The Ski Protein Family Is Required for MeCP2-mediated Transcriptional Repression*

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DNA methylation is essential for development in the mouse and plays an important role in inactivation of the X chromosome and genomic imprinting. MeCP2 is the founder member of a family of methyl-CpG-binding proteins. MeCP2 directly binds to the co-repressor mSin3, which interacts with class I histone deacetylase, recruiting them to methyl-CpG regions to suppress transcription. Here, we report that MeCP2 directly binds to two co-repressors, c-Ski and N-CoR, in addition to mSin3A, and that the c-Ski, which is encoded by the c-ski proto-oncogene, is required for MeCP2-mediated transcriptional repression. The two regions of c-Ski, including the C-terminal coiled-coil region, interact with the transcriptional repression domain in the center of the MeCP2 molecule. The immunostaining signals for c-Ski and MeCP2 overlap in the nuclear heterochromatin region, suggesting the co-localization of the two proteins. The degree of transcriptional repression mediated by a Gal4-MeCP2 fusion protein was abrogated by overexpression of the putative dominant negative form of c-Ski. Furthermore, injection of antibodies against c-Ski and Sno almost completely abolished the transcriptional repression mediated by the Gal4-MeCP2 fusion protein. These results suggest that the ski gene family is involved in methyl CpG-mediated transcriptional repression.

The ski gene was originally identified as an oncogene carried by avian Sloan-Kettering viruses (1). The ski gene family consists of two members, ski and sno (ski-related novel gene) (2), which share significant homology in their N- and C-terminal regions (2, 3). c-Ski/Sno act as co-repressors and directly bind to two other co-repressors, N-CoR/SMRT and mSin3A (4). N-CoR/SMRT is bound via its N-terminal cysteine-rich region, and mSin3A is bound by its C-terminal coiled-coil region. N-CoR and SMRT were originally identified as co-repressors that mediate transcriptional repression by nuclear hormone receptors (5, 6) and form a complex with the class II histone deacetylases (HDACs) (7–10). mSin3 (mSin3A and mSin3B) was originally shown to mediate transcriptional repression by basic helix-loop-helix proteins of the Mad family (11) and forms a complex with the class I HDACs (12, 13). These three co-repressors (N-CoR/SMRT, mSin3, and c-Ski/Sno) are necessary for the transcriptional repression mediated by nuclear hormone receptors, Mad, and possibly other repressors (4, 14–19). However, purified mSin3 complex contains neither N-CoR/SMRT nor c-Ski/Sno (12, 13), whereas the N-CoR/SMRT complex has neither mSin3 nor c-Ski/Sno as a component (7–10). Therefore, these three co-repressors may not always function together.

Cytosine residues in the sequence 5′-CpG (cytosine-guanine) are often post synthetically methylated in animal genomes. CpG methylation is essential for mouse development (20) and has been linked to long term silencing of certain genes during mammalian development (21, 22). For instance, CpG methylation is necessary both for inactivation of the X chromosome and for genomic imprinting. CpG methylation is also involved in repression of viral genomes (23, 24). Further, the methylation of tumor suppressor gene promoters contributes directly to the progression of some cancers (for review, see Ref. 25). The methyl-CpG signal is recognized by a group of methyl-CpG-binding proteins that share a well conserved methyl-CpG-binding domain (MBD) (for review, see Ref. 26). The recently reported solution structure of MBD indicated that the recognition of the methyl groups and CG sequence at the methylation site is due to five highly conserved residues that form a hydrophobic patch (27). MeCP2 is the best characterized member of this family and interacts specifically with methylated DNA to mediate transcriptional repression (28–30). The X-linked dominant disorder, Rett syndrome, which is a progressive neurodevelopmental disorder, was recently found to be caused by mutations in the MeCP2 gene on Xq28 (31). In fact, a mouse MeCP2-null mutation causes neurological symptoms that mimic Rett syndrome (32, 33). MeCP2 is an abundant 486-amino acid protein that contains two functional domains, a MBD essential for its binding to methyl-CpG, and a transcriptional repression domain (TRD) that mediates gene silencing. MeCP2 binds to the HDAC complex containing the co-repressor mSin3A via direct interaction between the TRD and mSin3A (34, 35). Recruitment of the HDAC complex by MeCP2 is thought to be an important mechanism of the transcriptional repression mediated by methyl-CpG. This is consistent with the observation that densely methylated DNA associates with transcriptionally repressed chromatin characterized by the presence of under-acetylated histones (36, 37). Although the class I HDACs were

