Schnurri-3 (KRC) Interacts with c-Jun to Regulate the IL-2 Gene in T Cells

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Abstract

The activator protein 1 (AP-1) transcription factor is a key participant in the control of T cell proliferation, cytokine production, and effector function. In the immune system, AP-1 activity is highest in T cells, suggesting that a subset of T cell–specific coactivator proteins exist to selectively potentiate AP-1 function. Here, we describe that the expression of *Schnurri-3*, also known as κ recognition component (KRC), is induced upon T cell receptor signaling in T cells and functions to regulate the expression of the interleukin 2 (IL-2) gene. Overexpression of KRC in transformed and primary T cells leads to increased IL-2 production, whereas dominant-negative KRC, or loss of KRC protein in KRC-null mice, results in diminished IL-2 production. KRC physically associates with the c-Jun transcription factor and serves as a coactivator to augment AP-1–dependent IL-2 gene transcription.

Key words: ZAS domain • coactivator • cytokines • AP-1 • TCR signaling

Introduction

The zinc finger transcription factor κ recognition component (KRC), a large protein composed of 2,282 amino acids, was originally isolated by virtue of its ability to bind to the recombination signal sequence (Rss) flanking the V, D, and J gene segments of the immunoglobulin gene (1–7). Sequence analysis revealed that KRC contains a zinc finger acidic domain structure (ZAS) composed of a pair of Cys2-His2 zinc fingers followed by a Glu- and Asp-rich acidic domain and five copies of the ser/Thr-Pro-X-Arg/Lys sequence thought to bind DNA. ZAS domain proteins are a family of enhancer binding proteins for the κB motif, and KRC recombinant proteins can bind in vitro to the κB motif as well as to the Rss sequence (2, 8) in highly ordered complexes (1, 9). KRC has been shown to regulate transcription of the mouse metastasis–associated gene S100A4/mts1 (10, 11), and down-regulation of KRC expression affects proliferation and cell death (12, 13). However, our recent studies have suggested that KRC can also act as an adaptor protein.

Recently, we described a novel function for KRC in regulating patterns of gene activation in response to proinflammatory stimuli (14). We demonstrated that KRC interacts with the adaptor protein TRAF2 to inhibit both nuclear factor (NF) κB and c-Jun NH2-terminal kinase (JNK)/SAPK-mediated responses, including apoptosis and TNFα

cytokine gene expression. Thus, overexpression of sense KRC inhibited, whereas antisense KRC or a dominant-negative KRC enhanced, NFκB-dependent transactivation and JNK phosphorylation. Although KRC has been shown to bind DNA, in our studies, its effect on gene activation could clearly be ascribed to indirect mechanisms.

KRC was isolated from a thymocyte cDNA library, and given the critical role of the NFκB and JNK pathways in regulating CD4 T cell activation, effector function, and cytokine gene expression as well as T cell survival and death (15–17); we searched for a function of KRC in T cells. Activator protein 1 (AP-1) function is critical for optimal T cell development, activation, and differentiation, as revealed by genetic evidence (18). Furthermore, loss of AP-1 transcriptional activity leads to T cell anergy, further highlighting the importance of this transcription factor in the immune system (19, 20). Here, we demonstrate that KRC enhances endogenous IL-2 production in both transformed and primary T cells. More importantly, CD4 T cells from KRC-deficient mice that we have generated produce significantly less IL-2 upon TCR stimulation compared with wild-type CD4 T cells. We show that KRC physically interacts with the tran-

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scription factor c-Jun and enhances AP-1 function in the context of the IL-2 promoter.

Materials and Methods

Reagents and Antibodies. PMA, ionomycin, rottlerin, and the MEK1 inhibitor were purchased from Calbiochem. Staphylococcus enterotoxin E (SEE) was purchased from Toxin Technology; anti-Myc, anti–c-Jun, anti-His, and anti-Gal4 antibodies were obtained from Santa Cruz Biotechnology, Inc.; and anti-Flag antibody was obtained from Sigma-Aldrich.

Transfection and Luciferase Assays. For each transfection, 5×10^6 Jurkat cells were incubated with either IL-2–Luc, NFAT/AP1-Luc, or AP1-Luc reporter DNA together with pEF vector or pEF-KRC and CMV-βGAL as normalization control in 0.4 ml of RPMI 1640 and transfected by electroporation (260 V, 975 μF). R. Flavell and R. Davis provided the AP-1 luciferase and N17R as dominant-negative and MKK7 constructs, respectively. Transfected cells were cultured at 37°C for 20 h in RPMI 1640 medium (GIBCO BRL) supplemented with 10% FBS. Transfected cells were stimulated with 50 ng/ml PMA and 2 μM ionomycin for 6 h before luciferase (Promega) and β-galactosidase assays (Galacton-PLUS substrate system; TROPIX, Inc.).

Western Blot Analysis and Reverse Transcription–PCR. Total RNA was isolated from T cells using TRIzol reagent (GIBCO BRL). 1 μg of total RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories). PCR was performed with 2 μM of each primer and 2.5 U of platinum high fidelity enzyme (Invitrogen) according to the manufacturer's instructions using the following primers: IL-2F, 5′-CAAGAATCCAAACTCACCAG-3′; IL-2R, 5′-TAGCAACCATACATTCAACAA-3′; KRCF, 5′-CTCCAATACAGAATTCAAGGGC-3′; and KRCR, 5′-TTTAGGTTGGCCAGTGTGTGTG-3′.

