Peptidyl-Prolyl Isomerase Pin1 Markedly Enhances the Oncogenic Activity of the Rel Proteins in the Nuclear Factor-κB Family

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Abstract

The peptidyl-prolyl isomerase Pin1 is frequently up-regulated in human cancers in which Rel/nuclear factor-κB (NF-κB) is constitutively activated, but its role in these cancers remains to be determined, and evidence is still lacking to show that Pin1 contributes to cell transformation by Rel/NF-κB. Rel/NF-κB transcriptional and oncogenic activities are modulated by several posttranslational modifications and coregulatory proteins, and previous studies showed that cytokine treatment induces binding of Pin1 to the RelA subunit of NF-κB, thereby enhancing RelA nuclear localization and stability. Here we show that Pin1 associates with the Rel subunits of NF-κB that are implicated in leukemia/lymphomagenesis and modulates their transcriptional and oncogenic activities. Pin1 markedly enhanced transformation of primary lymphocytes by the human c-Rel protein and also increased cell transformation by the potent viral Rel/NF-κB oncoprotein v-Rel, in contrast to a Pin1 mutant in the WW domain involved in interaction with NF-κB. Pin1 promoted nuclear accumulation of Rel proteins in the absence of activating stimuli. Importantly, inhibition of Pin1 function with the pharmacologic inhibitor juglone or with Pin1-specific shRNA led to cytoplasmic relocalization of endogenous c-Rel in human lymphoma-derived cell lines, markedly interfered with lymphoma cell proliferation, and suppressed endogenous Rel/NF-κB–dependent gene expression. Together, these results show that Pin1 is an important regulator of Rel/NF-κB transforming activity and suggest that Pin1 may be a potential therapeutic target in Rel/NF-κB–dependent leukemia/lymphomas. [Cancer Res 2009;69(11):4589–97]

Introduction

The Rel/nuclear factor-κB (NF-κB) family of inducible transcription factors play pivotal roles in innate and adaptive immunity, inflammation, and oncogenesis and include the c-Rel, RelA, RelB, NF-κB1 (p50/p105), and NF-κB2 (p52/p100) proteins (1). In most cells, NF-κB exists as latent cytoplasmic homo-/heterodimers, bound to inhibitory IκB proteins. Stimuli that activate the classic NF-κB cascade trigger the IκB kinase complex that mediates phosphorylation of IκBα, resulting in proteasomal degradation of IκBα and nuclear translocation of NF-κB dimers as well as their binding to κB DNA sites. This commonly results in transcriptional activation of genes important for the immune and inflammatory response, cell proliferation, adhesion, angiogenesis, and inhibition of apoptosis (1–6). Binding of NF-κB to the promoter for the *IκBα* gene triggers a negative feedback loop that terminates NF-κB activation (1). Hence, under normal conditions, activation of the classic Rel/NF-κB cascade is transient due to tight regulation of NF-κB subcellular localization by IκBα. Interference with this process can have severe consequences, as sustained activation of Rel/NF-κB is seen in many cancers where it promotes tumor cell survival, pathogenesis, and chemoresistance (7).

The peptidyl-prolyl isomerase Pin1 interacts specifically and exclusively with certain phospho-serine/threonine-proline (pSer/Thr-Pro) motifs in target proteins via its NH_2-terminal WW domain and catalyzes rapid *cis/trans* isomerization of proline amide bonds through its COOH-terminal domain (8–10). This commonly alters the conformation and biological function of substrates and can have profound physiologic relevance. Interaction of Pin1 with target proteins like p53, p73, cyclin D1, $p66^{shc}$, tau, APP, and IRF-3 has been implicated in cell cycle control, cellular stress, neuronal degeneration, and tumor progression (11).

Pin1 was shown to associate with the p65/RelA subunit of NF-κB and to promote RelA nuclear translocation and extend its half-life by blocking its inhibition by IκBα and its SOCS-1–dependent degradation (12). Interestingly, Pin1 is up-regulated in human breast cancer specimens and mouse mammary tumors and is correlated with nuclear accumulation of RelA (12, 13). Oncomine database analysis revealed markedly elevated Pin1 levels in breast carcinoma and in human B-cell chronic lymphocytic leukemia, diffuse large B-cell lymphoma, mantle cell lymphoma, and multiple myeloma in which constitutive nuclear activation of NF-κB is necessary for cell survival and proliferation (Supplementary Fig. S1; refs. 14–24). These observations suggest that Pin1 may contribute to Rel/NF-κB function in cancer. However, the role of Pin1 in oncogenesis has been a subject of controversy (25), and there is no direct evidence that Pin1 plays a role in Rel/NF-κB–dependent tumor cells. Here we show that Pin1 associates with Rel proteins and show that it significantly potentiates their transforming activity in primary lymphocytes, coincident with increased Rel nuclear accumulation. We show that knockdown of Pin1 prompts the cytoplasmic relocalization of endogenous c-Rel in multiple Rel/NF-κB–dependent human lymphoma-derived cell lines, resulting in inhibition of tumor cell proliferation and decreased

