Viral Ski Inhibits Retinoblastoma Protein (Rb)-mediated Transcriptional Repression in a Dominant Negative Fashion*

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The mechanism by which the viral oncogene ski (v-ski) transforms chicken embryo fibroblasts is currently unknown. Recently, the c-ski gene product (c-Ski) was found to bind to N-CoR (nuclear hormone receptor co-repressor), an element implicated in transcriptional repression mediated by multiple transcriptional repressors including the nuclear hormone receptors and Mad. c-Ski is required for transcriptional repression mediated by Mad involved in negative regulation of cellular proliferation. v-Ski abrogates Mad-induced transcriptional repression in a dominant negative fashion. Here we report that v-Ski also inhibits transcriptional repression mediated by Rb, another tumor suppressor gene product. Rb forms a complex with c-Ski, Sin3A, and histone deacetylase (HDAC) via direct binding to c-Ski and HDAC. c-Ski is required for the transcriptional repression mediated by Rb. These results suggest that inhibition of Rb activity contributes, at least partly, to transformation by v-Ski.

The oncogene v-ski was originally identified in avian Sloan-Kettering viruses and found to transform chicken embryo fibroblasts (1). The human c-ski proto-oncogene product (c-Ski) is a 728-amino acid nuclear protein, and the N- and C-terminal regions of c-Ski possess a cysteine-rich and a coiled-coil region, respectively (2, 3). The v-Ski protein lacks a 292-amino acid region from the C terminus of c-Ski, but still contains the N-proximal cysteine-rich region (4). This N-proximal region is responsible for the cellular transformation capacity of ski (5). The ski gene family comprises two members, ski and sno (ski-related novel gene) (2), and both have been shown to share clear homology in their N- and C-terminal regions (2, 6). Recently we found that c-Ski directly binds to N-CoR (nuclear hormone receptor co-repressor) (28). N-CoR was originally identified as a co-repressor that binds to and mediates transcriptional repression by nuclear hormone receptors (8). Another co-repressor, SMRT, shows striking homology to N-CoR (9). N-CoR also forms a complex with mammalian Sin3 orthologues (mSin3A and mSin3B). The binding of mSins to histone deacetylase (HDAC) suggested that transcriptional repression through N-CoR involves deacetylation of nucleosomal histones (10–14). The basic helix-loop-helix proteins of the Mad family act as transcriptional repressors after heterodimerization with Max (15). Mad interacts with the HDAC complex through direct binding to mSin3, and N-CoR is required for Mad-induced transcriptional repression (10–14). We demonstrated that N-CoR binds to the N-terminal region of c-Ski and that this interaction is also required for transcriptional repression mediated by Mad and the thyroid hormone receptor β (28). The same target sequence of Mad/Max, the so-called E-box, is also recognized by a heterodimer of Myc/Max that activates transcription. It is believed that Myc/Max enhances cellular proliferation or transformation, whereas Mad/Max leads to suppression of proliferation or induction of terminal differentiation in a wide range of cell types (16, 17). Our data indicated that v-Ski blocks Mad-induced transcriptional repression in a dominant negative fashion (28), suggesting that inhibition of Mad function contributes to transformation by v-Ski.

In addition to Mad, the retinoblastoma protein (Rb) encoded by another tumor suppressor gene also binds to HDAC (18–20). Rb regulates the G1/S transition in the cell cycle by silencing a group of target genes regulated by E2F transcription factors (21, 22). Rb binds to the activation domain of E2F and then actively represses the promoter by recruiting HDAC. The pocket region of Rb, which contains two subdomains, termed A and B, are responsible for interaction with HDAC (18–20). Although HDAC forms a complex with mSin3, N-CoR, and Ski, it remains unknown whether Rb can form a complex with any of these components of the N-CoR complex. To understand the molecular mechanism of transformation by v-Ski, we examined whether c-Ski forms a complex with Rb and whether v-Ski abrogates Rb-induced transcriptional activation as well as the case of Mad. Our results indicate that c-Ski is needed for the transcriptional repression mediated by Rb and that v-Ski abrogates Rb-induced transcriptional repression.