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shown to interact with MeCP2 via mSin3A (34, 35), other components that are required for the MeCP2-mediated repression remain elusive.

Here we have studied the role of c-Ski in MeCP2-mediated transcriptional repression. We have found that c-Ski directly binds to MeCP2 and is required for the transcriptional repression mediated by MeCP2.

MATERIALS AND METHODS

GST Pull-down Assays—GST pull-down assays were performed as described (4). Human MeCP2 cDNA was obtained from S. Kudo (38). The GST–MeCP2, GST–c-Ski, and GST–N-CoR fusion proteins contained the full-length human MeCP2, full-length human c-Ski, and N-terminal 427 amino acids of the mouse N-CoR protein, respectively. Approximately 10 μg of the GST fusion protein was used per assay. For the in vitro translation of c-Ski, MeCP2, and N-CoR, various cDNA fragments were cloned into a pSPUTK vector (Stratagene) containing the SP6 promoter and the Xenopus β-globin 5′-untranslated region. YBB buffer (20 mM Hepes, pH 7.5, 1 mM dithiothreitol, 0.1% Triton X-100, 10% glycerol, 0.5 mM bicine sodium salt, 1 mM EDTA, magnesium chloride, 12.5% glycerol, 1 mM dithiothreitol, 0.1% Nonidet P-40, protease inhibitor mixture). The nuclei were isolated and extracted in buffer E (35 mM Hepes, pH 7.9, 1.5 mM MgCl2, 12.5% glycerol, 1 mM dithiothreitol, 0.1% Nonidet P-40, protease inhibitor mixture). The nucleosomes were used for Western blotting with anti-c-Ski rabbit polyclonal antibodies raised against bacterially expressed human c-Ski and Snodrin were used to identify the amino acid 556–728 of c-Ski and the fragment overlapping the cysteine-rich N-CoR-binding domain (amino acids 197–330). To identify the specific region in the MeCP2 protein that interacts with c-Ski, we next performed similar GST pull-down assays using the GST-c-Ski fusion protein containing the full-length MeCP2 (Fig. 2). The 35S-MeCP2 translated in vitro efficiently bound to the GST-c-Ski fusion (Fig. 1A). To identify which region of c-Ski interacts with MeCP2, the GST pull-down assay was performed using various forms of in vitro translated c-Ski (Fig. 1A and B). To identify the specific region in the MeCP2 protein that interacts with c-Ski, we next performed similar GST pull-down assays using the GST-c-Ski fusion protein containing the full-length MeCP2 (Fig. 2). The 35S-MeCP2 translated in vitro efficiently bound to the GST-c-Ski fusion. Truncation to amino acid 309 from the C terminus of MeCP2 did not affect the affinity of binding with c-Ski, whereas truncation to amino acid 162 almost completely eliminated the interaction with c-Ski. This suggests that the region containing the TRD is responsible for binding to c-Ski, because deletion of the TRD abolished the interaction with c-Ski, whereas the fragment containing the TRD efficiently bound to c-Ski. The results are presented in Fig. 2B, left panel.

RESULTS

Two Regions of c-Ski Interact with the TRD of MeCP2—MeCP2 interacts directly with the co-repressor mSin3A (34, 35), raising the possibility that other co-repressors, such as c-Ski, also directly bind to MeCP2. To investigate this, we first performed GST pull-down assays using the GST–MeCP2 fusion protein containing full-length MeCP2 (Fig. 1). Wild-type c-Ski protein translated in vitro efficiently bound to the GST–MeCP2 fusion (Fig. 1A and B). To identify which region of c-Ski interacts with MeCP2, the GST pull-down assay was performed using various forms of in vitro translated c-Ski (Fig. 1A and C). The results indicated that both regions in the c-Ski protein bind to MeCP2: the C-terminal coiled-coil region (amino acids 556–728) and a region overlapping the cysteine-rich N-CoR-binding domain (amino acids 197–330).

