Ski Negatively Regulates Erythroid Differentiation through Its Interaction with GATA1

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Received 7 July 2004/Returned for modification 9 August 2004/Accepted 27 August 2004

The Ski oncoprotein dramatically affects cell growth, differentiation, and/or survival. Recently, Ski was shown to act in distinct signaling pathways including those involving nuclear receptors, transforming growth factor β, and tumor suppressors. These divergent roles of Ski are probably dependent on Ski’s capacity to bind multiple partners with disparate functions. In particular, Ski alters the growth and differentiation program of erythroid progenitor cells, leading to malignant leukemia. However, the mechanism underlying this important effect has remained elusive. Here we show that Ski interacts with GATA1, a transcription factor essential in erythropoiesis. Using a Ski mutant deficient in GATA1 binding, we show that this Ski-GATA1 interaction is critical for Ski’s ability to repress GATA1-mediated transcription and block erythroid differentiation. Furthermore, the repression of GATA1-mediated transcription involves Ski’s ability to block DNA binding of GATA1. This finding is in marked contrast to those in previous reports on the mechanism of repression by Ski, which have described a model involving the recruitment of corepressors into DNA-bound transcription complexes. We propose that Ski cooperates in the process of transformation in erythroid cells by interfering with GATA1 function, thereby contributing to erythroleukemia.

Cellular transformation frequently arises from defects in normal cell proliferation and differentiation. Many oncoproteins have been identified as having the capacity to alter proliferation and also differentiation programs, often leading to blockage at certain stages of terminal differentiation (14). Extensive studies have been carried out to understand how these proteins affect normal cellular events and thereby cause cancers. In particular, several nuclear oncoproteins have been found to impair the regulation of transcription factors which play key roles in controlling differentiation. In the case of normal hematopoiesis, this deregulation can contribute to the development of leukemia (22, 23, 26, 44).

v-Ski, a truncated form of the cellular homolog c-Ski, was originally identified as the oncoprotein from the avian Sloan-Kettering retroviruses, which can transform chicken embryonic fibroblasts (41). Cell transformation by Ski seems to require overexpression of this protein rather than mutation (6). Analysis of human tumors has determined that Ski is overexpressed in melanomas, esophageal carcinomas, and leukemias (12, 33, 36). Of relevance to Ski’s ability to cause leukemias are studies in which Ski was also shown to influence the growth and differentiation of hematopoietic cells (2, 8, 20, 21). These previous studies demonstrated that Ski can cause highly malignant erythroleukemia (20) and transform hematopoietic multipotent progenitors (8). In addition, Ski induces immortalization of primary multipotent progenitor cells from avian bone marrow (2). In all cases, it appears that Ski is functioning by blocking or delaying the ability of the cells to differentiate into mature hematopoietic cells. Such effects of Ski exhibit a very high degree of specificity for a particular hematopoietic lineage and stage of differentiation. However, the molecular mechanisms underlying this Ski-dependent block of differentiation are not understood.

Recently, Ski was shown to act in several distinct signaling pathways including those involving nuclear receptors (8, 29, 49), transforming growth factor β (TGF-β) (1, 24, 42, 48, 53), and tumor suppressors (17, 29, 45). Ski can act in all of these distinct signaling pathways by causing transcriptional repression. It appears that this is due to Ski’s ability to act as a transcriptional corepressor primarily due to multiple direct and indirect interactions with histone deacetylase complexes including N-CoR/SMRT/Sin3A corepressors (16, 29). These divergent roles of Ski are thought to reflect Ski’s capacity to bind to multiple protein partners that have disparate functions. However, it is not clear which interaction partner or signaling pathway is involved in the Ski-mediated inhibition of differentiation in hematopoietic cells.

Previous studies identified a point mutation in Ski (L110P) which severely affects its interaction with N-CoR and Smad2/3 but not with Sin3A, Smad4, and vitamin D receptor (48, 49). This mutation affects the repression activity of Ski in nuclear hormone receptor signaling but not in TGF-β signaling. The mutated residue is located at the highly conserved domain in the N terminus region of Ski/Sno/Dachshund family proteins (18, 52) that is implicated in the biological activities of Ski, including the induction of morphological transformation, anchorage-independent growth, and myogenic differentiation (57). Available structural and functional data suggest that this N-terminal domain of Ski mediates numerous signaling-dependent protein-protein interactions through common or distinct surfaces that may be partly disrupted by the L110P mutation (48, 49, 52).