MATERIALS AND METHODS
Co-immunoprecipitation—HeLa cells were lysed in lysis buffer consisting of PBS, 0.1% Nonidet P-40, 10% glycerol, and protease inhibitor mixture (Boehringer Mannheim). CV-1 cells were lysed in lysis buffer consisting of PBS, 1 mM NaF, 1 mM Na3VO4, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and protease inhibitor product; sno, ski-related novel gene; PBS, phosphate-buffered saline; GST, glutathione S-transferase.
v-Ski Inhibits Rb Function

RESULTS

To investigate whether Rb forms a complex with c-Ski in vivo, co-immunoprecipitation assays were performed (Fig. 1A). The cell lysates were prepared from HeLa cells and immunoprecipitated with anti-c-Ski, anti-Sno, or control anti-Gal4 antibody. Rb was co-immunoprecipitated with anti-c-Ski or anti-Sno antibodies but not with the control anti-Gal4 antibody. To examine complex formation between the endogenous Rb protein and mSin3A, co-immunoprecipitation was performed using CV-1 cells. The anti-Rb antibody XZ91 co-immunoprecipitated mSin3A, whereas the anti-Rb antibody G3-245 or control IgG did not (Fig. 1B). These results indicate that Rb forms a complex in vivo not only with HDAC but also with c-Ski and mSin3A. We could not detect N-CoR or SMRT in the complex immunoprecipitated with anti-Rb antibodies (data not shown). However, we cannot exclude the possibility that this is due to the low level of expression of N-CoR and SMRT in the complex. The amount of HDAC1 coprecipitated with Gal4-Rb in the presence of v-Ski was apparently less than that with c-Ski. These results suggest that v-Ski inhibits association between Rb and HDAC1 in a dominant negative fashion.

During the analysis of the interaction between Rb and the components of the N-CoR complex, we found that c-Ski directly binds to Rb in vitro. In the GST pull-down assays using in vitro translated Rb and the GST-c-Ski resin containing full-length c-Ski, a significant amount of in vitro translated Rb was found to bind to the GST-c-Ski resin (Fig. 2A). The results of binding assays using the different mutants of Rb indicated that the B subdomain in the pocket region of Rb is responsible for the interaction with c-Ski.
interaction with c-Ski. To identify the region in c-Ski that interacts with Rb, the GST pull-down assay was performed using the GST-Rb fusion protein resin and various forms of in vitro translated c-Ski protein (Fig. 2B). The results indicated that two regions in c-Ski efficiently interact with Rb; one is the region between amino acids 197 and 330, which includes a part of the N-CoR-binding domain (amino acids 99–274) and the other is the C-terminal coiled-coil region (amino acids 556–728). Thus, Rb directly binds to c-Ski and HDAC.

To further investigate whether c-Ski is required for transcriptional repression by Rb, antibody injection experiments were done (Fig. 3). Injection into Rat-1 cells of a lacZ reporter plasmid containing the lacZ gene linked to the TK promoter and Gal4-binding sites gave rise to many lacZ-positive cells. Co-injection of this lacZ reporter with a plasmid encoding the Gal4-Rb fusion protein made up of the Gal4 DNA-binding domain and the pocket region of Rb resulted in a decrease in the number of lacZ-positive cells. This decrease was relieved significantly by co-injection of anti-c-Ski antibody and partially by anti-Sno antibodies. Co-injection of both antibodies also significantly relieved the decrease in the number of lacZ-positive cells, but not completely. The incomplete abrogation of Gal-Rb function by co-injection of both antibodies may be due to the presence of other Ski-related protein(s) such as the third member of the ski gene family which we identified recently. As a control experiment, we used a Gal4 fusion protein containing the repressor domain of δEF1, which is thought not to utilize the N-CoR c-Ski complex. Co-injection of anti-c-Ski or anti-Sno antibodies did not alleviate the decrease in the number of lacZ-positive cells induced by Gal4-δEF1 (28), indicating that the effect of anti-Ski/Sno antibodies was specific for Rb.

