Biochemical and Biophysical Research Communications 471 (2016) 533–538

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Effect of the linkers between the zinc fingers in zinc finger protein 809 on gene silencing and nuclear localization

Yu Ichida*, Yuko Utsunomiya, Masafumi Onodera

Department of Human Genetics, National Center for Child Health and Development, 2-10-1 Okura, Setagaya, Tokyo 157-8535, Japan

ARTICLE INFO

Article history:
Received 25 January 2016
Accepted 11 February 2016
Available online 12 February 2016

Keywords:
Retroviral vector
Kruppel-associated box-containing zinc finger proteins (KRAB-ZFPs)
Zinc finger protein 809 (ZFP809)

ABSTRACT

Zinc finger protein 809 (ZFP809) belongs to the Kruppel-associated box-containing zinc finger protein (KRAB-ZFP) family and functions in repressing the expression of Moloney murine leukemia virus (MoMLV). ZFP809 binds to the primer-binding site (PBS)located downstream of the MoMLV-long terminal repeat (LTR) and induces epigenetic modifications at integration sites, such as repressive histone modifications and de novo DNA methylation. KRAB-ZFPs contain consensus TGEKP linkers between C2H2 zinc fingers. The phosphorylation of threonine residues within linkers leads to the inactivation of zinc finger binding to target sequences. ZFP809 also contains consensus linkers between zinc fingers. However, the function of ZFP809 linkers remains unknown.

In the present study, we constructed ZFP809 proteins containing mutated linkers and examined their ability to silence transgene expression driven by MLV, binding ability to MLV PBS, and cellular localization. The results of the present study revealed that the linkers affected the ability of ZFP809 to silence transgene expression. Furthermore, this effect could be partly attributed to changes in the localization of ZFP809 proteins containing mutated linkers. Further characterization of ZFP809 linkers is required for understanding the functions and features of KRAB-ZFP-containing linkers.

1. Introduction

Zinc finger protein 809 (ZFP809) is one of the most well-characterized Kruppel-associated box-containing zinc finger proteins (KRAB-ZFPs) that represent the largest single family of transcription factors in mammals, are exclusively found in tetrapedal vertebrates, and are involved in various cellular processes including apoptosis, cancer development, differentiation, immunity, and metabolism [1–9]. ZFP809 contains a KRAB domain at the N-terminus and seven zinc fingers at the C-terminus and is highly expressed in immature murine cells including embryonic stem cells and embryonic carcinoma cells [9]. ZFP809 inhibits the transcriptional expression of Moloney murine leukemia virus (MoMLV) [10] and binds to the MLV-primer binding site (PBS) located downstream of the 5′ MLV-long terminal repeat (LTR) and interacts with KAP1. ZFP809/KAP1 recruits heterochromatin protein 1 (HP1), an ERG-associated protein with a SET domain (ESET, a H3K9 methyltransferase), and the nucleosome remodeling and deacetylation (NuRD) complex. The NuRD complex represses the activity of LTR by inducing epigenetic silencing marks, such as histone modifications and DNA methylation at the MLV-LTR [11,12]. In addition, ZFP809 is required for the initiation of silencing endogenous retroviruses (ERVs) during mouse embryonic development [13]. Previously, we demonstrated that ZFP809 has functional domains required for the subcellular localization, gene silencing ability, and binding to MLV PBS [14,15].

KRAB-ZFPs have C2H2 zinc fingers and a consensus sequence (one or more TGEKP linkers between zinc fingers) [16–18]. Previous studies have demonstrated that the phosphorylation of this sequence (threonine) inactivates the function of C2H2 zinc fingers, such as their binding to target DNA sequences [16–18]. However, although we previously characterized the functional domains of ZFP809, the functions of the linkers within ZFP809 remain unknown. In the present study, we investigated the function of linkers within ZFP809 by constructing vectors that exogenously express

Abbreviations: KRAB-ZFP, Kruppel-associated box-containing zinc finger protein; ZFP809, zinc finger protein 809; PBS, primer-binding site; MoMLV, Moloney murine leukemia virus; LTR, long terminal repeat; HP1, heterochromatin protein 1; ESET, ERG-associated protein with a SET domain; NuRD, nucleosome remodeling and deacetylation; ERVs, endogenous retroviruses; PVDF, polyvinylidene fluoride; TBST, Tris-buffered saline-Tween; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus.