During erythroid cell development, several lineage-specific transcription factors including GATA1 regulate growth and differentiation (4). GATA1 is a zinc finger transcription factor...
that plays a central role in erythroid development (9, 10, 30, 46). GATA1 was identified as a protein that binds to a consensus (A/T)GATA(A/G) motif that is found in virtually all erythroid-specific genes (31, 51). Targeted disruption of the GATA1 gene leads to impairment of erythropoiesis in mice (11, 34). Moreover, GATA1 null embryonic stem cells fail to differentiate into mature erythroid cells (50).

The oncoprotein PU.1, a myeloid- and B-cell-specific ETS transcription factor, has been shown to inhibit GATA1 function and is implicated in Friend virus-induced murine erythroleukemia (26). Overexpression of PU.1 in mouse erythroleukemia cells results in a differentiation block (35). Furthermore, PU.1 has been shown to physically interact with GATA1 and represses GATA1 function (38, 56), suggesting the possibility that GATA1 is one of the critical targets for oncoproteins that interfere with erythropoiesis differentiation.

In this report, we address the hypothesis that Ski’s ability to block erythroid differentiation may be mediated in some manner via GATA1. Using a human erythroleukemia cell line K562 as a model system, we were able to identify GATA1, a transcription factor essential in erythropoiesis, as a target of Ski. Our results using a mutant of Ski with a point mutation show that direct physical interaction between Ski and GATA1 is essential for blocking of erythroid differentiation in K562 cells. Furthermore, this Ski-GATA1 interaction facilitates repression of GATA1-mediated transcriptional activation by interfering with GATA1 DNA binding. Our results provide a mechanism by which Ski contributes to erythroleukemia through antagonizing GATA1 function. This novel mode of transcriptional repression by Ski may also provide new insight into the divergent roles of Ski in the processes of normal cell proliferation and differentiation.

MATERIALS AND METHODS

Tissue culture and transfection. K562, HEL, COS-1, and QT6 cells were maintained in Dulbecco’s modified Eagle’s medium or RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 mg/ml). For QT6 cells, medium was additionally supplemented with 1% chicken serum. Expression plasmids were introduced into the cells by using FuGENE 6 as described by the manufacturer (Roche Applied Science). K562 and HEL cell lines stably expressing an empty vector, wild-type and Ski cDNA expression plasmids as described above. Oligonucleotides harboring two copies of GATA1 binding sequence (sense, 5′-CAC TGG ATA ACA CAA AGT GAT AAC TCT-3′; antisense, 5′-AGA GTT ATC CTC TGT TAT CAA GTG-3′) were end labeled (Biotin 3′ end DNA labeling kit; Pierce), after which electrophoretic mobility shift assays (EMSA) were performed using the LightShift chemiluminescent EMSA kit (Pierce). QT6 cells were transfected with 40 µl murine recombinant Ski (wt-Ski), or wt-Ski expression plasmids as described above. Oligonu

In vitro binding assay. The zinc finger domain of mGATA1 (NF plus CF; amino acids 178 to 300) was bacterially expressed as six-His-tagged protein, followed by purification using nickel-nitrilotriacetic acid resin (Qiagen). The GATA1 (NF-plus-CF)-bound resin was incubated with COS-1 cell lysate expressing wt-Ski prepared as described above in Nonidet P-40 buffer containing 20 mM imidazole for 8 h at 4°C on a rotating wheel. Protein complexes were recovered after four washings with Nonidet P-40 buffer containing 20 mM imidazole, followed by Western blotting as described above.