For Western blot analyses, 293 cells were transfected with Effectene (QIAGEN) according to the manufacturer's instructions. To prepare cell extracts, cells were washed twice with PBS and lysed for 20 min on ice in 1 ml Triton X-100 lysis buffer (25 mM Tris, pH 7.5, 250 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 1 mM EGTA, 0.5 mM DTT, and complete protease inhibitor mixture; Roche Molecular Biochemicals). Lysates were cleared by centrifugation for 10 min at 14,000 revolutions/min, precleared with 30 μl of protein A/G–Sepharose beads, and incubated overnight with the indicated antibody. Subsequently, 30 μl of protein A/G–Sepharose beads was added, and lysates were incubated at 4°C for an additional 2 h. The immunoprecipitates were washed five times with the lysis buffer, resuspended in SDS sample buffer, and heated at 95°C for 5 min. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories). Western blotting was performed by probing with primary antibody followed by horseradish peroxidase–conjugated and goat anti–rabbit IgG (Zymed Laboratories) and enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences).

Raji and SEE Stimulation. Jurkat cells were transfected by electroporation and incubated for 20 h at 37°C before stimulation for 8 h with the Raji B cell line and SEE using Raji cells (1:1 with Jurkat cells) and 200 ng/ml SEE.

In Vitro Kinase Assays. Jurkat cells were transfected with Flag-JNK2 together with pEF or pEF-KRC (KRC cDNA driven by the elongation factor promoter), and 24–48 h after transfection, anti-Flag antibody was used to immunoprecipitate JNK2.

The kinase reaction was performed at 30°C for 30 min with 1 μg GST–c-Jun, 10 μCi γ-[^{32}P]ATP, and 10 μm ATP in 30 μl of kinase buffer (20 mM Hepes, pH 7.4, 10 mM MgCl$_2$, 25 mM β-glycerophosphate, 50 μm Na$_3$VO$_4$, and 1 mM DTT).

Pull Down Assays. In vitro–translated c-Jun ([^{35}S]methionine labeled) and His-KRCtr were incubated for 2 h at 4°C in binding buffer (PBS, 0.25% NP-40, 1 mM PMSF, and 0.25 mM DTT), incubated for 2 h with the anti-HIS antibody (Santa Cruz Biotechnology, Inc.), 30 μl of protein A/G–Sepharose beads was added, and the reaction was incubated at 4°C for an additional 2 h. The immunoprecipitates were washed five times with the binding buffer, resuspended in SDS sample buffer, and heated at 95°C for 5 min.

Retroviral Gene Transduction. Activated CD4 T cells were transduced by RV, RV-KRC, or RV-ZAS2 as described previously (21).

Generation of KRC-deficient Mice and T Cell Stimulation. ES cells were generated in which the entire 5.4-kb exon 2 of KRC was replaced by a neomycin cassette, resulting in an allele that produces no KRC protein. KRC$^{+/-}$ ES cells transmitted the disrupted allele to 129/B6 offspring. Heterozygous pups were backcrossed to wild-type B6 mice. Mice analyzed were progeny of intercrosses between heterozygous F3 generation backcrossed 129/B6 mice. CD4 T cells were purified by positive selection from spleen and lymph nodes of 6–8-wk-old male KRC$^{+/+}$ and KRC$^{-/-}$ littermates using magnetic beads according to the manufacturer's instructions (Miltenyi Biotec). Cells were stimulated at 10^6 cells/ml with 1.0 μg/ml plate-bound anti-CD3 plus 0.5 μg/ml anti-CD28. 24 h later, supernatants were collected and analyzed for IL-2 levels by ELISA. Additionally, cells were stimulated for 72 h in the presence of 200 U/ml of human IL-2, and supernatants were collected and analyzed for IFNγ levels by ELISA.

Results

KRC Expression Is Induced upon Signals That Trigger Activation of CD4 T Cells. It was shown previously that KRC is expressed in cells of the immune system including T cells, B cells, and macrophages (unpublished data). To determine whether the level of KRC transcripts changes
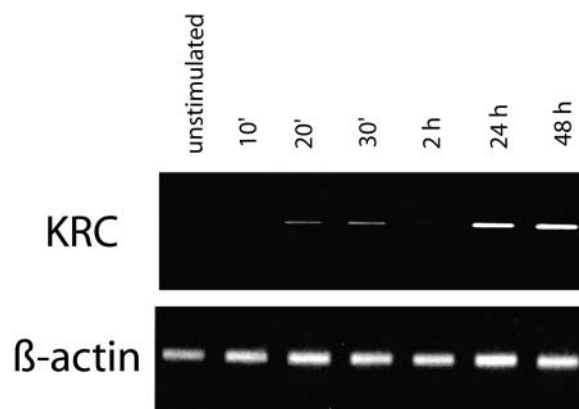