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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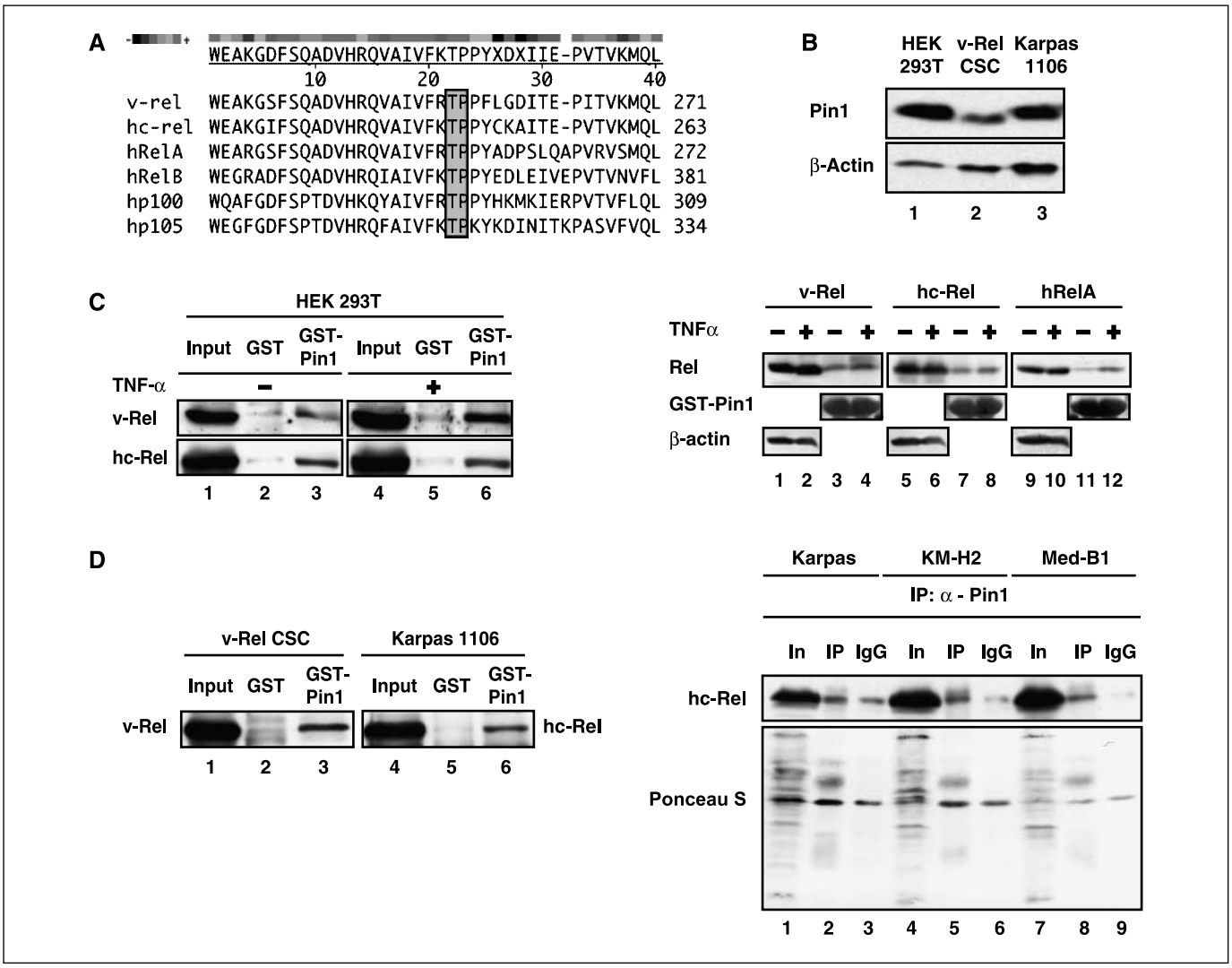


Figure 1. The c-Rel and v-Rel subunits of NF-κB associate with Pin1. *A,* sequence alignment of the vertebrate Rel/NF-κB family proteins, highlighting conservation of NH_2-terminal sequences flanking the Thr254-Pro Pin1 recognition motif in RelA, whereas sequences flanking its COOH-terminal end are more divergent. *B,* immunoblots showing endogenous expression of Pin1 in 293T cells, v-Rel–transformed chicken spleen cells, and the primary mediastinal B-cell lymphoma cell line Karpas 1106. The blot was probed with anti-Pin1 and reprobed for actin. *C, left,* pull-down of v-Rel or hc-Rel in extracts from transiently transfected 293T cells with GST-Pin1 or GST as control, followed by immunoblotting with anti-Rel. Where indicated, cells were treated with hTNFα before harvest (*lanes 4–6*). *Right,* pull-down of v-Rel, hc-Rel, or hRelA transfected in 293T cells with GST-Pin1, followed by immunoblotting with anti-Rel. Where indicated, cells were treated with hTNFα. Input (1/10 of lysate; *1–2, 5–6*, and *9–10*). The blot was reprobed for actin and Pin1. *D, left,* pull-down of endogenous v-Rel from v-Rel–transformed chicken spleen cells (*lanes 1–3*) or hc-Rel from human lymphoma-derived Karpas 1106 cells (*lanes 4–6*) with GST-Pin1 or GST, followed by immunoblotting with anti-Rel (v-Rel: #1691; hc-Rel: #265). *Right,* coimmunoprecipitation of endogenous hc-Rel with Pin1 in extracts from human lymphoma cell lines using anti-Pin1, followed by immunoblotting with anti–hc-Rel. Input (1/10 of lysate). The membrane was stained with Ponceau S (*bottom*).

Rel/NF-κB–dependent gene expression. These results identify Pin1 as a critical regulator of Rel transforming activity and point to Pin1 as a potential therapeutic target in Rel/NF-κB–dependent tumors.

Materials and Methods

Plasmids and cell culture. Viral (v-Rel) and cellular human c-Rel (hc-Rel) were expressed from pJDCMV19SV for glutathione *S*-transferase (GST) pull-downs, or in the spleen necrosis virus retroviral vector pUC19-pJD214 for immunofluorescence and transformation assays (26). GST-Pin1 and GST-v-Rel were expressed in pGEX-4T-1 (GE Healthcare). Pin1 tagged to green fluorescent protein (Pin1-GFP) or its mutants, Pin1(S16E)-GFP or Pin1(S16A)-GFP, were expressed from an internal ribosome entry site in bicistronic retroviral vectors pUC19-pJD214-vRel or pUC19-pJD214-hcRel. Human 293T cells and primary chicken embryonic fibroblasts were maintained as described (26). The human non-Hodgkin's primary mediastinal B-cell lymphoma cell line Karpas 1106 (a gift from Dr. A. Karpas, University of Cambridge, Cambridge, United Kingdom; ref. 27) and Hodgkin's lymphoma cell line KM-H2 (DSMZ) were maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% antibiotics. KM-H2 cells were supplemented with 2 mmol/L glutamine. Primary mediastinal large B-cell lymphoma MedB-1 cells (a gift from Drs. P. Moller and S. Bruderlein, Institute of Pathology, Ulm, Germany) were maintained in Iscove/RPMI 1640 (4:1) with 10% FBS, 2 mmol/L glutamine, and antibiotics. Rel-transformed chicken spleen cells were maintained as described (26).

