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Functional identification of the pro-apoptotic effector domain in human Sox4

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Abstract

Recent studies provide evidence that Sox4 is involved in regulating apoptosis as well as tumorigenesis of various human cancers; however, its role in the apoptotic machinery is not fully understood. Here we describe that the central domain containing glycine-rich region in Sox4, named CD, is a pivotal pro-apoptotic domain to induce apoptotic cell death. Deletion of the DNA-binding domain or *trans*-activation domain in Sox4 did not significantly affect pro-apoptotic activity, whereas transient transfection of the high mobility group box or the serine-rich region abrogated the apoptotic activity. Moreover, overexpression of the CD construct (aa 166–342) revealed the apoptotic activity comparable to that of wild-type Sox4, approximately 60% of cell death. Our data suggest that the apoptotic activity of Sox4 can be dissociated from its transcriptional *trans*-activation and is mediated through its CD.

Keywords: Sox4; Glycine-rich region; High mobility group; Serine-rich region; DNA-binding domain; *trans*-Activation domain; Apoptosis

Sox (SRY-related HMG-box) proteins play key roles in regulating transcription during such diverse developmental processes as early embryogenesis, sex determination, neural development, lens development, chondrogenesis, cardiac development, and hemopoiesis [1,2]. All Sox proteins share the highly conserved high mobility group (HMG) box region of the mammalian testis-determining gene sex-determining region Y (*SRY*). However, Sox proteins show diverse functions in mammals because the residues outside the HMG-box domain are variable and may influence selection of partner proteins and binding stability [1,3,4]. Recently, it was reported that human and mouse genomes possess more than 20 pairs of Sox family of transcription factor genes determined by sequence analysis, and each gene is allocated to one of the seven subgroups, A–G [3,5]. Among the members of the Sox family, Sox4 cloned from a thymus cDNAs belongs to the C subgroup [6]. The open reading frame of Sox4 encodes 474 amino acids, which includes an HMG-box domain (aa 57–135) as a DNA-binding region. SRR

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(serine-rich region, aa 333–397) that serves as a *trans*-activation domain is located at the C-terminus of Sox4, and GRR (glycine-rich region, aa 152–227) is located between the HMG-box domain and SRR [7,8]. Both SRR and HMG serve as a crucial role in regulating transcription by Sox; however, the function of GRR in Sox4 is virtually unknown.

Sox4 has been reported to play roles in development of endocardial ridge and other organs including CNS, lung, tooth buds, and gonads [9], as well as to regulate lymphocyte development and differentiation [9–11]. Moreover, recent studies provide evidence that Sox4 is highly expressed in several tumors including breast cancer, hepatocellular carcinoma, colon cancer, meduloblastoma, and lung cancer [12–16], suggesting that Sox4 may be involved in tumorigenesis. We have previously shown that Sox4 was highly expressed in hepatocellular carcinoma cells, Hep3B and HepG2, during apoptosis induced by prostaglandin (PG) A_2 and Δ^{12}-PGJ_2 as well as in subcutaneous tumors grown in Hep3B-xenografted nude mice [13]. Interestingly, Sox4 overexpressed in Hep3B and HepG3 cells promotes apoptosis [17]. Apoptosis is a physiological cell death program critical to the development and homeostasis of multicellular organisms [18]. In humans, both excessive and insufficient apoptosis can lead to severe pathological consequences: cancer, autoimmune diseases, and neurodegenerative disorders [19]. The previous results suggest that Sox4 might play a role in regulating an apoptotic pathway as well as in tumorigenesis; nonetheless, the mechanism by which Sox4 contributes to regulating apoptosis or to tumor development remains to be elucidated.

We describe here that the central domain (CD) of Sox4 plays a pivotal role in regulating apoptosis. Deletions that destroy the *trans*-activation activity of Sox4 have no effect on its ability to induce apoptosis, indicating that Sox4 directly functions on the induction of apoptosis through its CD.