Figure 1. KRC expression increases in CD4 cells upon activation. Primary C57/B6 CD4$^+$ T cells were stimulated with anti-CD3 (2.0 μg/ml)/anti-CD28 (1.0 μg/ml) antibodies for the indicated times. RNA was prepared and KRC expression was determined by RT-PCR, with β-actin as an internal control.
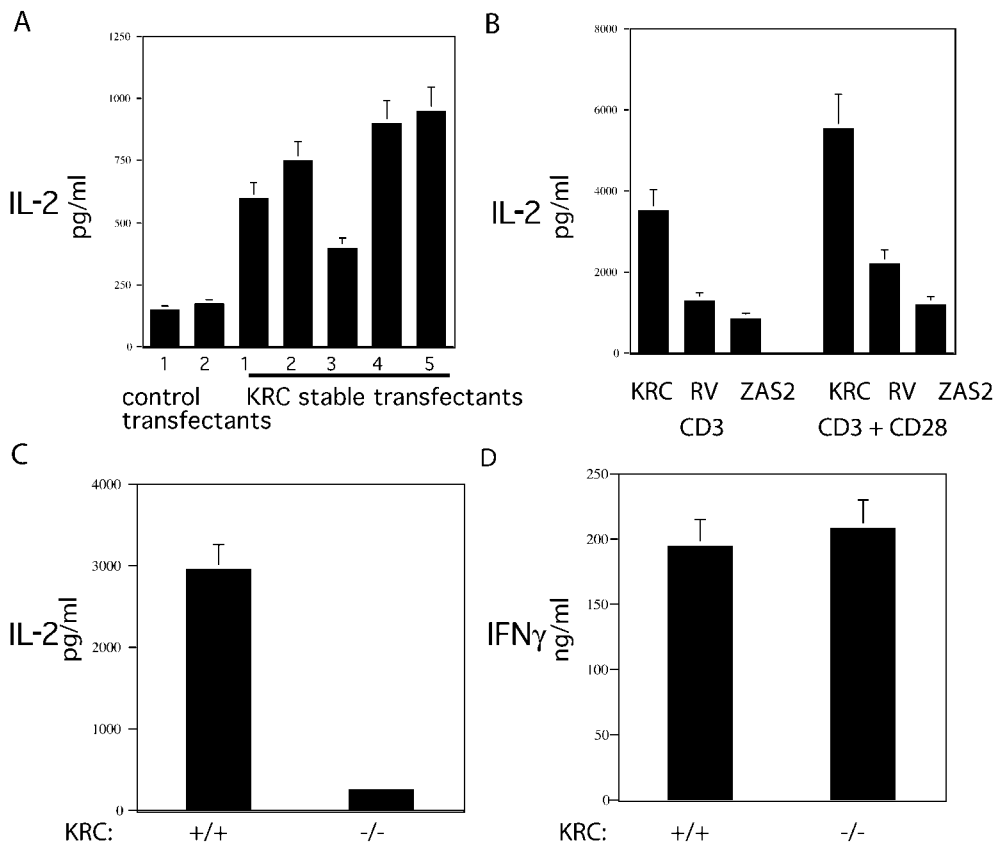
Figure 2. KRC overexpression increases, whereas KRC loss decreases endogenous IL-2 production. (A) Jurkat T cells were stably transfected with vector (pEF) or KRC expression plasmids. Stable clones were stimulated for 18 h with 50 ng/ml PMA plus 2 μM ionomycin, and IL-2 production was measured by ELISA. (B) Primary CD4^{+} T cells were activated for 36 h and subsequently transduced with control (RV), KRC, or KRC dominant-negative (ZAS2) bicistronic GFP-expressing retroviruses. GFP-positive cells were sorted and stimulated for 24 h with anti-CD3 or anti-CD3/anti-CD28 antibodies and IL-2 production was measured by ELISA. (C) CD4 T cells from KRC^{+/+} or [−/−] mice were stimulated with anti-CD3 (1.0 μg/ml)/CD28 (0.5 μg/ml) antibodies for 24 h, and IL-2 production was measured by ELISA. (D) CD4 T cells from KRC^{+/+} or [−/−] mice were stimulated with anti-CD3/CD28 antibodies for 72 h in the presence of 200 U/ml of human IL-2. IFNγ production was measured by ELISA. Data shown are representative of four independent experiments with similar results.

upon activation in CD4 T cells, RT-PCR analysis was performed (Fig. 1). KRC expression was induced with very rapid kinetics (within 20 min) in CD4^{+} T cells upon activation by CD3/CD28 stimulation, and increased levels of KRC transcripts were seen throughout the duration of primary CD3/CD28 stimulation, up to 48 h. Given its rapid pattern of induction after T cell activation and its sustained increase in expression, we searched for a potential role for KRC in events occurring downstream of early T cell activation, such as entry into the cell cycle and induction of the cytokine IL-2 (22).

KRC Overexpression Increases, whereas KRC Loss Decreases Endogenous IL-2 Production in Both Transformed and Primary T Cells. IL-2 promoter activation requires antigen receptor engagement plus an accessory signal usually supplied by an antigen-presenting cell (22). Agents that bypass these receptors, such as PMA and ionomycin, can mimic T cell activation in the human T cell lymphoma Jurkat. To assess the function of KRC in T cells, Jurkat cells, which express barely detectable levels of endogenous KRC protein by Western blot analysis, were stably transfected with a plasmid encoding full-length KRC (pEF-KRC) or with vector-only control (pEF). G418 drug-resistant Jurkat clones were expanded and analyzed for IL-2 secretion after activation. Clones stably expressing KRC showed clear increases in KRC protein levels, as detected by Western blotting (unpublished data). We observed that all clones expressing

pEF-KRC produced substantially greater amounts of IL-2 upon PMA and ionomycin treatment than activated Jurkat clones transfected with the control vector (Fig. 2 A). KRC overexpression alone was not sufficient to induce IL-2 secretion because no IL-2 was detected in the culture supernatants of unstimulated KRC-overexpressing clones (unpublished data). These results suggested that KRC is able to boost IL-2 secretion in concert with signals emanating from the TCR.

Although the Jurkat model has proved valuable to dissect pathways of T cell signaling, certain observations made in Jurkat cells are irreproducible in primary T cells (23, 61). Therefore, we studied the effects of KRC overexpression in primary CD4 T cells using a retroviral delivery system. We generated bicistronic retroviral vectors encoding full-length KRC, the KRC ZAS2 domain (which we have previously shown acts as a dominant negative in the context of KRC-mediated inhibition of TNF-induced NFκB activation; reference 14), and control GFP. Purified CD4 T cells were infected with these retroviruses 36 h after primary activation with both anti-CD3 and anti-CD28 and sorted by flow cytometry for GFP expression 24 h after infection. The ability of each population to produce IL-2 after subsequent activation by anti-CD3 or anti-CD3 plus CD28 was measured at 24 h after stimulation. As shown in Fig. 2 B, CD4 cells transduced with full-length KRC produced higher amounts (approximately three- to fourfold

increase) of IL-2 than CD4 cells infected with the GFP control retrovirus. Furthermore, CD4 cells transduced with the dominant negative KRC ZAS2 domain construct produced significantly less IL-2 than both the full-length KRC and GFP control transduced cells. These data are consistent with the notion that the ZAS2 domain interferes with endogenous KRC activity in T cells to prevent optimal expression of IL-2.