To identify the specific region in the MeCP2 protein that interacts with c-Ski, we next performed similar GST pull-down assays using the GST-c-Ski fusion protein containing the full-length c-Ski (Fig. 2). The 35S-MeCP2 translated in vitro efficiently bound to the GST-c-Ski fusion. Truncation to amino acid 309 from the C terminus of MeCP2 did not affect the affinity of binding with c-Ski, whereas truncation to amino acid 162 almost completely eliminated the interaction with c-Ski. This suggests that the region containing the TRD is responsible for binding to c-Ski, because deletion of the TRD abolished the interaction with c-Ski, whereas the fragment containing the TRD efficiently bound to c-Ski. The results are presented in Fig. 2B, left panel.
bound to GST-c-Ski, whereas other N-CoR fragments did not.

Further, to identify which region of the MeCP2 protein interacts with N-CoR, we also performed the GST pull-down assays using the GST-N-CoR and a series of MeCP2 deletion mutants translated in vitro (Fig. 4). The results indicated that the broad region containing both the MBD and the TRD of MeCP2 (amino acids 55–309) is required for efficient interaction with N-CoR. Thus, MeCP2 interacts with three co-repressors: mSin3A, c-Ski, and N-CoR.

**MeCP2 Directly Interacts with c-Ski and N-CoR**—The binding of MeCP2 with the two co-repressors, c-Ski and N-CoR, was shown using the in vitro translated proteins. However, it is possible that a protein in the reticulocyte lysates mediates an interaction between them. To confirm a direct interaction of MeCP2 with c-Ski and N-CoR, the His tag-linked MeCP2 protein was expressed in E. coli, purified, and used for the GST pull-down assay (Fig. 5). The purified His-MeCP2 bound to both GST-c-Ski and GST-N-CoR containing the N-terminal 427-amino acid fragment of N-CoR. Addition of the reticulocyte lysates to the binding reactions slightly enhanced the affinity of His-MeCP2 with the GST-c-Ski and GST-N-CoR resins, suggesting the presence of co-factor(s) in the reticulocyte lysates that stimulate an interaction between them. These results indicate that MeCP2 directly binds to both c-Ski and N-CoR, and probably other co-repressor such as mSin3A in the reticulocyte lysates may enhance the interaction between them.

**Co-immunoprecipitation of c-Ski and MeCP2**—The in vivo interaction between MeCP2 and the co-repressor c-Ski was...
confirmed by co-immunoprecipitation assay (Fig. 6). The expression vectors for both c-Ski and FLAG-linked MeCP2 were transfected into 293T cells, and whole cell lysates were prepared and used for co-immunoprecipitation. The antibody against c-Ski co-precipitated full-length MeCP2 but not the MeCP2 mutant lacking the TRD (MeCP2\(^{H9004\_TRD}\)) (Fig. 6A, left panel). The normal IgG control co-precipitated neither wild-type nor mutant MeCP2. When we used the anti-FLAG antibody for co-immunoprecipitation, wild-type c-Ski, but not the c-Ski mutant lacking the MeCP2-interacting regions (c-Ski\(^{H9004\_46\_260/H9004\_493\_728}\)), was co-immunoprecipitated (Fig. 6A, right panel). Again, the normal IgG control co-precipitated neither wild-type nor mutant c-Ski. We also performed the co-immunoprecipitation experiments of endogenous c-Ski and MeCP2 proteins. Nuclear extracts prepared from 293 cells were immunoprecipitated with the antibodies against MeCP2 or normal IgG as a control (Fig. 6B). c-Ski was co-immunoprecipitated with the anti-MeCP2 antibodies but not with control IgG. Thus, the results of the co-immunoprecipitation assays indicate the presence of an in vivo association between c-Ski and MeCP2.