Materials and methods

Plasmid construction. The cDNA encoding the N-terminal region of human Sox4 (amino acid residues 1–173 encompassing HMG-box domain) was generated by PCR amplification with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) from the pCDM7-Sox4 plasmid as a template [8]. The amplified fragments were inserted into the pGEX-4T-1 plasmid (Amersham Bioscience, UK), designated pGST-HMG. Plasmids encoding wild-type Sox4 with the C-terminal FLAG or GFP epitope tags were generated by modifying pGST-HMG and pCDM7-Sox4 [6], designated pFLAG-Sox4 (WT) and pEGFP-Sox4 (WT), respectively. The following truncated Sox4 constructs of various sizes were generated by digesting with appropriate unique restriction enzymes within the Sox4 cDNAs and inserting into the pFLAG-CMV5a (Sigma, St. Louis, MO) or the modified pFLAG plasmids: HMG (aa 1–173), ΔSRR (aa 1–342), ΔHMG (aa 166–474), SRR (aa 343–474), and CD (aa 166–342). The sequence integrity and expression of all plasmid constructs were verified by DNA sequencing with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and immunoblot (IB) analyses with the specific antibodies, respectively.

Cell culture, transfection, and X-gal staining. Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). For all transfections, 1×10^6 cells were plated on 100-mm culture dish, and 1.5 μg of the indicated plasmid was transfected into HEK293 cells with LipofectAMINE reagent according to the manufacturer's instructions (Invitrogen).

HEK293 cells (3×10^5 cells) grown on 35-mm culture dish were cotransfected with 0.6 μg each of the Sox4 expression plasmids and 0.3 μg pCMV β-gal. After 24 or 48 h, the cells were fixed with 2% paraformaldehyde/0.2% glutaraldehyde in PBS for 5 min on ice. After rinsing twice in PBS, cells were stained in X-gal solution (1 mM $MgCl_2$, 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6$, and 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)) for 6 h at 37 °C. Cell morphology was examined by light microscopy. The extent of cell death was measured by counting total blue cells and blue cells with round shape on 10 randomly selected fields under the inverted microscope at a magnification of 200× (Carl Zeiss, Oberkochen, Germany) [20].

Apoptotic cell death assays. For immunofluorescence staining, HEK293 cells were plated on coverslips and then transfected with 0.5 μg of the respective expression vectors in the same manner as mentioned above. After 24 h transfection, cells were rinsed in PBS and fixed with 4% paraformaldehyde in PBS for 10 min. Cells were blocked for 30 min with 5% normal goat serum (Jackson Lab, Baltimore, PA) in PBS and incubated with anti-FLAG antibody (1:200; Sigma) for 1 h at room temperature, followed by incubating with Cy3-conjugated secondary antibody (1:1000; Jackson Lab).

After fixation and permeabilization of cells, nuclei were stained with 1 μg/ml of 4,6-diamidino-2-phenylindole (DAPI; Sigma) for 1 min and subsequently washed as described above. Morphological changes of nuclei were analyzed by fluorescence microscopy (Olympus, Tokyo, Japan).

To verify the apoptotic features, translocation of phosphatidylserine (PS) to the outer membrane of apoptotic cells was detected by the annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA) according to the supplier's instructions. Images of the stained nuclei were visualized to determine the incidence of apoptotic changes under fluorescence microscopy.

Immunoblot analysis. At 24 h post-transfection, cells were washed twice with ice-cold PBS and were resuspended in RIPA buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Samples were centrifuged for 30 min at 4 °C, and the protein concentration of cell extracts was determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, CA). The protein extract (20 μg) was denatured in sample buffer (60 mM Tris–HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, and 0.1% bromophenol blue) and subjected to 12% SDS–PAGE. The resolved proteins were transferred electrically to the nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), and the membrane was blocked with 5% skim milk in Tris-buffered saline (10 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 (TTBS). The membrane was incubated with monoclonal mouse anti-FLAG (1:1000; Sigma), polyclonal rabbit anti-Sox4 (1:1000; Koma Biotech., Seoul, Korea), or anti-PARP antibodies (Roche, Mannheim, Germany), followed by incubating with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies, respectively (1:5000; Amersham). The specific protein bands were visualized with Enhanced Chemiluminescent (ECL; Amersham) IB system as described by the manufacturer.

Results

Sox4 induces apoptotic cell death in HEK293 cells

To ascertain a general mechanism of apoptosis induced by Sox4, we transiently transfected into HEK293 cells a plasmid encoding human Sox4 along with the pCMV β-gal plasmid (Fig. 1). Expression of endogenous Sox4 was not detectable in HEK293 cells by IB analysis with anti-Sox4 antibody (data not shown). At 24 to 48 h post-transfection, the cells were stained with X-gal, and we assessed the cytotoxic effects of Sox4 by comparing the number of round blue cells (dead cells) to the number of elongated forms (surviving cells) (Fig. 2). Morphological changes occurred in Sox4-expressing cells, showing cell shrinkage and chromatin condensation, while pFLAG-transfected cells grew normally well. Overexpression of Sox4 in HEK293 cells resulted in increasing the number of cell death, approximately 60%, compared with cells transfected with mock-vector alone (Fig. 2B).

