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γ-Synuclein: Cell-Type-Specific Promoter Activity and Binding to Transcription Factors

Irina Surgucheva · Andrei Surguchov

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Abstract γ-Synuclein, also referred to as breast-cancer-specific gene 1, is the third member of the neuronal protein family synuclein. Synucleins attracted the attention of many investigators because of their role in human diseases. γ-Synuclein participates in the pathogenesis of several types of cancer and some neurodegenerative diseases. Its role in tumorigenesis is due to the upregulation of transcription and the effect on downstream targets, including signaling pathways and transcription factors. γ-Synuclein is also expressed in neurons and glial cells, but the regulation of its expression, as well as the mechanism of transition from normal functions to pathology in these cell types, is not studied. Here, we examined how γ-synuclein promoter is regulated in neuronal and glial cells. We also show that γ-synuclein is able to bind directly to several transcription factors. These results are discussed in connection with the implication of γ-synuclein in diseases.

Keywords γ-Synuclein · Promoter · Transcription · Gene expression

Synucleins are a family of small proteins consisting of three known members, α-, β-, and γ-synuclein (George 2002). Synucleins have been specifically implicated in neurodegenerative diseases and cancer. γ-Synuclein is involved in human neoplastic diseases, particularly in breast cancer and ovarian cancer. Several studies have shown that γ-synuclein was abnormally expressed in a high percentage of advanced and metastatic breast tumors but not in normal or benign breast tissues (Ji et al. 1997; Bruening et al. 2000; Liu et al. 2000, 2002; Jiang et al. 2003, 2004; Wu et al. 2003). The high level of γ-synuclein expression in these cells is controlled on a transcriptional level by AP1 regulatory sequences located in intron 1 and exon 1 but not in the 5' flanking region of the gene. This regulatory mechanism is cell type specific (Lu et al. 2002).

Similar to the other members of the synuclein family, γ-synuclein is also expressed in neurons and glial cells (Buchman et al. 1998; Surguchov et al. 2001; Li et al. 2002). Abnormal accumulation of γ-synuclein in neuronal and glial cells causes different types of pathology, e.g., axonal spheroid-like lesions in Parkinson's disease and Dementia with Lewy bodies (Galvin et al. 1999), axonal spheroids in neurodegeneration with brain iron accumulation, type 1 (Galvin et al. 2000) or deposition in glial cells in glaucoma (Surgucheva et al. 2002). However, the mechanism of transcriptional regulation of γ-synuclein in neuronal and glial cells has not been investigated.

We used a 2,195-bp fragment of the γ-synuclein gene (Lu et al. 2001, 2002) including 1,260 bp of the noncoding 5'-flanking region upstream of the start ATG codon, exon 1, and intron 1 containing two AP-1 binding sites in forward and reverse orientation (Fig. 1f). We also generated three truncated fragments (1,967, 1,864, and 1,759 bp) and two fragments containing mutations in the AP-1 *cis*-element located at positions +599 and +612 (Mu1 and Mu2). These fragments were cloned upstream of the reporter luciferase (LUC) gene and used for transient transfection in human neurons, glial cells, and embryonic kidney cells. The generation of γ-synuclein promoter

I. Surgucheva · A. Surguchov
Retinal Biology Research Laboratory,
Veterans Administration Medical Center,
4801 East Linwood Boulevard,
Kansas City, MO 64128, USA

I. Surgucheva · A. Surguchov (✉)
Department of Neurology, Kansas University Medical Center,
3901 Rainbow Boulevard,
Kansas, KS 66160, USA
e-mail: asurguchov@kumc.edu