Partial Co-localization of c-Ski and MeCP2—To further confirm the in vivo association between c-Ski and MeCP2, we then...
examined the subcellular localization of c-Ski and MeCP2 in transfected CV-1 cells using laser confocal microscopy (Fig. 7A). As reported previously (4), c-Ski was localized in the nuclear dotlike structure when c-Ski alone was expressed (Fig. 7A, panel a). On the other hand, wild-type MeCP2 signals were found in both the fine microspeckle structure and the foci with dim edges in the nucleus when MeCP2 alone was expressed (Fig. 7A, panel b). As reported by Bird and co-workers (28, 42), the MeCP2 signals matched the heterochromatin regions that were stained with Hoechst 33258 (data not shown). When c-Ski was co-expressed with wild-type MeCP2 (Fig. 7A, panels d–f), the nuclear dotlike structure of c-Ski was partly disrupted. The c-Ski signals were detected not only in the dotlike structures (arrows 1 and 2 in the schematic in Fig. 7A) but also in the fine microspeckles and in the island-like area (arrows 3 and 4 in the schematic in Fig. 7A). MeCP2 signals were also found in some of the dotlike structures (arrow 1) and a part of the island-like area (arrow 3). The island-like area containing MeCP2 (arrow 3) was shown to be the heterochromatin region that was positive for Hoechst 33258 staining (data not shown). Thus, c-Ski was recruited to the heterochromatin region by MeCP2, whereas c-Ski moved some of the MeCP2 proteins to the c-Ski-containing dotlike structure from the heterochromatin region. Furthermore, co-expression of c-Ski and MeCP2 changed the shape of heterochromatin region from a fine microspeckle structure and foci with dim edges to an island-like structure, although the mechanism of this transformation is unknown. We also used the mutant MeCP2 lacking the TRD as a control in the immunostaining study. This mutant protein was localized in both the cytoplasm and the nucleus (Fig. 7A, panel c). This is consistent with the previous report that the sequence between amino acids 255–271 (RKAEADPQAIPKKRGRK) of the TRD of MeCP2 functions as a nuclear localization signal (42). Co-expression of the MeCP2 mutant lacking the TRD with c-Ski did not affect the nuclear dotlike structure of c-Ski (Fig. 7A, panels g–i). These results strongly suggest an in vivo association between c-Ski and MeCP2 in the nucleus via the TRD of MeCP2.

We also investigated the subcellular localization of the endogenous c-Ski and MeCP2 proteins in 293 cells using deconvolution microscopy. The endogenous c-Ski proteins were localized in the fine microspeckle structures in the nucleus and in the peripheral region of nuclei. The endogenous MeCP2 proteins were enriched in the heterochromatin region, which was positive for the Hoechst 33258 staining, and in the periphery of nuclei. Some of the c-Ski stainings overlapped with the MeCP2 staining, which was shown as the yellow color in the overlay of two signals. Thus, a significant amount of endogenous c-Ski associates with MeCP2 in the nuclei.

Abrogation of the MeCP2-dependent Transcriptional Repression by a Dominant Negative Form of c-Ski—The physical association between MeCP2 and c-Ski suggested that c-Ski may be required for the transcriptional repression mediated by MeCP2. To investigate this, we first examined the effect of overexpression of the putative dominant negative form of c-Ski on MeCP2-mediated repression in Ski-deficient MEFs (Fig. 8A). Small amounts of the Gal4-TRD fusion protein, which consists of the Gal4 DNA-binding domain and the Ski-interacting domain of MeCP2 (amino acids 163–320), repressed transcription from a Gal4 site-containing reporter. This repression was probably mediated by the Sno protein in the Ski-deficient MEFs. Overexpression of the c-Ski fragment (amino acids 556–728) linked to the nuclear localization signal of the SV40 T antigen (NLS-556–728) from the chicken cytoplasmic β-actin promoter, which contained the MeCP2-binding domain, abolished the Gal4-TRD-mediated repression as a dominant negative form. The c-Ski mutant, which lacked the MeCP2-binding domain (Δ46–260/Δ493–728) but contained the endogenous nuclear localization signal, did not affect the repression. Thus, the dominant negative form of c-Ski efficiently abrogated the MeCP2-mediated transcriptional repression.