GST pull-down assays and coimmunoprecipitation. Extracts from 293T cells transfected with pJDCMV19SV-Rel, hc-Rel, or hRelA, or with pPin1-GFP, pPin1(S16E)-GFP, or pPin1(S16A)-GFP, from v-Rel–transformed chicken spleen cells or Karpas 1106 cells, were quantitated for equal amounts of total protein (1 mg) and used in pull-downs with GST-Pin1, GST-v-Rel, or GST, as described (12). Antibodies were against the v-Rel transactivation domain (#1691; ref. 26), human c-Rel transactivation

domain (#265, a gift from M.K. Ernst and N.R. Rice, National Cancer Institute-Frederick, Frederick, MD), GFP (Torrey Pines Biolab), poly-ADP-ribose polymerase (PARP; Cell Signaling), or actin (Sigma). Endogenous Pin1 was detected with a monoclonal anti-Pin1 generated in the laboratory of KPL. Where indicated, 293T cells were treated with human tumor necrosis factor α (hTNFα; 10 ng/mL; Roche) for 40 min. Endogenous hc-Rel was coimmunoprecipitated with endogenous Pin1 in extracts (1 mg) from lymphoma cells pretreated with hTNFα, followed by immunoprecipitation with polyclonal anti-Pin1 (Santa Cruz) and immunoblotting with anti–hc-Rel (#265). The membrane was stained with Ponceau S.

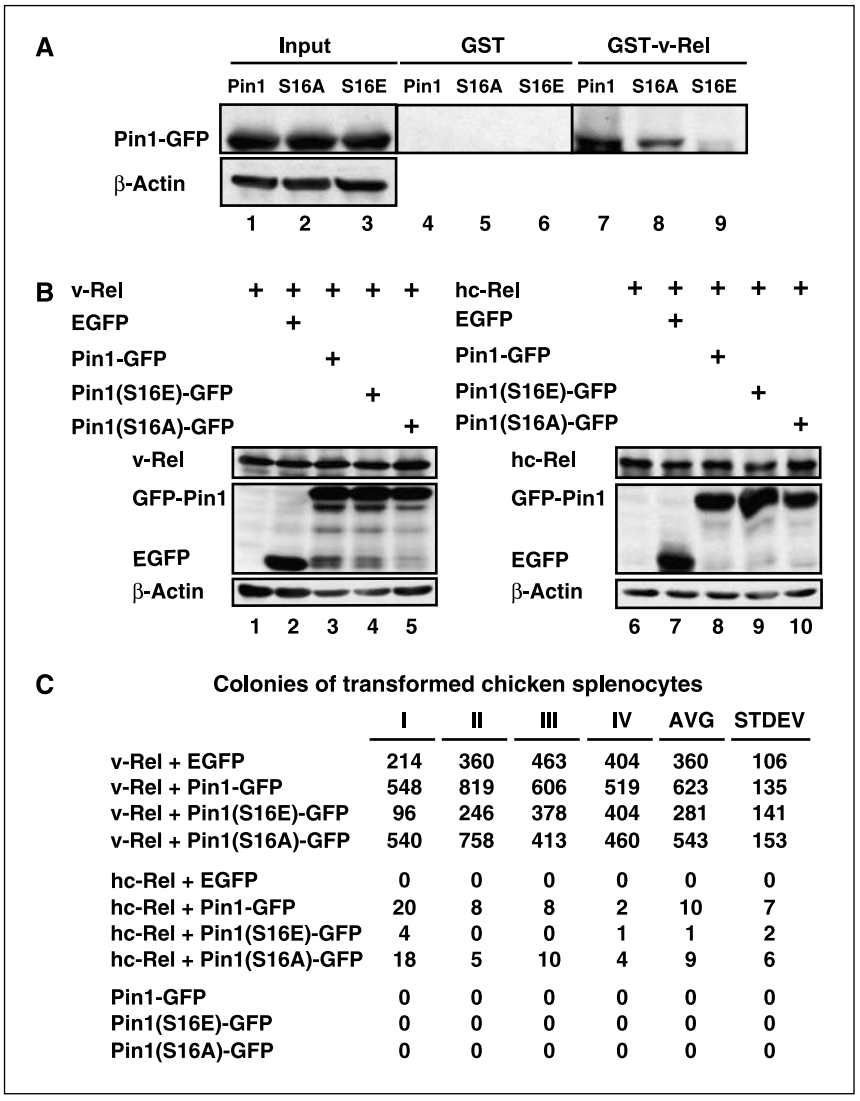
Transformation of primary chicken lymphoid cells. Primary chicken spleen cells were infected as described (3) with virus coexpressing v-Rel or hc-Rel along with wild-type or mutant Pin1. Transformed colonies in soft agar were scored after 2 wk. Animals were used according to the Institutional Animal Care and Use Committee guidelines under an approved protocol.

Immunofluorescence and cell fractionation. Cells (10^5) fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 were analyzed by immunofluorescence with anti–hc-Rel (Santa Cruz Biotechnology) and a rhodamine-conjugated secondary antibody (The Jackson Laboratory), followed by staining with Hoechst 33258 (Sigma). Where indicated, lymphoma cells were pretreated with juglone (0.1–10 μmol/L; Calbiochem) for 2 h or with DMSO as control.

Karpas 1106 cells treated with juglone (0.1–10 μmol/L) for 2 h were resuspended in hypotonic buffer [20 mmol/L HEPES (pH 7.6), 20% glycerol. 10 mmol/L KCl, 0.2 mmol/L EDTA, 0.1% Triton X-100, 5 mmol/L NaF, 10 mmol/L glycerophosphate, 1 mmol/L Na_3VO_4, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 1× PIC] on ice for 10 min, followed by centrifugation at 10,000 × *g* to recover the cytosolic fraction. Nuclear pellets resuspended in hypertonic buffer containing 600 mmol/L KCl were centrifuged at 10,000 × *g*. Extracts (40 μg) were analyzed by Western blotting with anti-Rel #265 and reprobed with anti-actin or anti-PARP (Cell Signaling) as cytosolic and nuclear markers.