Reverse transcription (RT)-PCR. Total RNA was isolated by using the RNeasy minikit (Qiagen). cDNA was synthesized with ThermoScript reverse transcriptase (Invitrogen) by using oligo(dT) primer. The PCR conditions were as follows: 95°C for 3 min and 25 cycles (γ-globin, erythroid-specific 5-aminovalylate synthase [Alas-E]; and β-actin genes) or 35 cycles (porphobilinogen deaminase [Pbgd] gene) of 30 s at 94°C, 30 s at 60°C, and 40 s at 72°C, followed by an extension time of 5 min at 72°C. The primer pairs used were as follows: γ-globin gene, 5′-GCC AAC CTG TCT TCT GGC-3′ and 5′-GAAG ATG GAT TGC CAA AAC GG-3′, yielding a 219-bp fragment; ALAS-E gene, 5′-GTT GCC TCC TCG AGT GTG-3′ and 5′-ATC TTC CAT CAT CTC AGG GC-3′, yielding a 581-bp fragment; Pbgd gene, 5′-TAC TGC AGC GGC AGC AAG AGG-3′ and 5′-AGG GGT TCT AGC GTC TTC CCA ACA-3′, yielding a 413-bp fragment; and β-actin gene, 5′-CAA GGC TGT GCT ACT CCT GTA-3′ and 5′-TGA CCT TCT CCT GCA TCC TGT C-3′, yielding a 545-bp fragment. The PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Band intensity was quantified by ImageQuant software (Amersham Biosciences).

EMSA. Cell extracts were prepared from COS-1 cells transfected with GATA1, wt-Ski, or wt-Ski expression plasmids as described above. Oligonucleotides harboring three copies of GATA1 binding sequence (sense, 5′-CAG TGG ATA ACA CAA AGT GAT AAC TCT-3′; antisense, 5′-AGA GTT ATC CTC TGT TAT CAA GTG-3′) were end labeled (Biotin 3′ end DNA labeling kit; Pierce), after which electrophoretic mobility shift assays (EMSA) were performed using the LightShift chemiluminescent EMSA kit (Pierce).

RESULTS

Erythroid differentiation of K562 cells is inhibited by wild-type Ski but not by Ski carrying a point mutation. To study the molecular mechanism underlying Ski-mediated alteration of erythroid differentiation, we used the human erythroleukemia dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose membranes (Protran; Schleicher & Schuell). For Western blotting, the primary antibodies used were antimethoglobin (ICN), anti-α-tubulin (Sigma), anti-β-T (Nuvagen), anti-GATA1 (N6), and anti-Myc (9E10). Proteins of interest were detected with appropriate secondary antibodies by chemiluminescence (ECL kit; Amer sham Biosciences).

In vitro binding assay. The zinc finger domain of mGATA1 (NF plus CF; amino acids 178 to 300) was bacterially expressed as Six-His-tagged protein, followed by purification using nickel-nitrilotriacetic acid resin (Qiagen). The GATA1 (NF-plus-CF)-bound resin was incubated with COS-1 cell lysate expressing wt-Ski prepared as described above in Nonidet P-40 buffer containing 20 mM imidazole for 8 h at 4°C on a rotating wheel. Protein complexes were recovered after four washings with Nonidet P-40 buffer containing 20 mM imidazole, followed by Western blotting as described above.
FIG. 1. Erythroid differentiation of K562 cells is inhibited by wild-type Ski but not by the L110P mutant. (A) Levels of Ski protein expression in K562 cells. WB, Western blotting. (B, C, and D) The L110P mutation impairs inhibition activity of Ski on K562 erythroid differentiation induced by hemin. Shown are representative data from K562 cells expressing wt-cSki and mt-cSki. (B) Benzidine staining before (- hemin) and after (+ hemin).
cell line K562 as a model system. K562 cells are precursors that proceed toward erythroid differentiation upon treatment with agents such as hemin (39). When induced to differentiate with hemin, K562 cells produce the embryonic and fetal hemoglobin, a property associated with the more mature erythroid cells (19). Several pools of K562 cells stably transfected with empty vector, wt-cSki, and mt-cSki (49) were established. The levels of Ski expression in these cells were assessed by Western blotting, which showed equal levels of expression and stability (Fig. 1A, wt-cSki and mt-cSki). Next, each pool was treated with or without hemin for 5 days and harvested for detection of hemoglobin production by benzidine staining and Western blotting (Fig. 1B and C). Upon stimulation with hemin, the vector control cells were differentiated, as judged by the increased number of benzidine-positive cells and the level of hemoglobin production (Fig. 1B and C, vector). In contrast, wt-cSki expression substantially reduced the number of benzidine-positive cells and the level of hemoglobin production (Fig. 1B and C, wt-cSki). However, mt-cSki-expressing K562 cells showed numbers of benzidine-positive cells and levels of hemoglobin production similar to those of the vector control (Fig. 1B and C, mt-cSki). The level of GATA1 protein expression during erythroid differentiation was not affected by wt-cSki (Fig. 1C, middle panel), thus excluding the possibility that Ski reduces GATA1 protein expression and thereby inhibits GATA1 function.