To indicate that the level of Ski expressed from the transfected DNA is not unusually high compared with endogenous c-Ski in wild-type MEFs, the Western blotting was performed (Fig. 8A, bottom right panel). The density of c-Ski band obtained by using lysates from the transfected Ski-deficient MEFs was approximately half of that obtained with the lysates from wild-type MEFs. Because the transfection efficiency into MEFs was about 5%, the level of Ski protein expressed from the transfected DNA was about 10-fold higher than the level of endogenous c-Ski in wild-type MEFs. However, we used 4-fold more c-Ski expression plasmid to detect c-Ski in Western blotting assays compared with the amount of c-Ski expression plasmid used in the luciferase reporter assays described above.
Role of c-Ski in MeCP2-dependent Repression

A. Abrogation of the MeCP2-mediated repression by a dominant negative form of c-Ski. MEFs lacking c-Ski were transfected with the Gal4 site-containing luciferase reporter together with either the Gal4-TRD or the Gal4 DBD expression plasmid and an increasing amount of either the plasmid encoding the nuclear localization signal (NLS)-linked c-Ski fragment containing MeCP2-binding domain (NLS-556–728) or mutant c-Ski lacking the MeCP2-binding domain (∆46–260/∆493–728). Luciferase activity was then measured, and the ratio of the luciferase activity with the Gal4-TRD expression plasmid relative to that with the Gal4 DBD plasmid was calculated. An average of three experiments with standard deviations is shown. Bottom right, nuclear extracts were prepared from wild-type and c-Ski-deficient MEFs, and the c-Ski-deficient MEFs transfected with c-Ski expression plasmid and used for Western blotting with the anti-c-Ski antibody. The amount of lysate proteins used are shown above each lane. In the far left lane, whole cell lysates prepared from the 293T cells transfected with the c-Ski expression plasmid were used as a control. B, abrogation of MeCP2-induced repression by anti-Ski and anti-Sno antibodies. The nuclei of Rat-1 cells were injected with the Gal4 site-containing lacZ reporter together with either the Gal4-MeCP2 or Gal4 expression plasmids and the GFP vector as a marker. The effect of antibodies against c-Ski and Sno on the number of LacZ-positive cells was examined by co-injection. The number of cells injected in each experiment ranged from 70 to 240. An average of three experiments with standard deviations is shown. WT, wild type.

FIG. 8. Role of c-Ski in the MeCP2-mediated transcriptional repression. A, a strong interaction between c-Ski and MeCP2, it was somewhat surprising that the injection of antibodies against c-Ski and Sno almost completely abrogated the MeCP2-mediated transcriptional repression. Injection of the same antibodies only partially inhibits the repression mediated by Mad, thyroid hormone receptor-β, or Rb only partially (30–50%) (4, 19). This may suggest that the role of c-Ski/Sno is different depending on the repressor with which it interacts. When MeCP2 and c-Ski were both overexpressed, the location of each within the cell was affected (Fig. 7A). The signal of endogenous MeCP2 proteins overlapped with the staining signal with Hoechst 33258, which indicates the heterochromatin (Fig. 7B). Localization of MeCP2 in the heterochromatin region is consistent with the fact that the heterochromatin contains a major satellite DNA sequence, which accounts for 25–50% of genomic 5-methylcytosine. In the heterochromatin region, the c-Ski signal was also observed, supporting the idea that c-Ski is involved in the MeCP2-mediated transcriptional repression. In addition to the inside region of nucleus, both endogenous c-Ski and MeCP2 are localized in the periphery of the nucleus, which is also positive for Hoechst 33258 staining (Fig. 7B). This localization could be consistent with the recent report that the perinuclear chromatin domains contain the telomeres and constitutive areas of transcriptional repression in yeast (43, 44).