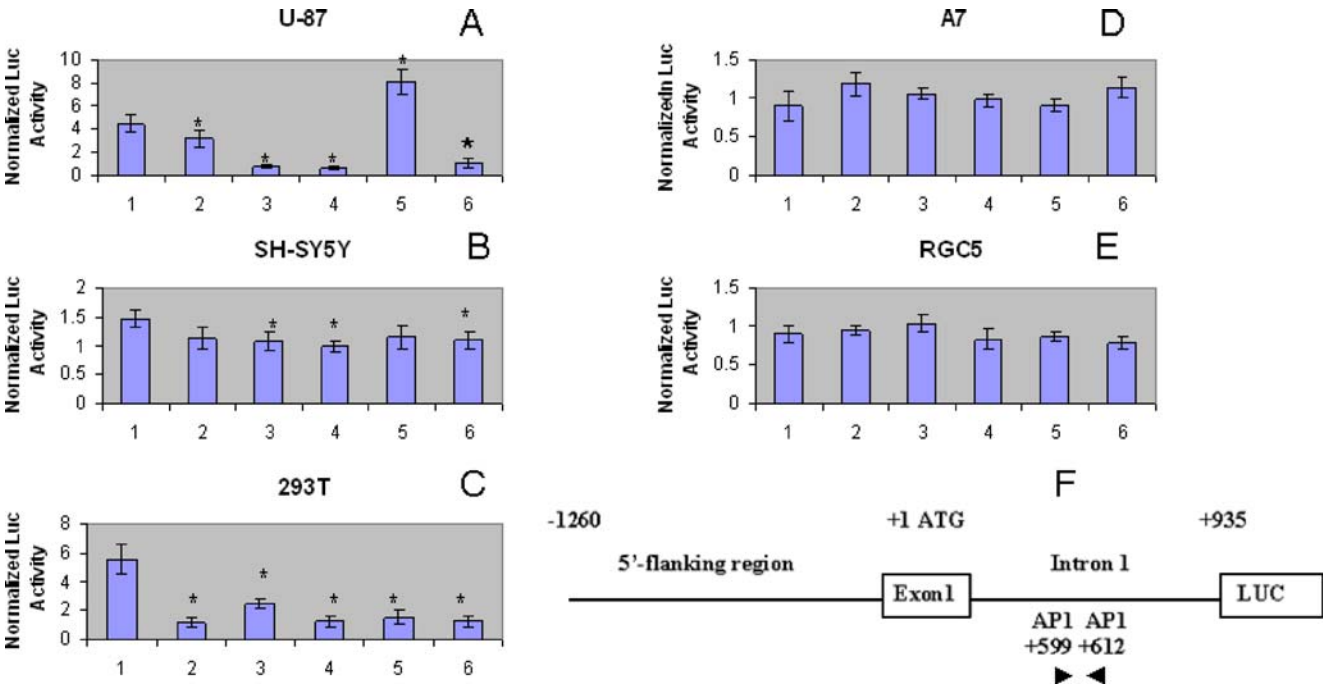

Figure 1 Cell-type-specific activity of γ-synuclein promoter. Human cell lines: astrocytoma U87, neuroblastoma SH-SY5Y, and 293T were purchased from ATCC, cultivated according to manufacturer's recommendations, and used for the transient transfection. Rat retinal ganglion cells RGC-5 (gift of Dr. Agarwal, Forth Worth, TX, USA) and rat astrocytes (gift of Dr. H. Geller, NHLBI, NIH, Bethesda, MD, USA) were grown as described previously (Surgucheva et al. 2006). *Transient expression analysis by dual LUC assays*. The cells were split into 24-well plates at density of $0.1–0.5\times10^6$ cells per milliliter. Twenty-four hours later, the cells were transiently transfected with a total of 500-ng reporter DNA and 10 ng of pRL-SV40. FuGENE-HD (Roche Applied Science) or Escort V (Sigma-Aldrich) were used as transfection agents. After 60 h, the cells were washed with phosphate-buffered saline and suspended into 100 μl of 1×PBL buffer (Promega). LUC activity was measured using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's recommendation. Luminescence was detected in Synergy HT (K24) plate reader modified for luminescence measurements. Firefly LUC activity was normalized to Renilla LUC activity. Each bar corresponds to the means±SE of four independent experiments. The figures for each construct were normalized to the results with promoterless LUC vector pGL3 basic. *Generation of constructs*. The human 2,195-bp DNA fragment (Lu et al. 2002) in pGL3 basic is a generous gift of Dr. Jingwen Liu (Palo Alto, CA, USA). To generate the truncated and mutated fragment, a "QuickChange Site Directed Mutagenesis kit" (Stratagene) and template pGL3 with 2,195-bp fragment cloned upstream of the LUC reporter gene were used. The 1,967-bp construct encompassing −1,260 or +707 bp was made by truncation of the intron 1. For generation of this construct, primers 1 and 2 were used (Table 1). On the next step, the ATG codon of γ-synuclein gene (position +1 of the first exon, E1) was deleted for easy translation of LUC gene. γ-Synuclein-1967 construct without ATG was used as a template for the generation of the MU1 and MU2 mutations in AP-1 binding sites. For the creation of MU1-1967, construct containing AP-1 at position +599, the AP-1 binding site GAGGTCA was changed to GAatTCA with primers 9 and 10 (Table 