To further analyze the role of KRC in regulating endogenous IL-2 expression, we analyzed CD4 cells purified from KRC-deficient mice. A detailed description of the generation and phenotypic characterization of these mice will be presented elsewhere (unpublished data). In brief, lymphoid development in these mice appears normal, with normal numbers of $CD4^+$ T cells isolated from spleen and lymph nodes. Additionally, resting CD4 cells recovered appeared phenotypically normal based on expression of maturation markers such as CD4, CD62L, CD25, CD69, and $TCR\beta$ (unpublished data). As shown in Fig. 2 C, $KRC^{-/-}$

CD4 cells activated in vitro for 24 h by CD3/CD28 stimulation produced 10-fold less IL-2 than CD4 cells from wild-type littermates. However, $IFN\gamma$ production by these cells after 72 h of primary stimulation in the presence of excess exogenous IL-2 was normal (Fig. 2 D), suggesting that the deficiency of KRC in these cells does not globally inhibit activation–induced cytokine production. We conclude that KRC is a positive regulator of IL-2 production both in Jurkat cells and, more importantly, in primary CD4 T cells.

KRC Overexpression Increases the Transcription of the IL-2 Gene. The production of IL-2 by T cells is regulated at multiple levels, including transcription, mRNA stability, and rate of protein secretion (24, 25). To define at which stages KRC acts, we first measured levels of IL-2 mRNA transcripts by semi-quantitative RT-PCR in Jurkat T cells stably transfected with full-length KRC. As seen in Fig. 3 A, Jurkat clones overexpressing KRC displayed higher levels of IL-2 transcripts when activated than Jurkat clones
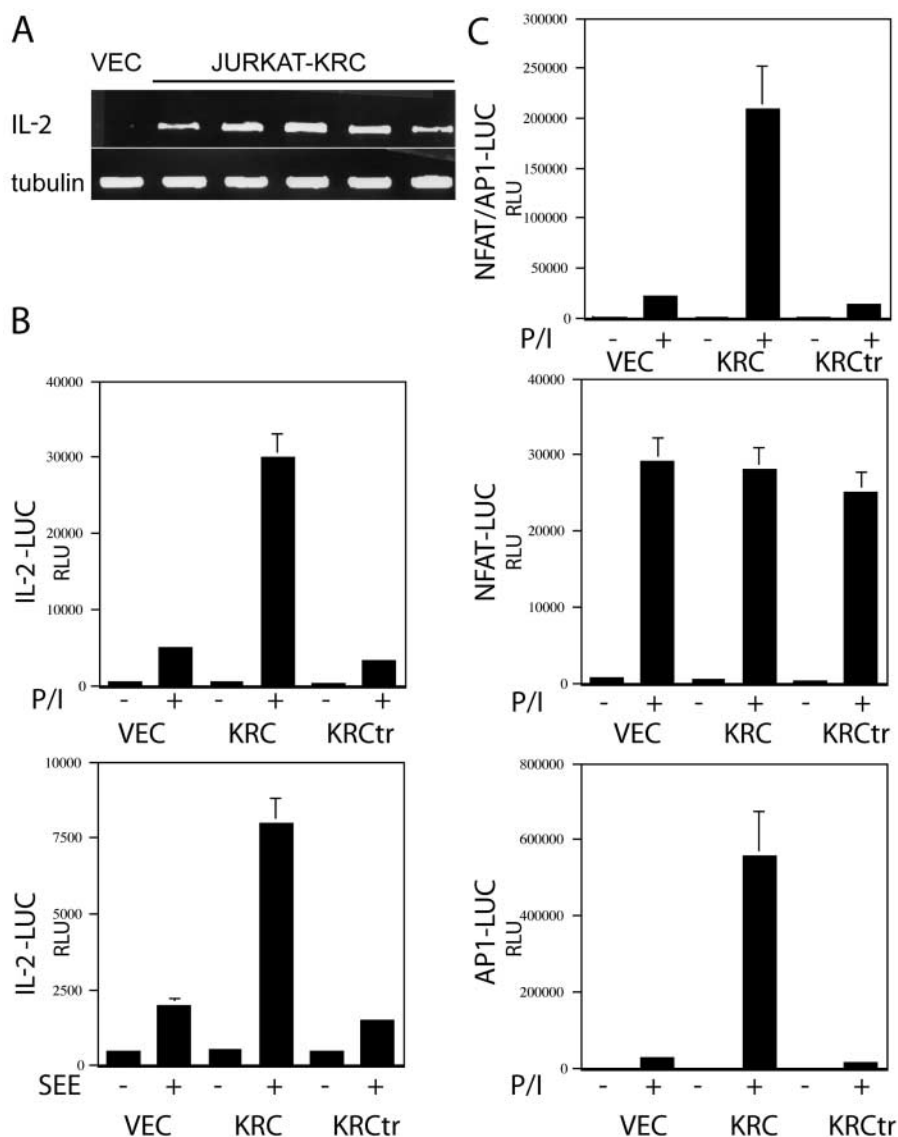