To test whether Ski inhibits the induction of genes associated with the erythroid differentiation program, we examined expression levels of mRNAs for fetal γ-globin, ALAS-E (7), and PBGD (5) in the K562 cells expressing vector, wt-cSki, and mt-cSki by using semiquantitative RT-PCR. Upon treatment with hemin, levels of γ-globin mRNA were upregulated about twofold compared to those in vector control cells (Fig. 1D, left panel, lane 4). Expression of wt-cSki blunted this induction by hemin (Fig. 1D, left panel, lane 5), whereas mt-cSki did not exhibit repression activity equivalent to that of wt-cSki (Fig. 1D, left panel, lane 6). Similar results were obtained for ALAS-E and PBGD (Fig. 1D), providing evidence that wt-cSki can block the induction of several erythroid-specific genes. Taken together, these results indicate that Ski inhibits hemin-induced erythroid differentiation of K562 cells and the L110P mutation practically eliminates Ski’s inhibitory activity.

GATA1 interacts with wild-type Ski but not with the L110P mutant. Ski can act as a transcriptional corepressor by directly interacting with several transcription factors (1, 8, 24, 29, 42, 49, 53). Therefore, since the lineage-specific transcription factor GATA1 is a master regulator of terminal differentiation of erythroid precursors (4) and regulates hemoglobin production (31), we investigated the possibility of a link between Ski and GATA1. Coimmunoprecipitation assays using cell lysates from the different K562 cells showed that endogenous GATA1 interacted efficiently with wt-cSki but only minimally with mt-cSki (Fig. 2A). The interaction between endogenous GATA1 and Ski was also observed in another human erythroleukemia cell line, HEL expressing wt-cSki (Fig. 2B). These properties of interaction between GATA1 and wt-cSki or mt-cSki were further confirmed by coimmunoprecipitation assays using COS-1 cell lysates expressing wt-cSki or mt-cSki in the presence of exogenously expressed GATA1 (Fig. 2C). wt-cSki clearly bound to GATA1, whereas the mt-cSki protein was impaired in its ability to interact with GATA1 (Fig. 2C). These results indicate that Ski interacts with GATA1 and that the L110P mutation significantly impairs Ski’s ability to interact with GATA1.

To map the GATA1 region critical for the physical interaction with Ski, we used GATA1 mutants which lacked the NF or CF (mutants ΔNF and ΔCF) (40). The N-terminal finger of GATA1 is required for binding to its cofactor FOG-1 (47). The C-terminal finger is essential for its DNA binding and interaction with other transcription factors such as PU.1 (38, 54). Coimmunoprecipitation assays using COS-1 cell lysates expressing wild-type GATA1, ΔNF, or ΔCF in the presence of wt-cSki showed that ΔCF clearly lost its ability to interact with Ski but that the wild type and ΔNF retained this interaction (Fig. 2D, top panels, lanes 3, 6, and 9). These results indicate that the CF is required for Ski binding. We next checked the interaction of the zinc finger domain of GATA1 (NF plus CF) with Ski by using coimmunoprecipitation and in vitro binding assays (Fig. 2D, bottom panels). The results showed that the zinc finger region of GATA1 is sufficient for Ski binding, suggesting that the CF of GATA1 facilitates a binding surface for Ski.

GATA1-mediated transcription is significantly repressed by wild-type Ski, but the L110P mutation partially reverses Ski’s repression activity. Having shown that GATA1 strongly interacts with wt-cSki but not with mt-cSki, we then examined the effect of wt-cSki or mt-cSki on GATA1-mediated transcriptional activation (15, 40). Transient transfection with wt-cSki revealed a significant repressive effect on GATA1-dependent transcription (Fig. 3A, wt-cSki). In contrast, transfection of mt-cSki resulted in only partial repression compared to that by wt-cSki (Fig. 3A, mt-cSki). Ski-mediated transcriptional repression was increased by increasing amounts of wt-cSki expression construct in a dose-dependent manner to a maximum repression of approximately 75% (Fig. 3B, wt-cSki). In contrast, increasing amounts of mt-cSki never gave rise to more than 40% repression (Fig. 3B, wt-cSki). When we used a reporter construct driven by the minimal thymidine kinase (TK) promoter, Ski-mediated transcriptional repression was not observed (Fig. 3C), excluding the concern that overexpression of Ski might have general nonspecific repression activity in the reporter assay. These data indicate that the transcriptional repression of GATA1 by wt-cSki is at least in part mediated by Ski-GATA1 interaction.