To further substantiate that Sox4-induced cell death is implicated in the induction of apoptosis, we stained the nuclei of cells with DAPI and analyzed GFP-positive cells under fluorescence microscope (Fig. 2C). No nuclear morphological alteration was observed in mock-transfected cells. In contrast, Sox4-expressing GFP-positive cells exhibited nuclear fragmentation, indicating that Sox4 promotes apoptotic cell death in a non-hepatocarcinoma cell, HEK293.

The Sox4 trans-activation activity does not modulate its pro-apoptotic activity

Sox transcription factors regulate a variety of cellular process in vertebrate development, either by activating or repressing target genes [21]. Likewise, Sox4 is a transcriptional factor with a separable DNA-binding and a *trans*-activation domains. To determine whether transcriptional activity of Sox4 is responsible for Sox4-mediated apoptosis, we generated Sox4 constructs, ΔHMG and ΔSRR, deleting the DNA-binding or *trans*-activation domains (Fig. 3A); both domains are indispensable for transcriptional activity of Sox4 [8]. The ΔHMG and ΔSRR constructs were tagged with the FLAG epitope to facilitate immunological detection of truncated Sox4 proteins with anti-FLAG antibody. Both proteins were expressed at levels comparable to wild-type (Fig. 3B). The extent of cell death was determined by monitoring the round cells (blue) from transiently transfected cells with the indicated plasmid along with the pCMV β-gal plasmid (Fig. 3C). The ΔHMG and ΔSRR, transcriptionally inactive Sox4 mutants, exhibited approximately 60% cell death as much as that of wild-type (Fig. 3D). The result indicates that the pro-apoptotic activity is separable from *trans*-activation activity in Sox4.

The central domain containing GRR has pro-apoptotic activity

To define the region responsible for the pro-apoptotic activity, we generated Sox4 truncated constructs based

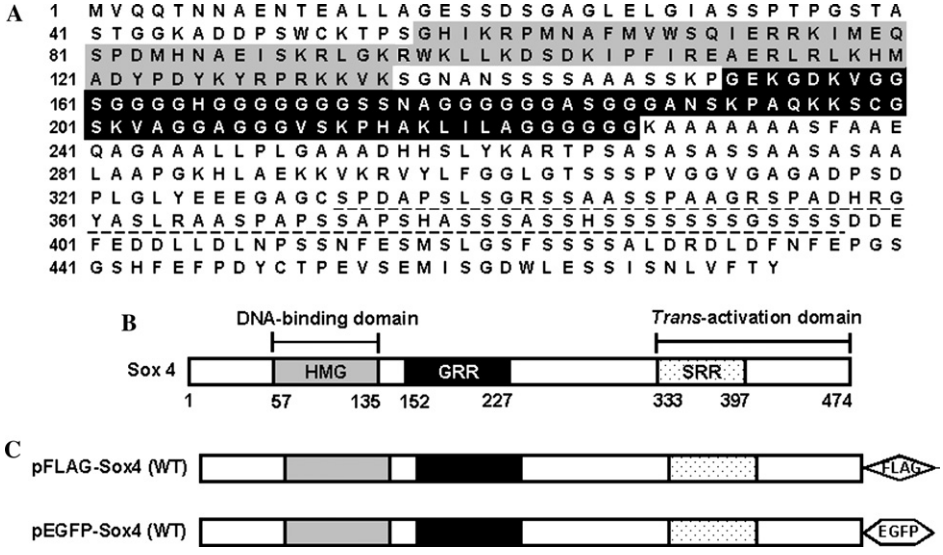

Fig. 1. Schematic diagram of Sox4 with its functional domains. (A) Amino acid sequence of human Sox4. The original cDNA clone encoded 474 amino acid residues (GenBank Accession No. NM_003107). High mobility group (HMG) box (aa 57–135) is in shaded box; glycine-rich region (GRR, aa 152–227) is in black box; and serine-rich region (SRR, aa 333–474) is in dashed underline. (B) Schematic diagram of the functional domains of Sox4. (C) Schematic diagrams of Sox4 expression constructs: the pFLAG-Sox4 (WT) and pEGFP-Sox4 (WT) plasmids encoding the C-terminal FLAG-tagged Sox4 and EGFP-tagged Sox4, respectively.