Figure 3. KRC overexpression increases the transcription of the IL-2 gene. (A) Stably transfected Jurkat T cell clones with vector (vec) or KRC (JURKAT-KRC) were stimulated with 50 ng/ml PMA plus 2 μM ionomycin for 6 h. IL-2 mRNA abundance was determined by RT-PCR with tubulin as an internal control. (B) Jurkat cells were transiently transfected with an IL-2–luciferase reporter (IL-2-LUC) along with Vector, KRC, or KRCtr (amino acids 204–1,055) and, in all cases, a CMV–β-Gal reporter as an internal control (Materials and Methods). 24 h later, cells were stimulated with PMA plus ionomycin for 6 h (top) or Raji cells loaded with SEE for 8 h (bottom). Luciferase activity was determined and normalized for β-galactosidase activity. (C) Jurkat cells were transiently transfected with NFAT/AP-1–, NFAT–, or AP-1–Luciferase reporters and treated as aforemtioned.

transfected with vector control. Next, we tested whether KRC was able to directly transactivate a 1.5-kb IL-2 promoter-luciferase reporter in Jurkat cells. Provision of KRC resulted in an ∼10-fold induction of luciferase activity in Jurkat cells treated with PMA plus ionomycin (Fig. 3 B, top). Just as KRC overexpression alone did not lead to spontaneous production of endogenous IL-2, no transactivation by KRC was observed in the absence of PMA/ionomycin in these luciferase reporter assays. To provide a more physiologic signal to activate Jurkat cells, we used a model system in which Raji B lymphoma cells act as antigen–presenting cells to present SEE to Jurkat. As shown in Fig. 3 B (bottom), provision of KRC substantially increased (∼10-fold) IL-2 promoter activity in this system. Interestingly, KRC had no effect on IL-2 promoter activity in the absence of Jurkat activation either by PMA/ionomycin or by antigen/APC. These data further suggest that KRC expression alone is not sufficient to induce IL-2 mRNA expression; instead, KRC's ability to enhance IL-2 production relies on endogenous factors found only in activated T cells.

KRC Augments IL-2 Promoter Activity via AP-1. KRC was originally cloned as a transcription factor, but in our previous analyses, its effect on gene activation could clearly be ascribed to its function as an adaptor protein. Nevertheless, KRC has been shown to bind both NFκB and Rss target sites in vitro, and an NFκB site is present in the IL-2 promoter that has been shown to bind the NFκB family member c-Rel (26). To test whether KRC overexpression leads to enhanced function of a specific site in the IL-2 promoter and to identify the site, we cotransfected Jurkat cells with KRC and various deletion constructs of the IL-2 promoter. In initial studies, KRC transactivated a luciferase reporter driven by only 200 bp of the IL-2 proximal promoter. The most prominent regulatory sequences in this region are cis elements that bind members of the NFAT, NFκB, and AP-1 transcription factor families (25, 27–29), although the NFAT and NFκB cis elements have been shown to overlap. Therefore, we tested whether KRC could transactivate a multimerized linked NFAT/AP-1 target site, or individual multimerized NFAT or AP-1 target sites. KRC enhanced PMA/ionomycin-induced transactivation of a multimerized linked NFAT/AP-1 element and the isolated, multimerized AP-1 element, but not the NFAT element (Fig. 3 C). In contrast to AP-1, the PMA/ionomycin-induced activity of NFAT was not further increased by coexpression of KRC. Therefore, KRC acts at the transcriptional level to increase expression of IL-2 through an AP-1 site–dependent mechanism. Preliminary results show that KRC overexpression enhances, and that KRC deficiency decreases, stimulation-induced up-regulation of CD69 (unpublished data), another AP-1 target gene in T cells (45).

KRC Does Not Increase AP-1 Expression, Bind the AP-1 Site, nor Alter TCR-mediated Mitogen-activated Protein Kinase (MAPK) Activity. It was unlikely that KRC, a zinc finger protein, transactivated the IL-2 promoter through direct binding to the AP-1 element, especially given our observa-

tion that KRC was able to enhance AP-1 activity only when Jurkat cells were simultaneously stimulated through the TCR pathway by PMA or antigen/APC. Indeed, in electrophoretic mobility shift assays, using extracts prepared from unstimulated Jurkat cells overexpressing KRC, no binding to a radiolabeled AP-1 site oligonucleotide was detected (unpublished data). Thus, KRC and AP-1 do not bind the same site within the IL-2 promoter to synergistically increase promoter activity. Additionally, we observed that KRC does not increase AP-1 activity by increasing the expression of c-Jun/c-Fos mRNA (unpublished data).

An alternative explanation was that KRC acts upstream to enhance posttranslational modifications of AP-1 that increase its activity. For example, NH_2-terminal phosphorylation of c-Jun or COOH-terminal phosphorylation of c-Fos have been shown to enhance AP-1 activation downstream of the Ras pathway (23, 30, 31). Overexpression of a dominant-negative Ras blocks TCR-induced AP-1 activity (32). More recently, it has been shown that mice deficient in PKC θ show defective TCR-induced AP-1 activation, suggesting a role for this kinase in Ras/MAPK/AP-1 activation (33, 34). We determined that both rottlerin, a PKC θ inhibitor, and overexpression of dominant-negative Ras (RasN17) abolished the ability of KRC to enhance AP-1 transactivation after PMA/ionomycin stimulation (Fig. 4 A). These data are consistent with the placement of KRC downstream of the Ras pathway or with a requirement for two distinct, but interconnected, signals for IL-2 promoter transactivation. We favor the latter explanation because KRC can increase AP-1 activation by Ras but cannot activate AP-1 on its own. Thus, KRC activation of AP-1 requires Ras, and KRC can substantially augment AP-1 activation by the Ras pathway.