* Corresponding author.
E-mail address: ichida-y@ncchd.go.jp (Y. Ichida).

http://dx.doi.org/10.1016/j.bbrc.2016.02.040
0006-291X/

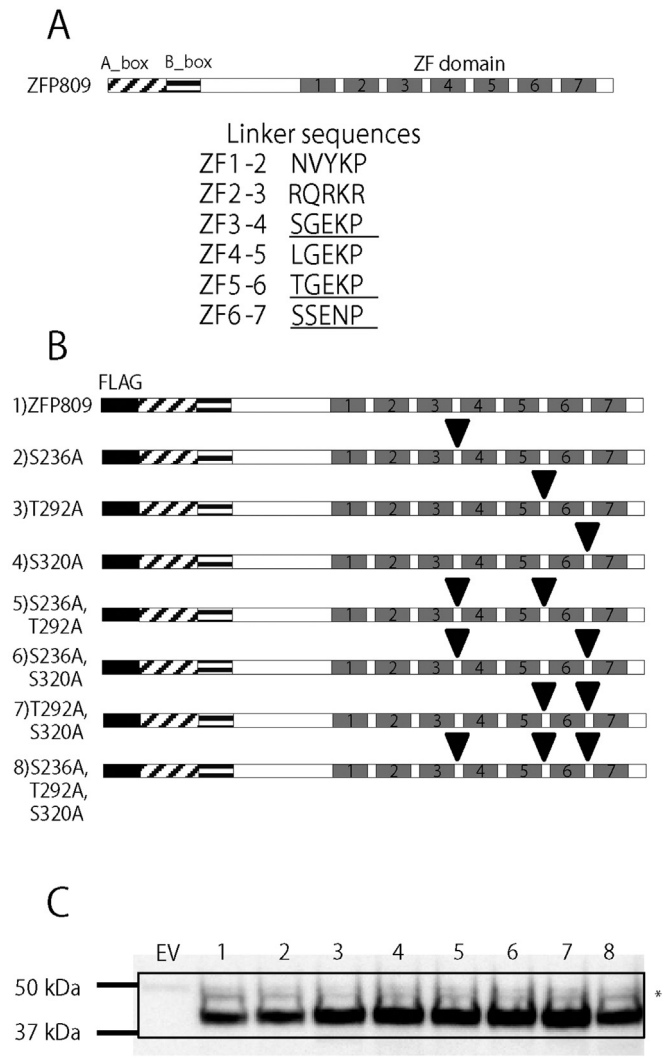

Fig. 1. Domain structures of mutated ZFP809 proteins. (A) Diagram of the domains within ZFP809 and the six linkers sequences between zinc fingers. Underlines indicate the sequence similar to TGEKP. (B) Schematic representation of the domain structures of the intact ZFP809 protein and mutated ZFP809 proteins (1–8). All constructs contained a FLAG tag at the N-terminus and were cloned into pLVSIN/IRES/mCherry vectors referred to as pLVSIN/CMV/flag-X/IRES/mCherry vectors (with X denoting either: ZFP809, S236A, T292A, S320A, S236-T292A, S236A-S320A, T292A-S320A, or S236A-T292A-S320A). (C) Confirmation of the expression of proteins from the eight vector constructs shown in Fig. 1B. 293FT cells transduced with one of the eight vector constructs were sorted based on mCherry expression. Sorted cells were subjected to Western blot analysis using the anti-FLAG antibody. The asterisk indicates non-specific bands.