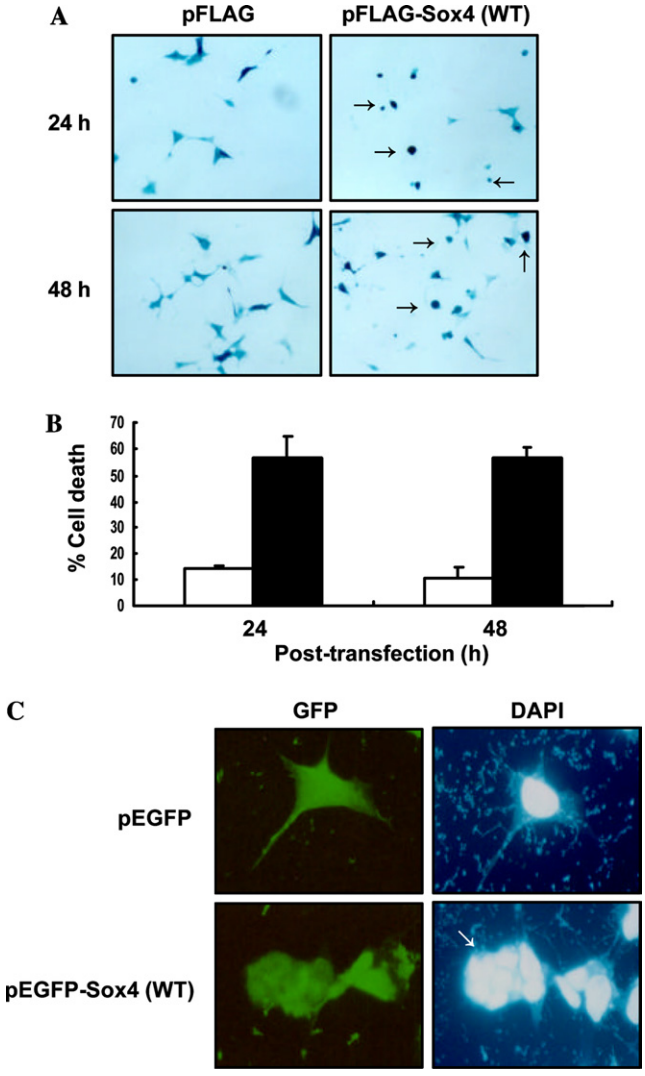

Fig. 2. Cytotoxicity induced by Sox4. (A) Representative morphological changes of Sox4-expressing HEK293 cells. pFLAG or pFLAG-Sox4 (WT) was cotransfected with pCMV β-gal into HEK293 cells. The cells were stained with X-gal at 24 or 48 h post-transfection. Morphological change was evaluated in terms of elongated blue cells (surviving cells) versus round cells indicated in arrows (dead cells). (B) Quantification of cytotoxicity induced by Sox4. Quantification of cell death was identified based on morphological changes of the blue cells. All values are expressed as percentages of cells with round morphology relative to total blue cells. Error bars represent standard deviation derived from the mean of three independent experiments. (C) Representative GFP and DAPI fluorescence images of cells expressing Sox4. HEK293 cells were transfected with pEGFP or pEGFP-Sox4 (WT). At 24 h post-transfection, the nuclear morphology of GFP-positive cells (green) was stained with DAPI (blue) and observed under Olympus 70AX fluorescence microscopy at a magnification of 400×; arrows indicate the apoptotic nuclei. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

on its functional domains: a DNA-binding domain (HMG), a central domain containing GRR (CD), and a *trans*-activation domain (SRR) (Fig. 4A). The indicated Sox4 constructs were co-transfected with the pCMV β-gal plasmid, and expression levels and the integrity of each of these Sox4 mutants were examined by IB analysis with anti-FLAG antibody (Fig. 4B). Cell death was determined by X-gal staining of transfectants (Fig. 4C) and quantified by counting the round blue cells (Fig. 4D). HMG showed approximately 13% cell death comparable to that of mock-vector alone; and SRR revealed nearly 20% cell death. In contrast, CD induced apoptotic cell death, apparently up to 60%, similar to that of the wild-type Sox4 protein. Moreover, CD is common to ΔHMG and ΔSRR that have pro-apoptotic activity. The CD is, therefore, a crucial determinant for Sox4-mediated apoptotic cell death.