KRC may enhance AP-1 function indirectly through the modulation of MAPK activity, kinases downstream of Ras that are known to potently stimulate AP-1 function (30, 31, 50). In T cells, stimulation via the TCR or with PMA/ionomycin induces the activation of three MAPKs: extracellular regulated kinase (ERK), p38, and JNK. The activation of these MAPKS is required for AP-1 transcriptional activity. In particular, JNK has been shown to increase AP-1 transcriptional activity by phosphorylating c-Jun (35). In initial studies, we determined that KRC overexpression did not alter levels of transcripts encoding a series of MAP3, MAP2, and MAP kinases as assessed by RNase protection assays (unpublished data). To test whether KRC had any effect on MAPK activity, we used a sensitive assay (the PathDetect reporting system) to evaluate the effect of KRC on ERK-mediated ELK-1 transactivation and p38-mediated ATF2 transactivation. Jurkat cells were cotransfected with a pGAL4-UAS-LUC reporter and expression plasmids encoding GAL4-Elk1 and GAL4-ATF2 fusion proteins, respectively. KRC was unable to modulate either MAPK or p38 activity in this assay (Fig. 4 B). We coexpressed KRC with HA-ERK1, myc-ERK2, Flag-P38, and Flag-JNK2, and measured the activity of each kinase using an immunoprecipitation kinase assay with
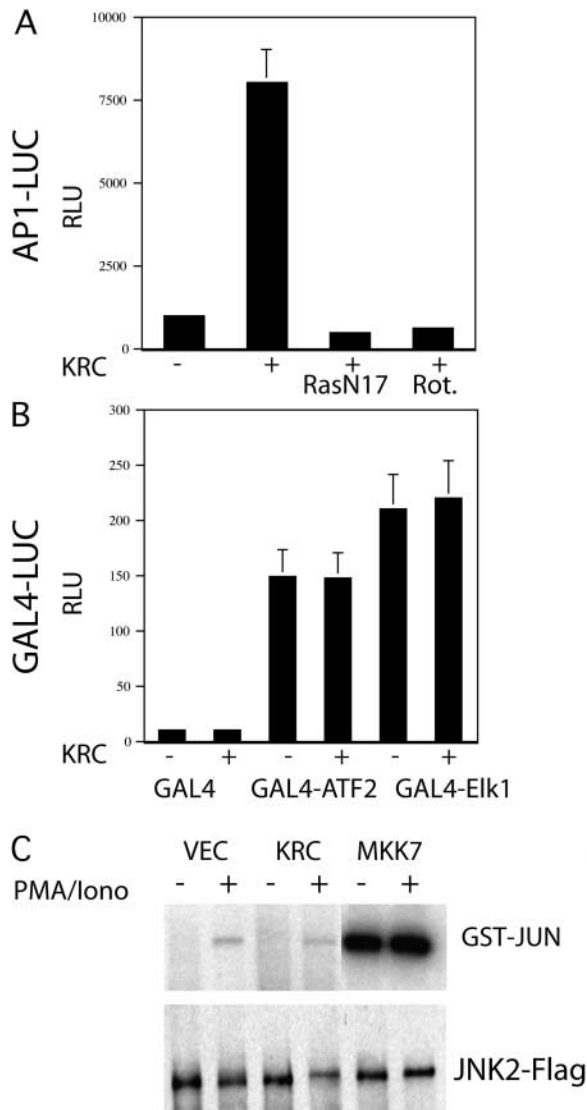
Figure 4. KRC does not modulate MAPK activity. (A) Jurkat cells were transiently transfected with AP-1–luciferase reporter along with KRC and RasN17 DN vectors. 24 h later, cells were pretreated with 10 μM rottlerin and stimulated for 6 h with PMA plus ionomycin. Luciferase activity was measured as described in Materials and Methods. (B) Jurkat cells were transfected with a GAL4 luciferase reporter along with a GAL4 DNA binding domain, GAL4-ATF2, or GAL4-ELK1 with or without KRC. 24 h later, cells were stimulated with PMA plus ionomycin and analyzed for luciferase activity as described in Materials and Methods. (C) Jurkat cells were transiently transfected with FLAG-JNK2, and either Vector, KRC, or MKK7. 48 h later, cells were stimulated with PMA plus ionomycin for 6 h, and JNK activity was determined by immunoprecipitation/kinase assay. Equal amounts of FLAG-Jnk2 protein were immunoprecipitated, as judged by anti-FLAG Western blot (bottom).

specific substrates: GST-Elk1, GST-ATF2, and GST-Jun for each MAPK. KRC had no detectable effect on any of the MAPKs in this assay (Fig. 4 C, results for JNK). We conclude that KRC does not increase AP-1 activity through increasing TCR-mediated MAPK activity, although we have observed earlier that KRC down-regulates TRAF2-mediated JNK activation after TNFα stimulation in macrophage cell lines (14). Because PMA/ionomycin is

a very poor inducer of JNK activation in T cells, we cannot rule out that KRC might also down-regulate JNK in T cells under different circumstances (e.g., CD28 stimulation). However, the ability of KRC to inhibit low levels of JNK activity after prolonged CD3/CD28 stimulation of naive Thp cells is unlikely to account for its ability to dramatically enhance AP-1 function and IL-2 production.

