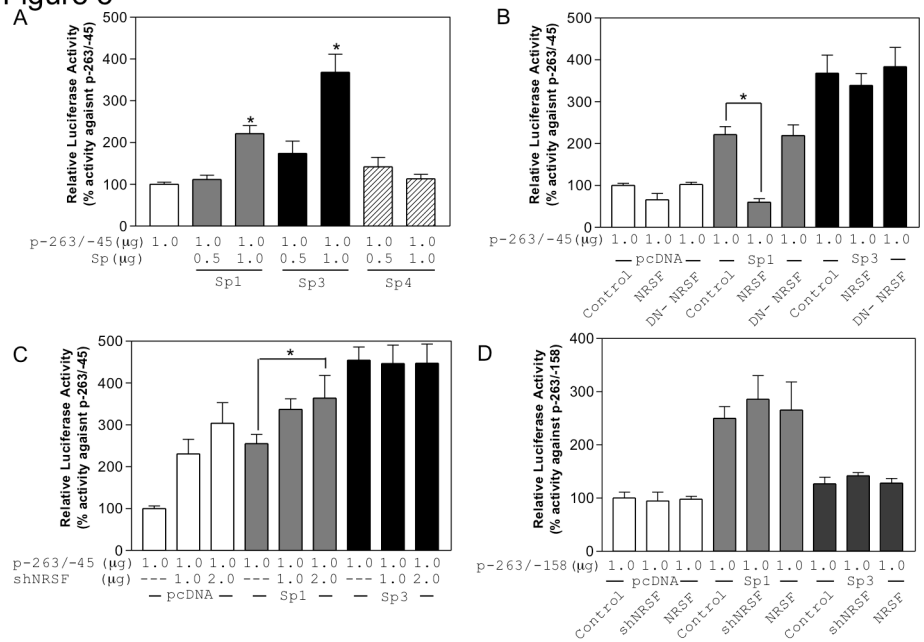
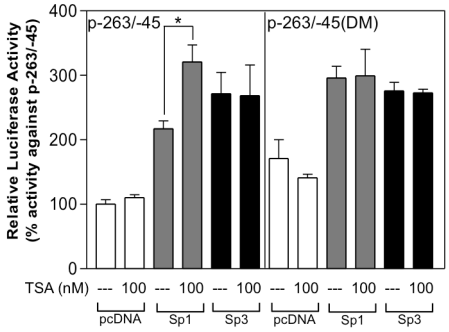

A
Relative Luciferase Activity (% activity agaisnt p-263/-45)
p-263/-45(μg) 1.0 1.0 1.0 1.0 1.0 1.0 1.0
Sp(μg) 0.5 1.0 0.5 1.0 0.5 1.0
Sp1
Sp3
Sp4
B
Relative Luciferase Activity (% activity agaisnt p-263/-45)
p-263/-45(μg) 1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0
pcDNA
Sp1
Sp3
Control
NRSF
DN-NRSF
C
Relative Luciferase Activity (% activity agaisnt p-263/-45)
p-263/-45 (μg) 1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0
shNRSF (μg) --- 1.0 2.0 --- 1.0 2.0 --- 1.0 2.0
pcDNA
Sp1
Sp3
D
Relative Luciferase Activity (% activity against p-263/-158)
p-263/-158(μg) 1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0
pcDNA
Sp1
Sp3
Control
shNRSF
NRSF

Figure 6


Relative Luciferase Activity
(% activity against p-263/-45)
400
300
200
100
0
p-263/-45
*
p-263/-45(DM)
TSA (nM)
--- 100 --- 100 --- 100 --- 100 --- 100 --- 100
pcDNA
Sp1
Sp3
pcDNA
Sp1
Sp3