To verify the apoptotic features, we monitored the key apoptotic events in the CD-expressing cells (Fig. 5). Morphological changes in the nuclei of CD-expressing cells were characterized by DAPI staining, showing the fragmented nuclei with highly condensed chromatin (Fig. 5A, upper panel). Cell surface exposure of PS during apoptosis was also detected by annexin V binding to PS, as an early marker of apoptosis (Fig. 5A, bottom panel).

Caspase-3 is one of the key executioners of apoptosis and can act on the proteolytic cleavage of many important cellular proteins, such as poly(ADP-ribose) polymerase (PARP), a 113-kDa nuclear enzyme [22–24]. To assess whether Sox4-induced apoptotic cell death is associated with activation of caspase-3, we investigated the cleavage of PARP by IB analysis (Fig. 5B). The pro-apoptotic protein Bax-expressing cells revealed more effective activation of caspases, resulting in the cleavage of the PARP to fragments of 89 and 24 kDa. In contrast, cleavage of PARP was not detected in any of Sox4-expressing cells. Taken together, the results suggest that the central domain of Sox4 is likely to induce apoptosis via a caspase-3-independent pathway.

Discussion

In the present study, we have described that the Sox4's central domain containing GRR (aa 166–342), named CD, serves as a novel functional domain for promoting apoptotic cell death. Several studies provide evidence that the transcriptional activator p53 possesses an additional extranuclear role directly engaging the apoptotic cascade in a p53 transcription-independent way, albeit an intact p53 exerts the full apoptotic capacity [25–27]. Likewise, the pro-apoptotic activity of the transcription factor Sox4 may have resulted from either transcriptional regulating the expression of pro- and anti-apoptotic genes or executing Sox4 in a transcription-independent manner. To assess this possibility, we generated transcriptional incompetent Sox4 proteins by deletion and investigated their pro-apoptotic activity. Deletion of domains that are indispensable for transcriptional regulation did not abolish any effects