KRC Physically Interacts with c-Jun and Acts as a Transcriptional Coactivator. We have demonstrated recently that KRC interacts with the adaptor protein TRAF2 to inhibit both NFκB and JNK/SAPK-mediated responses, including apoptosis and TNFα cytokine gene expression (14). We asked whether KRC might, therefore, physically associate with c-Jun. Expression vectors encoded c-Jun and a truncated myc-tagged version of KRC encoding amino acids 204–1,055 (KRC tr), which includes the third zinc finger domain, one of the three acidic domains and the putative NLS sequence that were overexpressed in the 293T kidney epithelial cell line. Coimmunoprecipitation using a monoclonal anti-myc antibody revealed that KRC physically associated with c-Jun (Fig. 5 A). Furthermore, it demonstrated that the region of KRC shown to associate with TRAF2 (amino acids 204–1,055) also interacted with c-Jun. Similar results were obtained in coimmunoprecipitation of overexpressed full-length KRC with c-Jun, although the absolute amounts of c-Jun obtained were less, presumably because the full-length KRC protein is poorly expressed due to its large size (Fig. 5 B). Further mapping of c-Jun to delineate its interaction site with KRC revealed that KRC interacts with c-Jun amino acids 1–224 fused to the DNA binding domain of GAL4, which includes the transactivation domain (unpublished data). Furthermore, this association is direct and does not require posttranslational modifications as shown by the interaction of in vitro translated KRC and c-Jun proteins (Fig. 5 B, right). Finally, it was important to demonstrate that this association occurred under physiologic conditions. Untransfected Jurkat or EL4 T cell lines were stimulated with PMA/ionomycin for 45 min, and AP-1 complexes were purified by immunoprecipitating c-Jun. Fig. 5 C shows that endogenous KRC is readily detected in these complexes obtained from stimulated cells.

To further investigate the mechanism via which KRC serves as an AP-1 coactivator, we activated AP-1 by overexpressing c-Jun or c-Jun and c-Fos in 293T cells with an AP-1 luciferase reporter. In this system, overexpression of KRC enhances both c-Jun and c-Jun plus c-Fos AP-1 activity (Fig. 5 d, approximately fivefold). However, the presence of endogenous AP-1 proteins might complicate interpretation of these results. Therefore, we fused the Gal4 DNA binding domain to the c-Jun or c-Fos transactivation domains and cotransfected these chimeric cDNAs with KRC and a Gal4 binding site–luciferase reporter construct into 293T cells. The chimeric GAL4-c-Jun, but not GAL4-c-Fos, protein potently transactivated the reporter construct in the presence of KRC, demonstrating that KRC indeed acts as a transcriptional coactivator (Fig. 5 D).
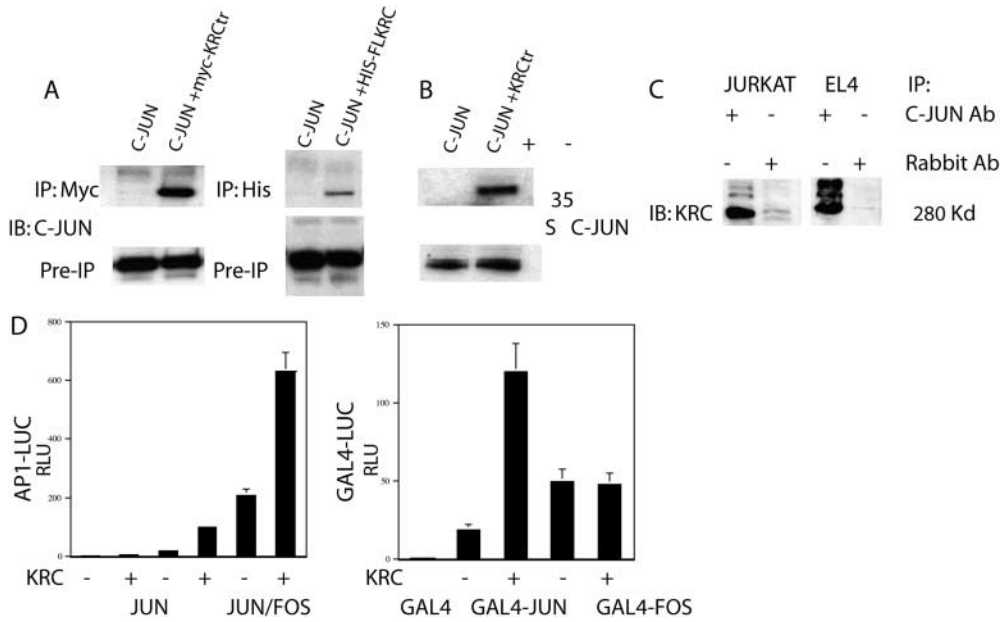
Figure 5. KRC physically interacts with c-Jun and acts as a transcriptional coactivator. (A) 293T cells were transfected with c-Jun and myc-KRCtr. 48 h later, lysates were immunoprecipitated with anti-Myc antibody. Immunoprecipitates were probed by Western blotting with anti–c-Jun antibody. (B) 293T cells were cotransfected with c-Jun and full length His-KRC (left). 48 h later, lysates were immunoprecipitated with anti-His antibody (DE8 Omniprobe), and precipitates were probed by Western blotting with anti–c-Jun antibody. In vitro–translated and S35-labeled c-Jun and His-KRCtr were mixed and immunoprecipitated with anti-His antibody (right). Recovered c-Jun protein was visualized by autoradiography. (C) Jurkat or EL4 T cells were stimulated with PMA plus ionomycin for 45 min. Lysates were immunoprecipitated with anti–c-Jun antibody, and immunoprecipitates were probed with specific anti-KRC rabbit antisera. (D) 293T cells were transfected with AP-1 luciferase along with c-Jun, c-Fos, and KRC (top). 24 h later, luciferase activity was determined as aforementioned. 293T cells were transfected with GAL4 luciferase along with GAL4, GAL4–c-Jun 1-224, or GAL4–c-Fos 208-313 (bottom). 24 h later, luciferase activity was determined as aforementioned.

In summary, KRC specifically associates with c-Jun under physiologic conditions and this association augments AP-1 transcriptional activity.