1). For the generation of MU2-1967, construct containing AP-1 in position +612, the AP-1 binding site TGACTCA was changed to TcctaCA. To create γ-synuclein-1864 construct, a part of intron 1 was deleted including both AP1 binding sites. γ-Synuclein-1967 construct was used as a template with primers 5 and 6. A similar approach was used for the generation of the shortest construct γ-synuclein-1759, from which the intron 1 was completely deleted. This construct was prepared with primers 7 and 8. All these constructs were amplified, Qiagen-purified, and sequenced. **a** Human astrocytoma U-87; **b** human neuronal cells SH-SY5Y; **c** human kidney 293T cells; **d** rat cell line A7 from the neonatal rat optic nerve; **e** rat retinal ganglion cell line. *1*—2,195-bp construct; *2*—1,967-bp construct; *3*—1,864-bp construct; *4*—1,759-bp construct; *5*—construct containing mutation in AP1 binding site (+599; MU1-1967 construct); *6*—construct containing mutation in AP1 binding site (+612; MU2–1967 construct). *Columns* represent the mean of at least four individual experiments; *bars* are SD. A paired Student's *t* test was used to assess significant differences between groups. The significant changes compared with 2,195-bp construct (*bar 1*) are indicated by *asterisks* ($p<0.05$)

fragments in the LUC reporter constructs were done basically as described elsewhere (Lu et al. 2001, 2002) with some modifications described in the legend of Fig. 1.

The modifications of γ-synuclein promoter affected its activity in a cell-specific manner (Fig. 1). Truncation of the promoter region from 2,195 to 1,759 bp monotonously reduces LUC expression in astrocytoma U87 cells (Fig. 1a; bars 1–4). Mutation of the AP-1 binding site TGACTCA to TcctaCA (MU2) also markedly reduces the promoter activity (bar 6). Unexpectedly, the mutation of the second AP-1 binding site GAGGTCA to GAatTCA results in the twofold increase of the promoter activity (Fig. 1a, bar 5) suggesting that the mutated AP-1 binding site is more efficient in U87 cells when compared to the wild-type sequence. This effect may be due to the partial overlap of the positive and negative DNA elements (Goodbourn and Maniatis 1988; Benko et al. 2003) in this site so that modifications we used destroyed the site for a *trans*-acting

Table 1 Sequences of γ-synuclein-specific primers.