RNA interference and reverse transcription-PCR. Pin1 shRNA in pSuper or a scrambled nontargeting shRNA control from KPL was delivered into human lymphoma cells by Amaxa nucleofection using Kit V (Amaxa AG), and programs were optimized for each cell line for transfection efficiencies >90% with pMaxGFP (Karpas 1106: X-001; KM-H2 and MedB-1: T-001). Reverse transcription-PCR (RT-PCR) was done within the linear range of the PCR cycle using primers specific for *pin1* (tgatcaacggctacatccag, caaacgaggcgtcttcaaat), *cyclin D1* (ctggagcccgtgaaaaagagc, ctggagaggaagcgtgtgagg), *vegf* (ccctgatgagatcgagtacatctt, aacgctccaggacttataccg), *Bfl-1* (caggctggctcaggactatc, cccagttaatgatgccgtct), and *actin* (tgacccaccagcacattta, tgaaagcagggcctgagg).

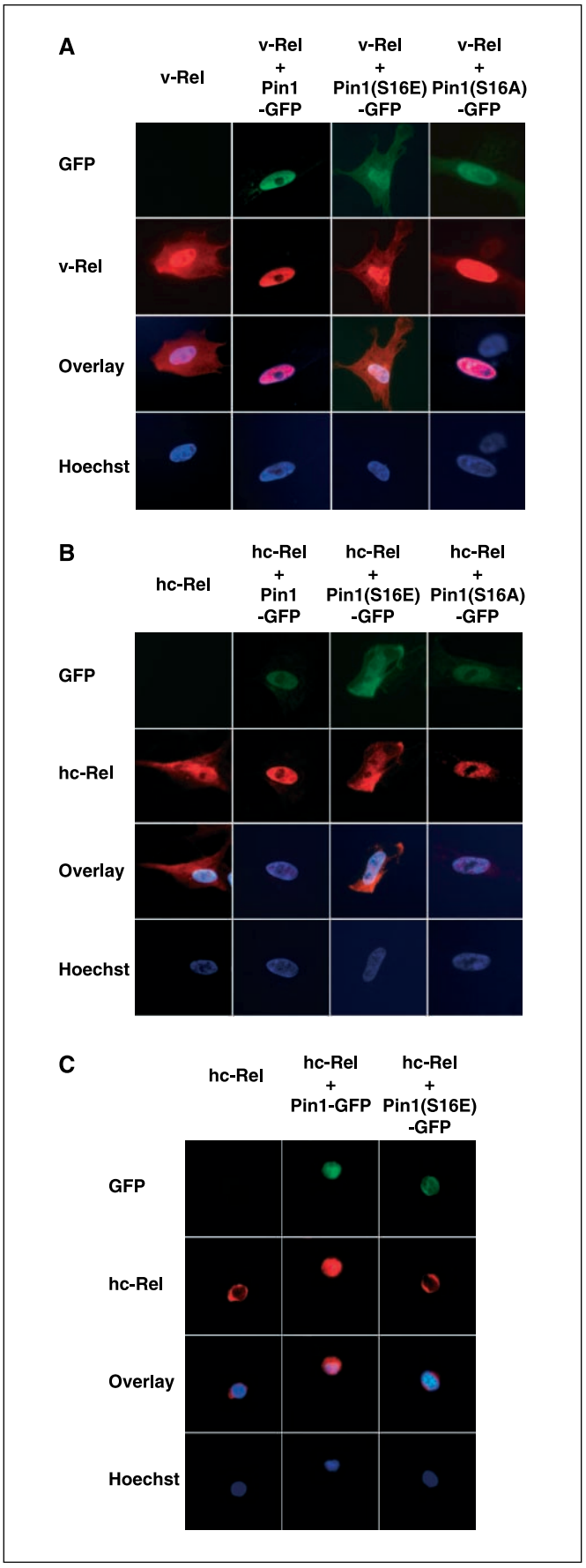
Cell proliferation and survival assays. The effects of juglone (0.1 μmol/L) or Pin1 shRNA on the proliferation and survival of lymphoma cells were determined by cell counting in the presence of trypan blue exclusion dye during a time course (0–24 h for juglone versus DMSO control or 0–144 h for Pin1 shRNA versus scrambled shRNA control).



C Colonies of transformed chicken splenocytes

	I	II	III	IV	AVG	STDEV
v-Rel + EGFP	214	360	463	404	360	106
v-Rel + Pin1-GFP	548	819	606	519	623	135
v-Rel + Pin1(S16E)-GFP	96	246	378	404	281	141
v-Rel + Pin1(S16A)-GFP	540	758	413	460	543	153
hc-Rel + EGFP	0	0	0	0	0	0
hc-Rel + Pin1-GFP	20	8	8	2	10	7
hc-Rel + Pin1(S16E)-GFP	4	0	0	1	1	2
hc-Rel + Pin1(S16A)-GFP	18	5	10	4	9	6
Pin1-GFP	0	0	0	0	0	0
Pin1(S16E)-GFP	0	0	0	0	0	0
Pin1(S16A)-GFP	0	0	0	0	0	0

Figure 2. Pin1 markedly increases the transforming activity of Rel proteins. *A,* Pin1 and mutant S16A associate with v-Rel in GST pull-down assays significantly more than mutant S16E. Pull-downs were carried out as in Fig. 1*C*, with extracts from 293T cells transfected with Pin1-GFP, Pin1(S16E)-GFP, or Pin1(S16A)-GFP and GST-v-Rel or GST, followed by Western blot with anti-Pin1. Input (1/10 of lysate). *B,* immunoblots showing efficient coexpression of v-Rel or hc-Rel with GFP-tagged Pin1, Pin1(S16E), Pin1(S16A), or GFP control in chicken embryonic fibroblasts used as the source of virus to infect primary chicken spleen cells. The blots were probed with antibodies to v-Rel (#2716) or hc-Rel (#265; *top*) or GFP (*middle*), and reprobed for actin as control (*bottom*). *C,* coexpression of Pin1 or mutant S16A markedly increases the transforming activity of hc-Rel and also enhances that of v-Rel in primary chicken spleen cells, in contrast to Pin1(S16E), as detected by colony formation in soft agar. The results of four independent experiments are shown along with averages and SDs.