Discussion

Here, we have demonstrated a role for the KRC protein, a *Drosophila schnurri* family member, in regulating expression of the IL-2 gene in T cells. *D. schnurri* (Shn) protein functions as a cofactor of the *D. schnurri* Smad homologue, dpp, to effect Decapentaplegic signaling during embryogenesis (36–41). *Schnurri* interacts with Mad in a Dpp-dependent manner, is required for dpp-dependent patterning of the *D. schnurri* wing, and restricts proliferation of committed progenitors in the germline (36, 42–44). However, the role of the three known vertebrate Shn orthologues (Shn-1, Shn-2, and Shn-3/KRC) in the context of BMP/TGFβ/activin signaling in development is unknown. Smad family members have been shown to interact with AP-1, raising the possibility that KRC may form a tripartite complex (46, 47). Indeed, c-Jun has been shown to associate with the oncoprotein Ski to suppress Smad2 transcriptional activity (48). However, it may be that the signaling pathways involved with *Schnurri* proteins in the fly and in mammalian cells are distinct because we have demonstrated that KRC is downstream of both the T cell and TNF receptors. In addition, another member of the *D. schnurri* family, *Schnurri-2*, is required for efficient thymic positive selection (49). Mice lacking Shn-2 had severely impaired positive selection of both CD4^{+} and CD8^{+} T cells, although no mechanism for this phenotype was presented. Thymic development appears normal in mice lacking KRC (unpublished data), suggesting that distinct Shn factors may serve distinct roles at different time points in T cell development and function. It will be interesting to determine whether Shn family members other than KRC/Shn-3 serve as AP-1 coactivators. Thus, although *D. schnurri* partners with members of the TGFβ/activin/dpp pathway, it may be that mammalian *Schnurri* proteins function downstream of different signaling pathways. One such signaling pathway is the T cell receptor. Upon TCR stimulation, KRC expression dramatically increases, and KRC physically associates with c-Jun to serve as a coactivator of AP-1–dependent IL-2 gene transcription.

Coactivator proteins that potentiate transcriptional responses mediated by AP-1 have been reported previously and include p300/CREB-binding protein (CBP), SRC-1, ASC-2, and the α chain of the nascent polypeptide-associated complex (α-NAC), among others (35, 51–55). It is likely that multiple coactivators contribute to the tissue specificity of target gene activation by AP-1 proteins through different mechanisms. For example, α-NAC is a c-Jun homodimer-specific coactivator protein, whose tissue distribution during embryogenesis is restricted to osteoblasts (54). In contrast, KRC coactivates both c-Jun homodimers and the c-Jun/c-Fos heterodimer. Furthermore, unlike α-NAC, which appears to stabilize the interaction of the Jun homodimer to its cognate DNA binding site, KRC does not affect AP-1 DNA binding activity in electrophoretic mobility shift assays (unpublished data).

The Jun-activating binding protein 1 (JAB-1), isolated in a yeast two-hybrid screen, selectively coactivates c-Jun and

JunD (58). JAB-1 stabilizes complexes of c-Jun and JunD at their individual AP-1 binding sites, increasing the specificity of target gene activation. In contrast with other AP-1 coactivators, signals upstream of JAB1 under physiological conditions have been identified. JAB1 appears to be required for the AP-1 transcriptional activity that is triggered upon engagement of the LFA1 accessory receptor (59). Interestingly, JAB-1 does not appear to potentiate AP-1 activity after TCR stimulation (unpublished data). In contrast, we have shown that KRC coactivates AP-1 in the context of the IL-2 gene after TCR ligation. Thus, it appears that different extracellular stimuli can recruit different AP-1 coactivators.

Additional specificity of AP-1 coactivators may be provided by posttranslational modifications. CBP, for example, interacts only with the NH_2-terminal phosphorylated form of c-Jun (35). However, JNK expression is very low in naive Thp cells, and JNK1/2-deficient or c-Jun S63A/S73A mutant CD4 T cells show no defect in IL-2 production after initial TCR stimulation, suggesting that AP-1 complexes in naive Thp cells use a coactivator distinct from CBP (56, 57, 61). Although many proteins have been identified that can serve as AP-1 coactivators in various overexpression systems, none have been studied under physiological conditions with respect to interactions with AP-1 complexes in the context of specific target genes. Furthermore, the tissue distribution and cell type–specific signals regulating the activity of these factors are unclear. Here, we present clear gain- and loss-of-function data demonstrating that KRC is both necessary and sufficient to potently regulate AP-1 activity in the context of the IL-2 promoter in $CD4^+$ T cells after CD3/CD28-mediated stimulation.

Other groups have reported a function for KRC as a DNA-binding transcription factor (11). In our studies, KRC appears to promote IL-2 gene expression via its interactions with c-Jun, as a coactivator. Indeed, certain DNA-binding transcription factors, such as c-Jun itself, can also function as coactivators for specific target genes demonstrating that the role assigned to a nuclear factor must be carefully designated based on functional evidence in a promoter-specific fashion (60).

KRC may be both upstream and downstream of AP-1 because signaling through the TCR, which induces KRC expression, acts in part through inducing AP-1 itself. It is tempting to postulate the existence of a self-reinforcing feedback loop between KRC and AP-1 that serves to amplify membrane signaling. This type of positive regulatory loop in the context of mechanisms regulating AP-1 function is not unprecedented; sustained, but not transient, ERK activation downstream of platelet-derived growth factor signaling in fibroblasts results in both c-Fos mRNA induction and stabilization of c-Fos protein, greatly potentiating AP-1 activity (50). Future work will be necessary to define the precise mechanism via which KRC acts as an AP-1 coactivator in the context of the IL-2 gene in T cells. Additionally, the use of KRC-null mice will allow a more global analysis of the role of KRC in regulating AP-1–dependent transcription in all cell types.

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