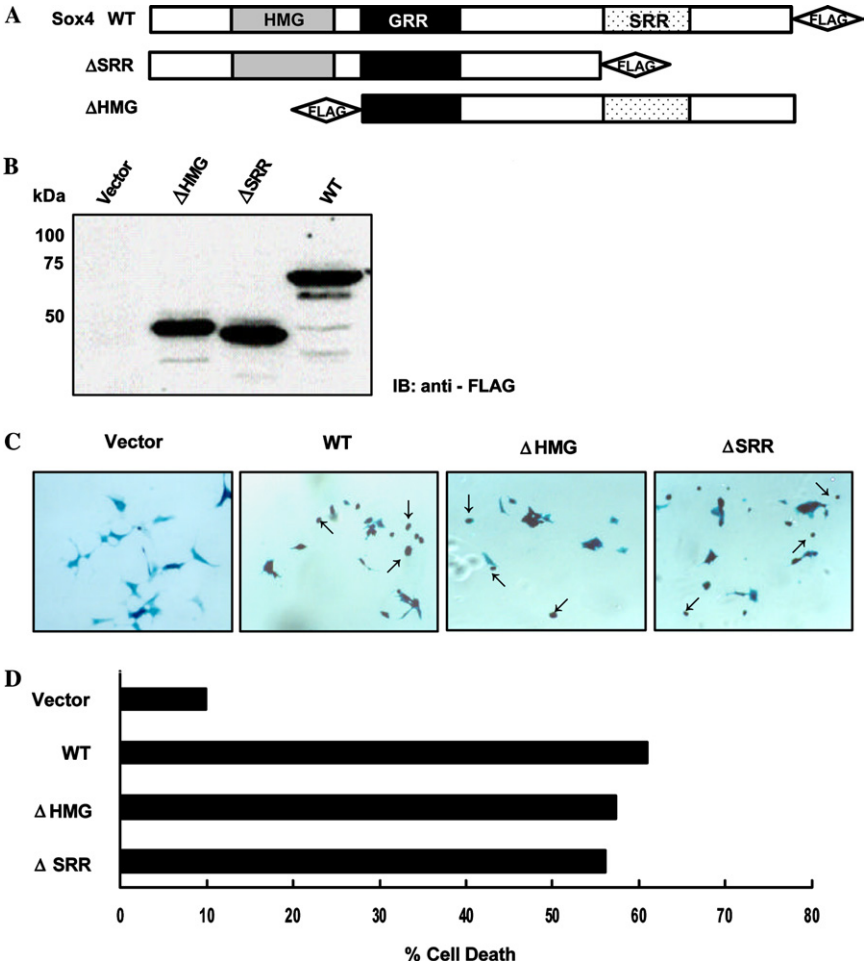

Fig. 3. Apoptotic cell death induced by transcriptionally defective Sox4. (A) Schematic representations of the truncated Sox4 constructs. Both constructs were tagged at the N- or C-terminus with the FLAG epitope: ΔSRR (aa 1–342) and ΔHMG (aa 166–474). (B) Expression of the truncated Sox4 proteins. Expression of Sox4 proteins was directed by the control of CMV promoter. Protein extracts isolated from transiently transfected cells were resolved by 12% SDS–PAGE, followed by IB analysis with anti-FLAG antibody. (C) Representative X-gal staining of HEK293 cells expressing transcriptionally defective Sox4. The indicated plasmids encoding transcriptionally defective Sox4 were cotransfected with pCMV β-gal into HEK293 cells. At 24 h post-transfection, cell death was evaluated by counting the numbers of elongated blue cells (surviving cells) versus round cells indicated in arrows (dead cells). (D) Quantification of cell death. All values are expressed as percentages of cells with round morphology relative to total blue cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

on the induction of apoptosis, suggesting that Sox4 is a pro-apoptotic factor that is directly associated with the apoptotic machinery in a transcription-independent manner.

The CD alone revealed an apoptotic activity similar to that of wild-type Sox4, as well as direct a similar apoptotic activity when it is inserted into an unrelated protein, GFP. The results suggest that the CD is necessary and sufficient for the Sox4's apoptotic induction and is therefore an independent signal for promoting apoptosis. However, we were not able to detect the cleavage of PARP, which is activated by caspases, during apoptosis induced by CD or wild-type Sox4. The results suggest that caspase-independent pathway may be attributable to nuclear chromatin condensation and DNA fragmentation observed in apoptosis induced by Sox4 [28–30]. The apoptosis initiation factor (AIF) and endonuclease G, which are involved in a caspase-independent pathway, need to be further characterized to elucidate the mechanisms by which Sox4 contributes to regulating apoptotic cell death.

A glycine-rich region (GRR) was identified in a class of RNA-binding proteins involved in post-transcriptional regulatory mechanisms and mRNA metabolism [31,32]. Additionally, GRR is found in the transcription factor NF-κB precursor p105 and mitochondrial processing peptidase (MPP) α-subunit [33,34]. Deletions or mutations of amino acids within GRR significantly reduced the processing activity. The GRR structural motif serves as a processing signal for the cleavage of p105 and is essential for recognition and cleavage of substrate proteins catalyzed by MPP [33–35]. Moreover, a glycine-rich epitope coding region (aa 204–218) in the bovine herpesvirus 5 (BHV-5) gE ectodomain is the