Results

Pin1 associates with the v-Rel and c-Rel subunits of NF-κB. Human RelA was shown to interact with Pin1 via the Thr254-Pro motif in its Rel-homology domain (12). Sequence alignment revealed conservation of this motif among vertebrate Rel/NF-κB family members. NH_2-terminal sequences flanking this motif share a high degree of similarity and are in some cases identical, whereas those flanking its COOH-terminal end are significantly more divergent (Fig. 1*A*). Because overexpression of the Rel proteins can malignantly transform primary lymphocytes *in vitro* and induce fatal leukemia/lymphoma and mammary adenocarcinomas in animal models, unlike RelA (26, 28–31), we directly addressed the role of Pin1 in Rel/NF-κB–mediated oncogenesis by investigating its effects on the transforming activity of the v-Rel and hc-Rel proteins.

We used GST pull-downs to determine if Pin1 could engage in physical interaction with Rel proteins. As expected, Pin1 is ubiquitously expressed in human 293T cells, in primary chicken spleen cells transformed by v-Rel, and in human lymphoma Karpas 1106 cells in which NF-κB is markedly activated due to nuclear accumulation of hc-Rel (ref. 32; Fig. 1*B*). GST-Pin1 interacted both with v-Rel and its cellular homologue hc-Rel in human 293T cells, compared with the GST control (Fig. 1*C, left, lane 3* versus *lane 2*). Interestingly, whereas association of RelA with Pin1 depends on cell stimulation with cytokine TNFα that triggers phosphorylation and activation of RelA (12), TNFα treatment had little or no effect on the interaction of either v-Rel or hc-Rel with GST-Pin1 (Fig. 1*C, left, lane 6* versus *lane 3*; *right, lanes 4, 8, 12* versus *lanes 3, 7, 11*). GST-Pin1 also interacted with endogenous v-Rel from v-Rel–transformed chicken spleen cells and with endogenous hc-Rel in extracts from the human lymphoma cell line Karpas 1106 (Fig. 1*D, left, lanes 3, 6* versus *lanes 2, 5*). Importantly, coimmunoprecipitation assays verified association between endogenous Pin1 and endogenous hc-Rel in lymphoma-derived cell lines, particularly evident in KM-H2 and MedB-1 cells (Fig. 1*D, right, lane 2* versus *lane 3, lane 5* versus *lane 6*, and *lane 8* versus *lane 9*). These results show that v-Rel and hc-Rel can specifically associate with Pin1.

Conversely, Pin1 transfected in 293T cells was pulled down with GST-v-Rel, confirming their interaction (Fig. 2*A, lane 7* versus *lane 4*). In contrast, a Pin1 mutant with a serine to glutamate substitution at position 16 (S16E) in the NH_2-terminal WW domain that interacts with pSer/Thr-Pro motifs showed significantly less interaction with GST-v-Rel (*lane 9*). This is consistent with the failure of this mutant to efficiently associate with RelA (12). A mutant with substitution of serine 16 to alanine (S16A) retained the ability to interact with GST-v-Rel, although with somewhat reduced efficiency (Fig. 2*A, lane 2*). Consistent with previous findings that Pin1(S16A) constitutively binds to Pin1 substrates, in contrast to S16E (33), our data show that v-Rel preferentially associates with Pin1 and Pin1(S16A).

Pin1 markedly enhances the transforming activities of hc-Rel and v-Rel. We investigated the ability of Pin1 to modulate the oncogenic activity of Rel proteins in primary lymphocytes,

Figure 3. Pin1 and Pin1(S16A) promote relocalization of v-Rel and hc-Rel to the nucleus in the absence of stimuli. *A,* immunofluorescence showing that coexpression of Pin1 or Pin1(S16A) provokes nuclear translocation of v-Rel in chicken embryonic fibroblasts infected with bicistronic retroviral vectors, in contrast to Pin1(S16E). *B,* Pin1 or Pin1(S16A) similarly induces accumulation of hc-Rel in the nucleus of infected chicken embryonic fibroblasts. *C,* immunofluorescence showing relocalization of hc-Rel to the nucleus of transformed chicken spleen cells coexpressing hc-Rel proteins with Pin1, but not in those coexpressing Pin1(S16E).