Although the ski gene was originally identified as an oncogene carried by avian Sloan-Kettering viruses (1), it was recently reported that c-Ski binds directly to Smad proteins, which are key regulators in TGF-β signaling (45–48). c-Ski inhibits the TGF-β-induced transcriptional activation by recruiting the HDAC complex to Smad proteins. Therefore, ski experiments were performed using a Gal4-lacZ reporter containing Gal4-binding sites (Fig. 8B). Injection of the reporter into Rat-1 cells gave rise to many lacZ-positive cells, whereas co-injection of the lacZ reporter with the plasmid encoding the Gal4-MeCP2, which consisted of the DNA-binding domain of Gal4 and full-length MeCP2, resulted in a decrease in the number of lacZ-positive cells. This decrease was partially reversed by co-injection with anti-c-Ski antibody or anti-Sno antibody. Furthermore, co-injection of both anti-c-Ski and anti-Sno antibodies almost completely abolished the Gal4-MeCP2-mediated decrease in the number of lacZ-positive cells. The specificity of the anti-c-Ski and anti-Sno antibodies used here has previously been shown; co-injection of the c-Ski and Sno expression vectors blocks the activity of these antibodies, but the antibodies do not affect the repression mediated by another repressor (δEF1) that does not use the Ski complex (4). The results of co-transfection assays and the antibody injection assays indicate that c-Ski and Sno are required for the MeCP2-mediated transcriptional repression.

DISCUSSION

An increasing number of molecules are understood to be involved in MeCP2-mediated transcriptional repression. Here, we have shown that c-Ski binds directly to MeCP2 and is necessary for its proper function. We have demonstrated that MeCP2 binds directly to two co-repressors, c-Ski and N-CoR. Because previous reports by Nan et al. (34) and by Jones et al. (35) showed that MeCP2 binds directly to mSin3A, our results bring the number of known co-repressors for MeCP2 to three. It is unlikely, however, that one macromolecular complex contains all three. Recent biochemical studies of the co-repressor complex indicate that the purified mSin3 complex contains neither N-CoR/SMRT nor c-Ski/Sno (12, 13), and the N-CoR/SMRT complex involves neither mSin3 nor c-Ski/Sno as a component (7–10). Therefore, there are probably separate complexes for each of the co-repressors that may associate with MeCP2 either sequentially or at the same time. Although we found a strong interaction between c-Ski and MeCP2, it was somewhat surprising that the injection of antibodies against c-Ski and Sno almost completely abrogated the MeCP2-mediated transcriptional repression. Injection of the same antibodies only partially inhibits the repression mediated by Mad, thyroid hormone receptor-β, or Rb only partially (30–50%) (4, 19). This may suggest that the role of c-Ski/Sno is different depending on the repressor with which it interacts.
may act in part as an oncogene by inhibiting TGF-β signaling in TGF-β-responsive cells. In addition to this mechanism, Ski may also enhance cellular proliferation by enhancing the methyl CpG-mediated silencing. The recent discovery of numerous hypermethylated promoters of tumor suppressor genes indicates that inactivation of tumor suppressor genes by DNA methylation is an important mechanism of tumor formation (for review, see Ref. 25). Therefore, overexpression of c-Ski may lead to silencing some tumor suppressor genes, resulting in cellular transformation.

Our results indicate that c-Ski and Sno are needed for MeCP2-mediated transcriptional repression. This may suggest that the ski gene family is also required for genome imprinting. We have investigated whether some imprinting genes such as H19 and Igf2 are ectopically expressed in ski-deficient embryos because of reduction in methyl-CpG-dependent transcriptional repression. However, we found no difference in the expression levels of these genes between wild-type and ski-deficient embryos. This is consistent with the recent report that the hypermethylated genes such as MLH1 and TIMP3 cannot be transcriptionally reactivated with the HDAC inhibitor trichostatin A (A49).

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