Primer	Nucleotide sequence
F-1967	5'- ccctgggtctagccagtgtccctaccatggaagacgccaaaaacataaag-3'
Compl.-1967	5'- ctttatgtttttggcgtcttccatggtagggacactggctagacccaggg-3'
F-1864	5'- ccagaggaggaagggatggaagacgccaaaaacataaag-3'
Compl. 1864	5'- ctttatgtttttggcgtcttccatcccttcctcctctgg-3'
F-1759	5'- tacagccaggtcacggatccctccacagatggaagacgcc-3'
Compl. 1759	5'- ggcgtcttccatctgtggagggatccgtgacctggctgta-3'
F-MU 1	5'-agaggaggaaggggaattcaagccaatgactcagctctggcc-3'
Compl.-MU1	5'- ggccagagctgagtcattggcttgaattcccctcctcctct-3'
F-MU2	5'- agaggaggaaggggaggtcaagccaatcctacagctctggcc-3'
Compl.-MU2	5'- ggccagagctgtaggattggcttgacctccccttcctcctct-3'

repressor specific for U87 cells. Alternatively, the increase of luciferase activity in U87 due to the heptanucleotide GAATTCA may be explained as follows. GAATTCA is a part of the promoter (*cis*-element) in several genes, for example, bmp2b that is involved in the signaling during embryogenesis (Leung et al. 2003). The introduction of this sequence into upstream regions of several genes acquires new regulatory properties, for example, sensitivity to retinoic acid (Larose et al. 1996) or growth factors (Varghese et al. 2005). The new regulatory properties of the gene expression due to this heptanucleotide are cell type specific. In U87 astrocytoma cell line, retinoic acid is an important regulator of cell growth and apoptosis (Teelt-

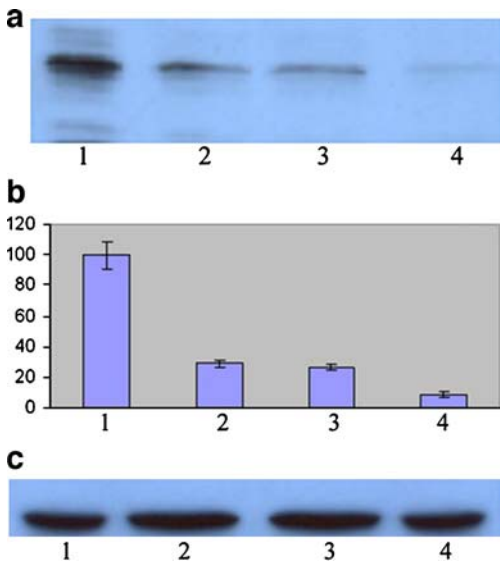

Figure 2 Regulated expression of γ-synuclein by OM. RGC-5 cells were grown for 2 days (*lanes 1* and *2*) or 3 days (*lanes 3* and *4*) and either treated with 25 ng/ml (*lane 2*) and 50 ng/ml (*lane 4*) of OM or used without treatment (*1*, *3*). **a** γ-Synuclein revealed by Western blotting with monoclonal antibody (Mab-606071, Antagene). **b** Bands shown in **a** were scanned and the average of three experiments presented. **c** Actin was revealed with the monoclonal antibody MAB1501 (Chemicon International)

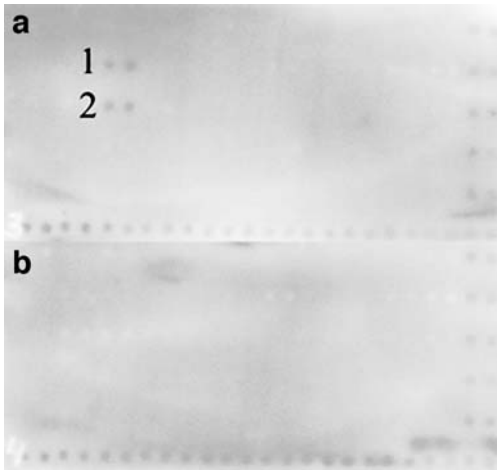

Figure 3 Binding of γ-synuclein to TFs. γ-Synuclein binding partners were identified using TransSignal TF Protein Array Version 2 (Panomics). **a** *1*—JunB, *2*—MECP2. **b** Control to array II incubated without γ-synuclein. Purified recombinant γ-synuclein (**a**) or buffer (**b**) were incubated with the TransSignal TF Protein Array membrane, washed, and detected by γ-synuclein-specific monoclonal antibody (Mab-606071, Antagene). Membranes were treated as recommended by the manufacturer and exposed to Hyperfilm ECL. The *spots* at the *bottom* and at the *right end* of the blots are positive controls. The experiments were repeated three times

Hansen et al. 2004) which may explain a different effect of this *cis*-element in the U87 cell line compared to other cells.