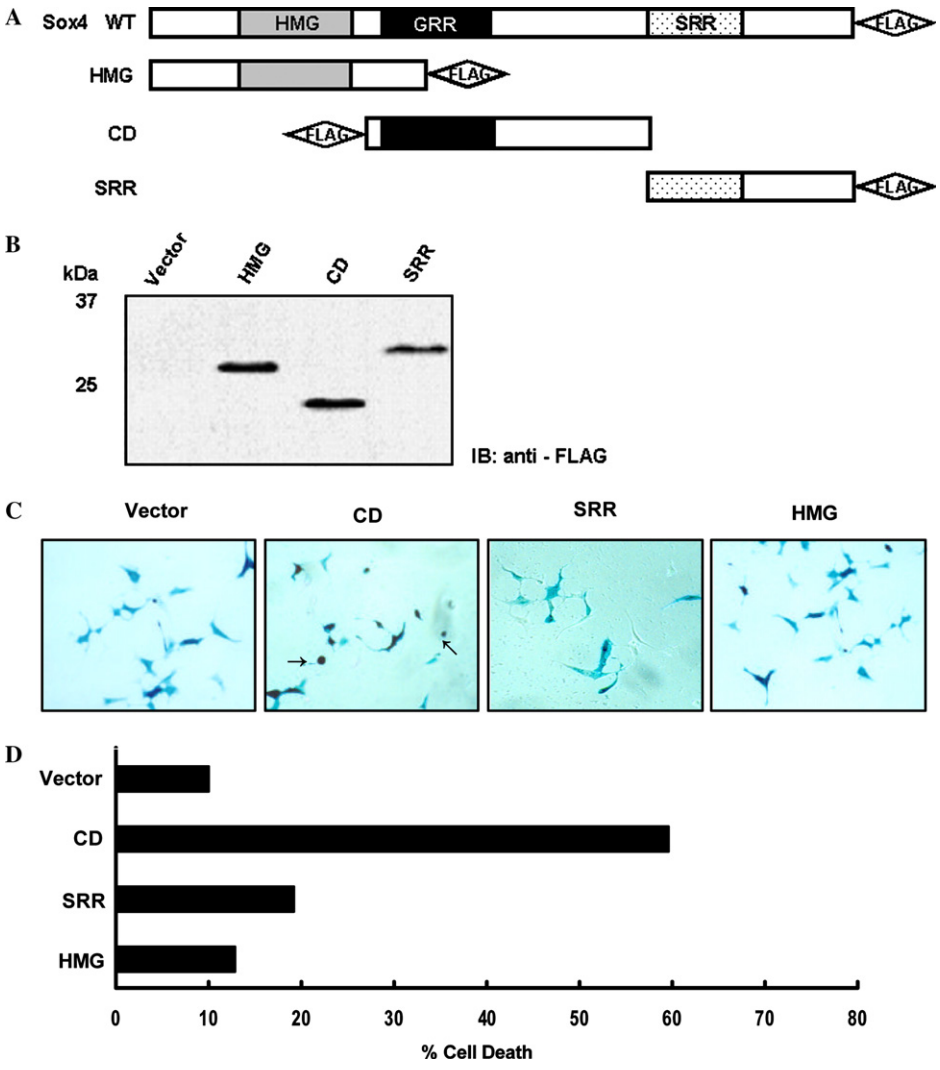

Fig. 4. The region responsible for cell death is the central domain containing GRR. (A) Schematic representations of the truncated Sox4 constructs. All constructs were tagged at the N- or C-terminus with the FLAG epitope: HMG (aa 1–173), CD (aa 166–342), and SRR (aa 343–474). (B) Expression of the truncated Sox4 proteins was analyzed by IB analysis with anti-FLAG antibody. (C) Representative X-gal staining of HEK293 cells expressing transcriptional defective Sox4. At 24 h post-transfection, cell death was evaluated by counting the numbers of elongated blue cells (surviving cells) versus round cells indicated in arrows (dead cells). (D) Quantification of cell death. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

region required for expression of the full virulence of BHV-5 [36]. These studies described that GRR plays important roles as diverse as RNA-binding, protein–protein interaction, transcriptional regulation, processing events, and nucleolar targeting [31–33,37,38]. The GRR (aa 152–227) in Sox4 is composed of approximately 47% glycine residues (amino acids 36 among 76). A variety of functions of GRR raise the possibility that the GRR within the CD of Sox4 might be a crucial domain involved in the apoptotic regulation, although we could not determine the pro-apoptotic activity of the GRR itself. Since no unique restriction enzyme sites exist around the GRR, and G+C content in GRR (aa 152–227) is over 75%, it makes it more difficult to subclone GRR into the expression vector either by a standard cloning method or PCR amplification.

The full-length Sox4 protein has a calculated molecular mass of 47.3 kDa, however, it migrates at approximately 70 kDa through SDS–PAGE (Fig. 3B). SRR and ΔHMG proteins that share SRR anomalously migrated through the SDS–PAGE system than was expected by their calculated molecular mass (Figs. 3 and 4B). The isoelectric points (p*I*) of the SRR and ΔHMG proteins are 4.09 and 5.43, respectively. These two protein fragments are highly acidic and thus had largely negative charges at the working pH of the SDS–PAGE system. These highly negative charges may influence the mobility of the proteins on SDS–PAGE by the inefficient binding to SDS. Furthermore, the C-terminal SRR (aa 333–397) in Sox4 consists of approximately 42% serine residues (amino acids 27 among 65). We analyzed amino acid residues of Sox4 with the ExPASy