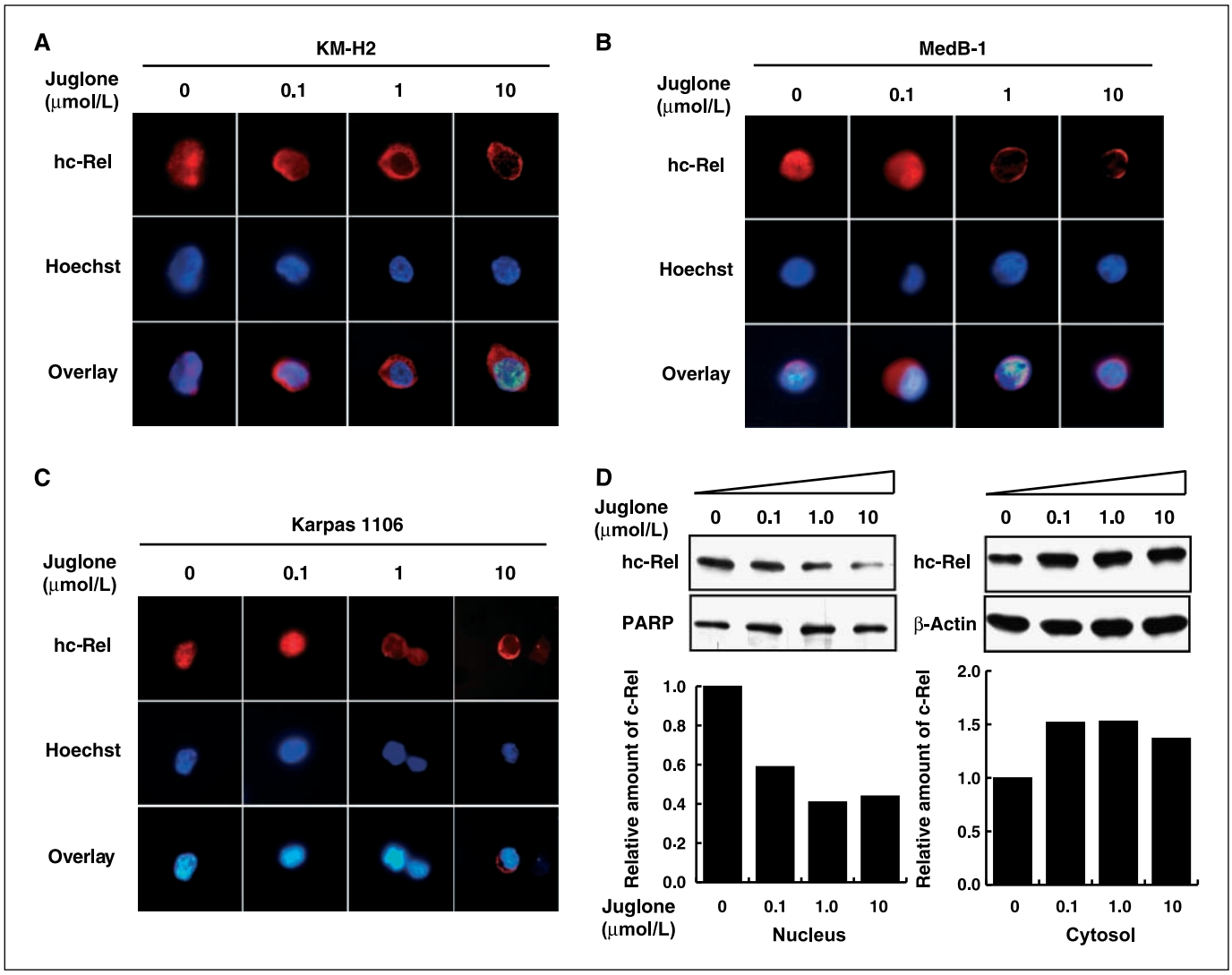


Figure 4. Juglone induces cytoplasmic relocalization of endogenous hc-Rel in human lymphoma cells. Immunofluorescence analysis of endogenous hc-Rel subcellular localization in the presence of increasing concentrations of juglone (0.1, 1, or 10 μmol/L) for 2 h in human lymphoma-derived KM-H2 (*A*), MedB-1 (*B*), or Karpas 1106 (*C*) cell lines, compared with DMSO control, using anti–hc-Rel and a rhodamine-conjugated secondary antibody. Nuclei were stained with Hoechst 33258 dye. *D,* fractionation of Karpas 1106 cells treated with juglone for 2 h confirmed rapid redistribution of endogenous hc-Rel from the nucleus to the cytoplasm. The blots were probed with anti–hc-Rel and reprobed for uncleaved PARP (~115 kDa) or actin as nuclear and cytosolic markers. Histograms show quantitation of hc-Rel band intensities in the nucleus and cytoplasm normalized to those of PARP and actin.

using a bicistronic retroviral vector to coexpress v-Rel or hc-Rel along with GFP-tagged Pin1 or mutants S16A and S16E. Western blots confirmed equivalent expression of all proteins (Fig. 2*B*). Coexpression of Pin1-GFP significantly increased the already potent transforming activity of v-Rel in primary lymphocytes nearly 2-fold, as seen by colony formation in soft agar (Fig. 2*C*). In contrast, Pin1(S16E) failed to do so, consistent with its very weak association with v-Rel (Fig. 2*C*). Coexpression of Pin1(S16A) generally enhanced the efficiency of v-Rel–mediated transformation, albeit less than wild-type Pin1. Importantly, the effects of Pin1 on lymphocyte transformation by the cellular hc-Rel protein were even more pronounced, despite variability in the efficiency of individual transformation assays that is commonly observed in these assays with primary chicken splenocytes, particularly with more weakly transforming proteins, like hc-Rel. Whereas hc-Rel coexpressed with GFP failed to transform cells, Pin1 and Pin1(S16A) markedly boosted hc-Rel transforming activity, yielding up to 20 colonies that could be picked and propagated in liquid culture, in contrast to Pin1(S16E) (Fig. 2*C*). Enhancement of Rel-mediated transformation did not result from Pin1 acting as an oncogene because neither Pin1-GFP nor its mutants could transform splenocytes on their own (Fig. 2*C*). These results show that Pin1 markedly increases Rel-mediated transformation. Given the weak transforming activity of hc-Rel (26, 31), these data suggest that Pin1 may play a significant role in enhancing hc-Rel oncogenic activity.

Pin1 increases the nuclear distribution of v-Rel and hc-Rel. The ability of Pin1 to enhance the transforming activity of hc-Rel and v-Rel led us to investigate its effect on their subcellular localization. Previous studies showed that Pin1 and Pin1(S16A) localize to the nucleus, whereas Pin1(S16E) is diffused throughout the cell (33). Immunofluorescence showed that Pin1-GFP localizes to the nucleus of infected chicken embryonic fibroblasts, and its coexpression with either v-Rel or hc-Rel prompted their relocalization to the nucleus (Fig. 3*A* and *B*). This is consistent with the