In neuronal cells, SH-SY5Y, both truncations and mutations in AP-1 element causes 25–33% reduction of the promoter activity (Fig. 1b). Truncations and modifications of AP-1 binding sites causes a more significant decrease in the promoter activity in 293 cells compared to that in SH-SY5Y cells (Fig. 1c). In rat cell lines (A7 and retinal ganglion cells 5 (RGC5)), we did not observe a noticeable promoter activity (Fig. 1d, e). Since parallel transfection by pGL3 control used as a positive control plasmid results in high LUC activity in rat cell lines, we conclude that the promoter of human γ-synuclein is species specific and is not recognized in heterologous rat cells.

Thus, the modifications by deletions or insertion of mutations in AP-1 binding sites of γ-synuclein promoter differentially affect its activity in human cell types that we tested. The effect of promoter modifications on its activity in cell lines that we used is different than in cancer cell lines where the most significant reduction of the promoter activity was observed as a result of the truncation of γ-synuclein-1967 fragment to γ-synuclein-1864 (approximately fivefold reduction; Liu et al. 2000).

Regulated expression of γ-synuclein in RGC-5 Earlier transcriptional downregulation of γ-synuclein by oncostatin M (OM) was found in H3922, a cell line derived from an infiltrating ductal carcinoma (Liu et al. 2000). We decided to determine whether or not similar type of regulation

occurs in neuronal cell lines. RGC were treated by 25 and 50 ng/ml OM for 2–3 days and the effect of such treatment on γ-synuclein level was determined by Western blot (Fig. 2). After 2 days of OM treatment, γ-synuclein expression was reduced by more than 65%. At the same time, the expression level of a housekeeping gene α-tubulin revealed with DM1a antibody was not changed (not shown).

γ-Synuclein binding to TFs As shown earlier, γ-synuclein is a transcriptional regulator able to affect the efficiency of transcription of other genes (Surgucheva et al. 2003; Jiang et al. 2004). This property of γ-synuclein may play an important role in cancer progression and neurodegenerative diseases. Transcription factor (TF) AP-1 was identified as one of the targets of γ-synuclein (Surgucheva et al. 2003). To identify other factors which may be targets for γ-synuclein, we tested the ability of γ-synuclein to bind to TFs using TransSignal TF Protein Array (Panomics).

The results presented in Fig. 3a demonstrate that γ-synuclein binds to TFs JunB and MECP2. The appearance of a signal cannot be explained by unspecific binding of antibodies to TFs since the signal disappeared when γ-synuclein was replaced by a buffer (Fig. 3b). Analysis with other arrays identified the following TFs interacting with γ-synuclein: CREB1 (array 1), PPAR-gamma and TCEA1 (array 3), and ATF3 and Jun/JunB (array 4).

These results are consistent with our previous data pointing to the role of AP-1 in the upregulation of MMP-9 by γ-synuclein (Surgucheva et al. 2003). AP-1 collectively describes a group of structurally and functionally related members of the Jun protein family that share structural similarities and form heterodimeric complexes with members of the ATF family, including ATF-3.

Thus, transcriptional control of γ-synuclein expression in glial and neuronal cells is different compared to that described for cancer cells earlier. These differences might be due to the different pattern of trans-acting factors ensuring cell-type-specific transcriptional regulation. On the other hand, in cells of neuronal origin RGC5, the expression of γ-synuclein is downregulated by oncostatin M in a similar way to that in cancer cells. The ability of γ-synuclein to bind specifically to several TFs may explain the mechanism of the downstream effects of γ-synuclein. It is a subject for further investigations to determine the functional role of such binding.

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