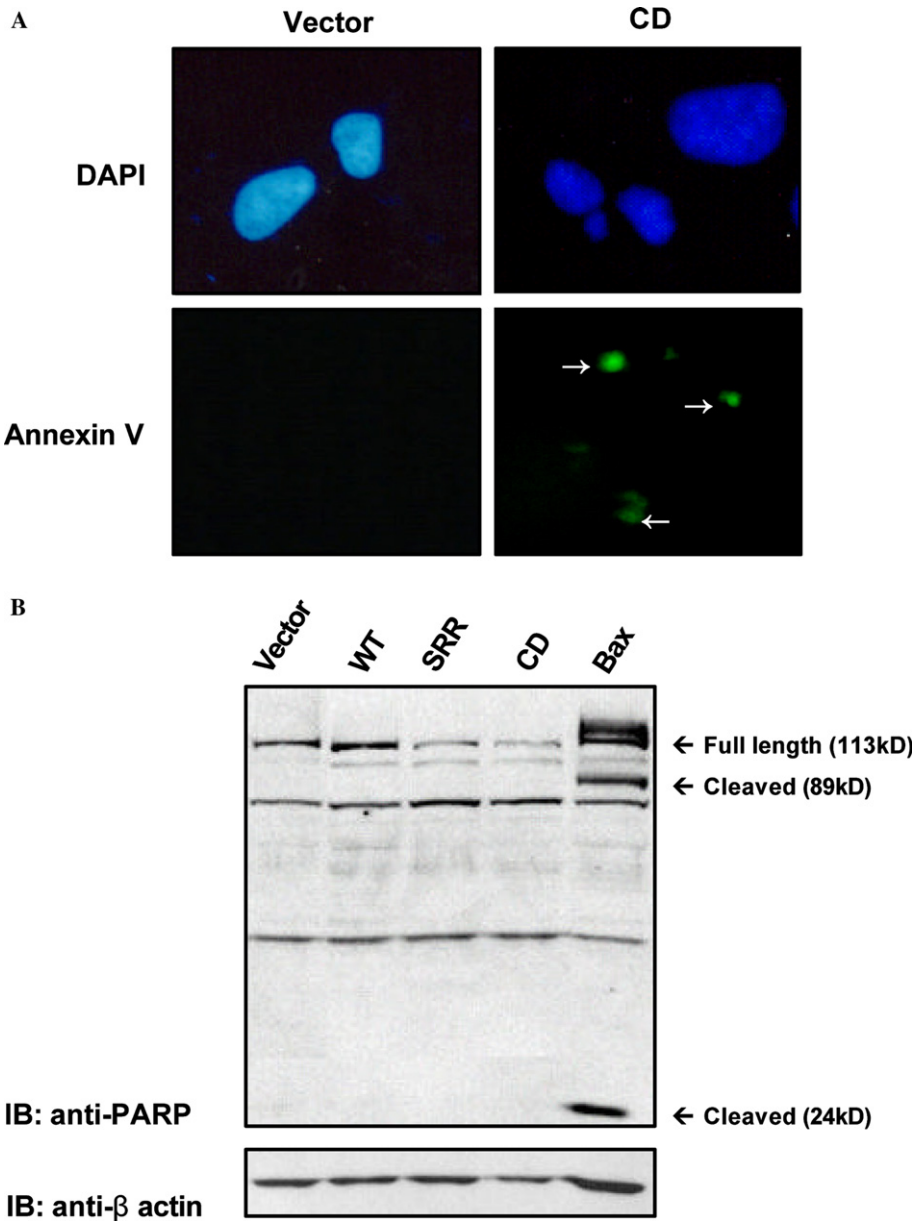

Fig. 5. Apoptotic cell death induced by the Sox4's CD. (A) Representative immunofluorescence images of annexin V staining. At 24 h post-transfection, the cells transfected with CD were stained with DAPI (upper panel) or annexin V (lower panel). (B) Effect of CD on the cleavage of PARP. Cell lysates isolated from cells transfected with the indicated plasmids were analyzed by IB analysis with anti-PARP antibody.

Proteomics Server program (the NetPhos 2.0 server; http://www.expasy.ch) to predict serine phosphorylation sites in the Sox4 protein. The total numbers of predicted serine phosphorylation sites are 44. Among them, 29 serine residues have a score of above 0.9 indicating as potential phosphorylation sites, and approximately 72% of these serine residues (amino acids 21 among 29 serine residues) are clustered within the SRR. Multiple phosphorylation on the several serine residues of Sox4 may further cause gel mobility changes in SDS–PAGE, as evidenced by several studies [39,40]. Although the hyper-phosphorylation of the SRR has not been yet identified, IB analysis with a phosphospecific antiserum against the Sox4's SRR or mapping of the phosphorylation sites with tandem mass spectrometry should provide key information for future studies on post-translational modifications of Sox4 [41]. Various Sox4 constructs used in this study can facilitate numerous types of molecular characterization of Sox4, as well as lead to uncovering of a new molecular target triggering apoptosis and eventually to the establishment of newly potent tools against human cancer.

Acknowledgments

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