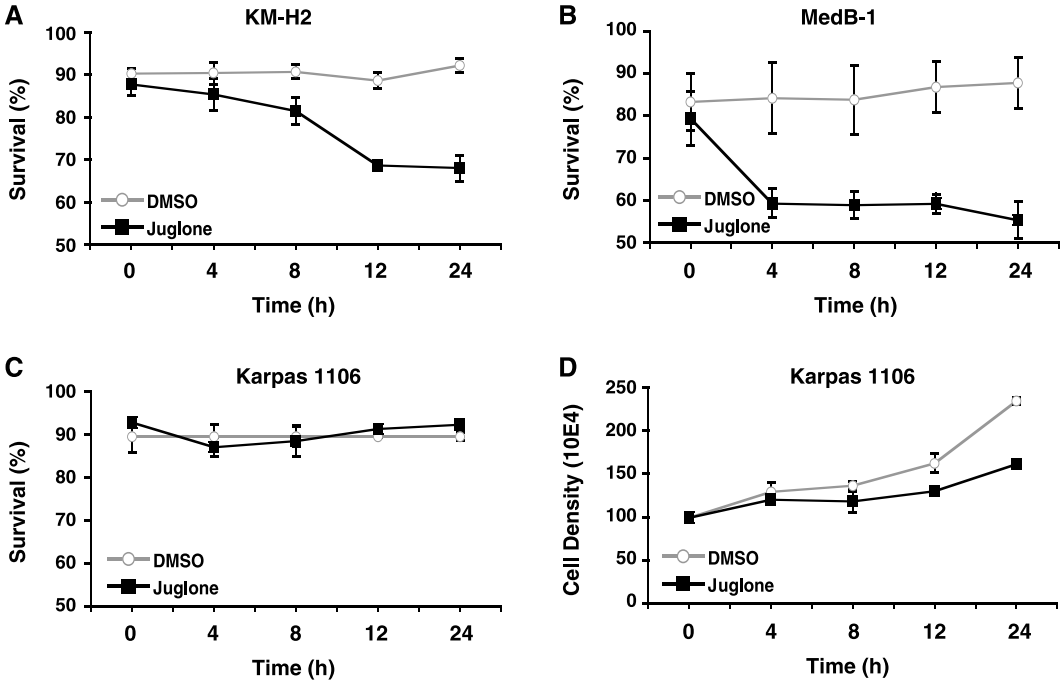


Figure 5. Juglone interferes with cell survival and/or growth in Rel-dependent human lymphoma cells. Treatment of human lymphoma-derived KM-H2 (*A*) or MedB-1 (*B*) cells with 0.1 μmol/L juglone during a time course induces cell death compared with the DMSO control, as determined by live cell count following trypan blue exclusion staining. Whereas this treatment did not affect the survival of Karpas 1106 cells (*C*), juglone significantly interfered with Karpas 1106 cell growth compared with the control (*D*). *Points,* average of three independent experiments.

ability of Pin1 to enhance their transforming potential. In contrast, Pin1(S16E) expressed alone was found in both the cytoplasm and the nucleus and was unable to induce nuclear accumulation of v-Rel or hc-Rel. Pin1(S16A) was primarily nuclear, with low-level residual localization to the cytoplasm, and effectively prompted nuclear accumulation of v-Rel and hc-Rel in infected chicken embryonic fibroblasts. This agrees with the capacity of Pin1(S16A) to retain association with Rel proteins and enhance their transforming activity. These results indicate that Pin1 can promote nuclear localization of Rel factors and that this is correlated with enhanced Rel transforming activity.

We investigated the relevance of these observations in the physiologic setting of transformed primary lymphocytes by probing the effects of Pin1 or its mutants on the subcellular localization of hc-Rel in chicken spleen cells. As anticipated, hc-Rel was predominantly cytoplasmic in primary splenocytes transformed by hc-Rel alone, as previously reported (Fig. 3*C, left*; refs. 26, 31). However, hc-Rel was predominantly nuclear in splenocytes transformed by its coexpression with Pin1, consistent with their increased transforming efficiency. In contrast, Pin1(S16E), which showed very weak association with Rel, was unable to promote nuclear localization of hc-Rel, in agreement with its inability to enhance hc-Rel-mediated transformation. These data suggest that Rel proteins are substrates for the peptidyl-prolyl isomerase activity of Pin1, and that Pin1 may significantly enhance their oncogenic activity by changing the equilibrium between their localization to the nucleus versus cytoplasm.

Juglone induces cytoplasmic relocalization of endogenous hc-Rel in lymphoma cells, coincident with lymphoma cell death and/or growth inhibition. Because Pin1 significantly increased the transforming activity of hc-Rel, we asked if endogenous Pin1 plays a role in the subcellular distribution of endogenous hc-Rel in human lymphoma-derived cells because Pin1 is highly expressed in lymphoma cells in which Rel/NF-κB is activated (Fig. 1*B, lane 3*; Supplementary Fig. S1). Juglone (5-hydroxy-1,4-naphthoquinone) is a natural and irreversible inhibitor of the parvulin family of peptidyl-prolyl isomerases that block the interaction of Pin1 with its substrates by covalently modifying its only two cysteine residues (Cys57 and Cys113; ref. 34). Juglone is frequently used to study the relevance of Pin1 function in cells and *in vivo* because it can give rise to a similar phenotype as Pin1 dominant negative mutants or Pin1 knockdown (35–37). We analyzed the effects of juglone on the distribution of endogenous hc-Rel in human lymphoma cell lines in which Rel/NF-κB is constitutively activated, including KM-H2, MedB-1, and Karpas 1106 cells (32, 38, 39). Treatment with increasing concentrations of juglone for 2 hours provoked rapid redistribution of predominantly nuclear hc-Rel to the cytoplasm in a dose-dependent manner compared with DMSO control (Fig. 4*A–C*). hc-Rel was efficiently excluded from the nucleus with 1 μmol/L juglone, and its cytoplasmic relocalization was detected with as little as 0.1 μmol/L juglone in KM-H2 cells. Fractionation of Karpas 1106 cells confirmed the significant redistribution of hc-Rel on treatment with juglone in a dose-dependent manner (Fig. 4*D*). These data suggest that Pin1 may play a role in modulating the localization of endogenous hc-Rel in human lymphoma cells.

Given the important implications of our finding that Pin1 can markedly enhance the transforming activity of hc-Rel, we investigated the effect of inhibiting Pin1 function with juglone on the biological properties of human lymphoma cells in which Rel/NF-κB is constitutively activated. We found that significant

detrimental effects on tumor cell survival and/or proliferation accompanied the rapid relocalization of hc-Rel to the cytoplasm of human lymphoma cell lines treated with juglone. Treatment of either KM-H2 or MedB-1 cells with 0.1 μmol/L juglone, the lowest concentration that provoked cytoplasmic relocalization of hc-Rel within 2 hours of treatment, triggered 20% to 30% cell death within 4 hours of treatment compared with DMSO (Fig. 5*A* and *B*). Whereas there was no significant difference in the survival for Karpas 1106 cells subjected to the same treatment (Fig. 5*C*), these cells displayed a noticeable decrease in growth rate starting at 6 hours posttreatment. By 24 hours, the growth of Karpas 1106 cells was severely blunted compared with those treated with DMSO (Fig. 5*D*). These data indicate that juglone restricts hyperproliferation and compromises the survival of tumor-derived cells.