To investigate whether v-Ski mutants that lack the C-terminal region of c-Ski could abrogate transcriptional repression by Rb in a dominant negative fashion as in the case of Mad, we examined the effect of overexpression of v-Ski on Gal4-Rb-induced transcriptional repression (Fig. 4, A and B). Gal4-Rb containing the pocket region of Rb strongly repressed transcription from the Gal4 site-containing reporter. This Gal4-Rb-induced repression was abrogated by v-Ski in a dose-dependent manner. Furthermore, wild type c-Ski partly abrogated Gal4-Rb-induced transcriptional repression. We observed that the microspeckle pattern of N-CoR was disrupted by coexpression of a high amount of c-Ski but not by a low amount of c-Ski. These two observations are consistent with the idea that over-
expression of wild type c-Ski abrogates transcriptional repression by creating an imbalance between the components of the co-repressor complex rather than potentiating transcriptional repression. In control experiments, repression by the Gal4-deleted fusion protein was not abolished by co-expression of either v-Ski or wild type c-Ski (28). Using the E2F1 site-containing luciferase reporter, we also examined the effect of v-Ski on E2F1-mediated transcriptional activation (Fig. 4C). v-Ski was also found to enhance E2F1-induced transcriptional activation in a dose-dependent manner. These results indicate that v-Ski inhibits Rb-dependent transcriptional repression.

**DISCUSSION**

The oncogene v-ski can transform chicken embryo fibroblasts. Our results indicate that v-Ski abrogates transcriptional repression mediated not only by Mad but also by Rb. c-Ski has two regions that are conserved in related proteins, the N-terminal cysteine-rich region and the C-terminal coiled-coil region. N-CoR binds to the N-terminal cysteine-rich region, while the C-terminal coiled-coil region binds to mSin3 (28). The C-truncated c-Ski protein lacking the coiled-coil region cannot bind to mSin3 and disrupts the dot-like structure of N-CoR (28), suggesting that this form of c-Ski acts as in a dominant negative fashion. Because v-ski also lacks the C-terminal coiled-coil region, v-Ski probably inhibits Mad- and Rb-mediated transcriptional repression in a dominant negative fashion. In our co-transfection assay, overexpression of normal c-Ski also partly abrogated the transcriptional repression mediated by Rb (Fig. 4). This is consistent with the fact that overexpression of wild type c-Ski also leads to transformation (24). Mutation of the human Rb gene occurs in a wide variety of tumors (25). In addition, one of the mad-related genes, mxi1, was recently demonstrated to act as a tumor suppressor using mutant mice (26). Therefore, abrogation of Rb and Mad activity by v-Ski may contribute, at least partly, to transformation by v-ski.

Rb was recently reported to directly bind to HDAC (18–20). Our results indicate that Rb also directly interacts with c-Ski. Furthermore, Rb forms a complex with mSin3, although it is not clear whether the Rb-HDAC-mSin3A-Ski complex contains N-CoR. The antibody injection experiments showed that c-Ski is required for Rb-mediated transcriptional repression (Fig. 3). At present, it remains unknown whether N-CoR and mSin3 are needed for the transcriptional repression mediated by Rb. Thus, c-Ski is required for the transcriptional repression mediated by at least Mad, thyroid receptor, and Rb. It is possible that other transcriptional repressors that utilize the N-CoR-mSin3-HDAC complex also require c-Ski. The complex containing mSin3 consists of multiple proteins such as SAP30 and the histone-binding proteins RbAp46 and RbAp48 (27). Interestingly, SAP30 is required for the transcriptional repression mediated by the estrogen receptor but not by thyroid receptor or the retinoic acid receptor (7). To understand the molecular mechanism of v-Ski-induced transcriptional repression, it will be important to determine whether c-Ski acts in specific transcriptional repression mediated by a limited number of repressors or in transcriptional repression in general.

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