ZFP809 containing mutated linkers in cultured cells.

2. Materials and methods

2.1. Cell culture

The 293FT cell line was purchased from Thermo Fisher Scientific Inc. 293FT cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS, Thermo scientific), 2 mM L-glutamine, 0.1 mM sodium pyruvate, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate at 37 °C in an atmosphere containing 5% carbon dioxide (CO2).

2.2. Vector construction

Zfp809 was amplified by reverse transcription-polymerase chain reaction (RT)-PCR using the total RNA extracted from F9 cells. The pCMV/flag-Zfp809 vector was constructed as described previously [14].

For the preparation of lentiviral vectors, pCMV/flag-Zfp809 vectors were digested with XhoI and BamHI and inserted into XhoI/BamHI-digested pLVSIN/IRES/mCherry vectors, which were constructed as described previously [14].

Nucleotide substitutions were introduced into pLVSIN/CMV/flag-ZFP809/IRES/mCherry vectors using PrimeSTAR Mutagenesis Basal Kits (Takara Bio) and mutagenic primers (Supplementary Table S1) or were synthesized by gBlocks Gene Fragments (Integrated DNA Technologies, IDT) such that the serine or threonine residues within linkers were substituted for alanine. The resultant vectors were referred to as pLVSIN/CMV/flag-X/IRES/mCherry (with X denoting S236A, T292A, S320A, S236A-T292A, S236A-S320A, T292A-S320A, or S236A-T292A-S320A).

For the generation of lentiviruses, each of the pLVSIN/CMV/flag-X/IRES/mCherry vectors was co-transfected with packaging vectors (ViraPower Packaging Mix; Life Technologies) into 293FT cells using Lipofectamine LTX (Life Technologies). Culture supernatants were harvested on days 1, 2, and 3 and were transduced into target cells with 4 μg/mL of polybrene by spinoculation at 1000× g for 1 h at 32 °C [19].

2.3. Western blotting

Cell lysates were extracted from the 293FT cells transduced with one of the pLVSIN/CMV/flag-X/IRES/mCherry vectors using Nuclear/Cytosol Fractionation Kits (BioVision). The extracted samples were electrophoresed on 4%–20% Mini-Protean TGX Precast Gels (Bio-Rad) and were transferred to polyvinylidene fluoride (PVDF) membranes using Trans-Blot Turbo Mini PVDF Transfer Packs (Bio-Rad). PVDF membranes were blocked with Blocking One (Nacalai Tesque) for 30 min at room temperature. The membranes were then incubated overnight at 4 °C with an anti-FLAG monoclonal antibody (F1804, Sigma–Aldrich; 1:1000 dilution in Tris-buffered saline-Tween; TBST) solution containing 1X TBS and 0.1% Tween 20/Blocking One (1:20). The membranes were washed in TBST for 5 min and then incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000 dilution in TBST/Blocking One; GE Healthcare Life Sciences) for 2 h at room temperature. After a final wash with TBST for 5 min, signals were detected using the ECL system (GE Healthcare Life Sciences).

2.4. Flow cytometry analysis

Cells were washed, resuspended in PBS containing 2% FCS, and analyzed or sorted based on EGFP or mCherry expression using FACSAria™ III (BD Biosciences).

2.5. Nuclear extracts and electrophoretic mobility shift assays (EMSA)

Nuclear extracts were obtained from 293FT cells transduced with pLVSIN/CMV/flag-X/IRES/mCherry vectors using Nuclear/Cytosol Fractionation Kits (BioVision). The sense sequence of the oligonucleotide probe containing the MLV PBS sequence (underlined) was as follows: 5′-TTTGGGGGCTCGTCCGGGATTT-3′.

Double-stranded probes were prepared by annealing sense and antisense single-stranded oligonucleotides, end-labeled with [γ-32P]dATP (PerkinElmer) using T4 polynucleotide kinase (New