GATA1 DNA binding is blocked by wild-type Ski but not so efficiently by the L110P mutant. Ski has been shown to be...
FIG. 2. GATA1 interacts with wild-type Ski but not with the L110P mutant. (A) GATA1-bound Ski was detected by immunoprecipitation (IP). Cell extracts from K562 cells expressing vector control, wt-cSki, or mt-cSki were immunoprecipitated by endogenous GATA1, followed by Western blotting (WB) with anti-T7 (Ski; top panel) and anti-GATA1 (middle panel) antibody. Equivalent amounts of wt-cSki and mt-cSki proteins were presented in the input control (10%; bottom panel). (B) Cell extract from HEL cells expressing wt-cSki was immunoprecipitated by anti-GATA1 antibody or normal rat IgG (negative control), followed by Western blotting as described for panel A. The asterisk indicates a nonspecific signal. (C) Cell extracts from COS-1 cells expressing wt-cSki or mt-cSki were immunoprecipitated by exogenously expressed GATA1, followed by Western blotting as described for panel A. (D) The CF of GATA1 is required for interaction with wt-cSki. (D, top panels) Cell extracts from COS-1 cells expressing wild-type GATA1 (WT), ΔNF, and ΔCF plus wt-cSki were immunoprecipitated by anti-GATA1 antibody or normal rat IgG (negative control), followed by Western blotting as described for panel A. (D, bottom panels) The zinc finger domain of GATA1 (NF plus CF) is sufficient for interaction with wt-cSki. (D, bottom left panel) Cell extract from COS-1 cells expressing wt-cSki and GATA1 (NF plus CF) was immunoprecipitated by anti-Myc antibody or normal mouse IgG (negative control), followed by Western blotting as described for panel A. (D, bottom right panel) Cell extract from COS-1 cells expressing wt-cSki was incubated with bacterially expressed GATA1 (NF plus CF) protein on nickel-nitrilotriacetic acid resin, followed by Western blotting as described for panel A. GATA1 (NF plus CF) protein was detected by Coomassie brilliant blue (CBB) staining. +, present; −, absent.
tethered on several distinct DNA elements through its interaction with DNA binding proteins (1, 8, 24, 42, 53). To see whether Ski is recruited onto DNA through binding to GATA1, EMSA were performed (Fig. 4). GATA1 efficiently bound to double-stranded DNA harboring canonical GATA binding sequences (Fig. 4A, lane 2). Unexpectedly, coexpression of wt-cSki dramatically reduced the amount of GATA1 bound to the DNA (Fig. 4A, lane 5), whereas coexpression of mt-cSki resulted in only a partial reduction (Fig. 4A, lane 6).

The specificity of GATA1-DNA complex formation was confirmed by adding an excess amount of unlabeled DNA, which practically competed out binding (Fig. 4A, lanes 8, 11, and 12). The level of protein expression in each cell lysate was assessed by Western blotting and found to be equal and stable (Fig. 4B). These data strongly suggest that Ski interferes with GATA1 DNA binding through interaction with GATA1.

To assess whether Ski affects promoter occupancy of GATA1 in living cells, we performed a ChIP assay using the K562 cells expressing vector, wt-cSki, or mt-cSki. We monitored GATA1 occupancy in several promoter and enhancer regions of erythroid-specific genes, including those of HS2 within the β-globin locus control region (13), ALAS-E (43), and PBGD (25), which contain potential GATA1 elements and have been shown to be repressed by Ski in this study (Fig. 1D).

Vector control cells were specifically enriched with chromatin fragments containing HS2, ALAS-E, and PBGD genes (Fig. 5, lane 3), consistent with earlier reports. In contrast, wt-cSki expression significantly reduced the chromatin enrichment for all three genes (Fig. 5, lane 6). mt-cSki expression did not have
living cells, further supporting the hypothesis that Ski blocks occupancy of GATA1 on several erythroid-specific genes in lane 9). These results show that Ski can affect the promoter as strong an effect on chromatin enrichment as wt-cSki (Fig. 5, results of a ChIP assay of GATA1 binding to promoter and enhancer regions (HS2, ALAS-E, and PBGD genes) in K562 cells expressing vector, wt-cSki, and mt-cSki treated with 40 μM hemin for 48 h. Input, 1% of chromatin lysate subjected to immunoprecipitation; IgG, normal IgG as a negative control; N6, anti-GATA1 antibody.

as strong an effect on chromatin enrichment as wt-cSki (Fig. 5, lane 9). These results show that Ski can affect the promoter occupancy of GATA1 on several erythroid-specific genes in living cells, further supporting the hypothesis that Ski blocks GATA1-mediated transcriptional activation through its interference with GATA1 DNA binding.

DISCUSSION

Our findings provide new insight into the mechanisms of Ski-mediated transcriptional repression. Previous reports proposed a classical model for the mechanism of repression by Ski in which Ski acts on DNA by recruiting histone deacetylase-containing corepressor complexes through interaction with DNA binding transcription factors (1, 29, 42, 49). However, in the present study, we have provided evidence that Ski can act off DNA by blocking DNA binding through interaction with GATA1 transcription factor, resulting in repression of GATA1 function. Intriguingly, a similar mechanism of repression of GATA1 function was proposed for the lineage-specific ETS family transcription factor PU.1, which can also block erythroid cell differentiation (38, 56). We propose a new mode of repression by Ski, which may partly address how Ski’s disparate activities are modulated.

The model proposed here for Ski-dependent inhibition of GATA1 function does not exclude contributions from the other mechanisms that have been proposed. For instance, it is reported that activity of GATA1 is regulated by protein modification such as acetylation and phosphorylation and that these modifications enhance GATA1 DNA binding (3, 32). Also, another report showed that self-association of GATA1 regulates its activity (28). Thus, it is possible that the effects of Ski on GATA1 function may also involve these mechanisms.

It was previously reported that the L110P mutation in Ski does not affect Ski’s repression activity in TGF-β signaling (49). Therefore, our data together with the previous results suggest that Ski-mediated repression of GATA1 is most likely carried out in a TGF-β signaling-independent manner.

The expression profile of endogenous Ski mRNA has been examined previously in hematopoietic lineage cells (27, 33). Ski is expressed in murine fetal liver-derived mature B and T lineage cells, as well as macrophages and mast cells in the myeloid lineage. In normal murine bone marrow-derived progenitors, however, Ski mRNA expression appears to be limited to dual-lineage megakaryocyte-erythrocyte colony starts, indicating a potential role in erythroid differentiation and also an overlap with GATA1 mRNA expression in these cells. Our findings, along with these previously reported data, suggest that Ski contributes to the regulation of GATA1 function in normal hematopoiesis.

Current models suggest that GATA1 and PU.1 antagonize each other’s activity for lineage specification in the hematopoietic cell system (55, 56). Based on our present data, Ski interferes with GATA1 in a way mechanistically similar to that in which PU.1 interferes with GATA1 function. However, over-expression of Ski in K562 cells does not induce reprogramming from erythroid to myeloid or monocytic lineage or megakaryocytic differentiation (data not shown). Therefore, it does not appear to effect lineage decisions like PU.1. However, since K562 cells constitute a cell line, it is possible that they have lost this lineage plasticity; thus, conclusions on Ski’s ability to effect lineage specification should await further experimentation with more appropriate cell systems.

Our results show that Ski regulates transcriptional-activator function of GATA1 by interfering with its DNA binding, leading to repression of the erythroid differentiation program controlled by GATA1 and a subsequent block in terminal erythroid differentiation. These findings contribute to our understanding of how Ski acts in hematopoietic cell differentiation and malignant transformation in leukemia and further underline the complexity and interdependence of the mechanisms governing Ski functions.

ACKNOWLEDGMENTS

We thank J. D. Engel, K. Ohneda, and M. Yamamoto for valuable reagents; K. Donnelly for supporting our research project; and the members of the Hayman laboratory for helpful discussions and criticisms on the manuscript.

This work was supported by grant CA42573 from the National Institutes of Health to M.J.H.

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