Pin1 knockdown prompts cytoplasmic relocalization of hc-Rel, interferes with lymphoma cell growth, and suppresses Rel/NF-κB–dependent gene expression. Because pharmacologic inhibition of Pin1 with juglone could have some indirect and/or off-target effects, we used a Pin1-specific shRNA to verify our hypothesis. Nucleofection of Pin1 shRNA significantly reduced Pin1 protein levels at 96 hours in all lymphoma cell lines tested, compared with the nontargeting shRNA control (Fig. 6*A, lane 2* versus *lane 1*). Consistent with our findings with juglone, Pin1 knockdown prompted redistribution of endogenous hc-Rel to the cytoplasm compared with the shRNA control, as seen by immunofluorescence (Fig. 6*B*). Furthermore, Pin1 shRNA significantly interfered with the proliferation of all lymphoma cell lines compared with the shRNA control (Fig. 6*C*) but did not affect their viability (data not shown). This indicates that juglone has some off-target effects. Importantly, Pin1 knockdown with shRNA significantly reduced the expression of known endogenous Rel/NF-κB target genes including *cyclin D1, VEGF*, and *Bfl-1* (Fig. 6*D*). Together with our finding that Pin1 promotes lymphocyte transformation by v-Rel and hc-Rel, these results show that Pin1 is an important modulator of Rel/NF-κB function in transcription and oncogenesis.

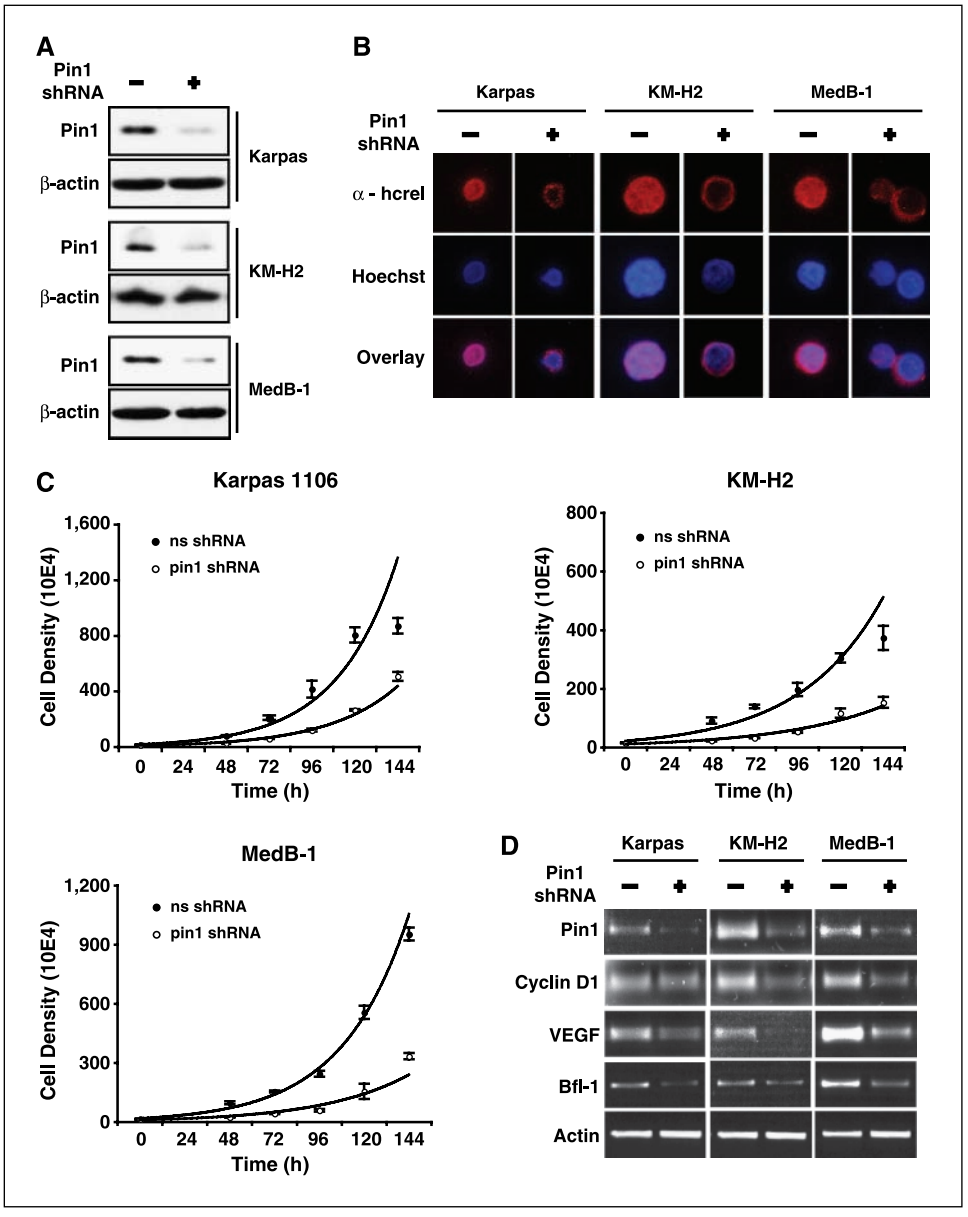


Figure 6. Pin1 knockdown prompts cytoplasmic relocalization of hc-Rel, interferes with lymphoma cell growth, and suppresses expression of Rel/NF-κB target genes. *A,* immunoblot of lymphoma-derived Karpas 1106, KM-H2, or MedB-1 cells harvested at 96 h postnucleofection with Pin1 shRNA or a scrambled shRNA control, using monoclonal anti-Pin1 or anti-actin. *B,* immunofluorescence of endogenous hc-Rel subcellular localization in lymphoma cells lines transfected with Pin1 shRNA or shRNA control, using anti–hc-Rel and a rhodamine-conjugated secondary antibody. Nuclei were stained with Hoechst 33258 dye. *C,* Pin1 knockdown with shRNA (○) significantly delays the growth of lymphoma cell lines, compared with a scrambled shRNA control (●). *Points,* average of three independent experiments. *D,* RNA was analyzed by RT-PCR at 48 h postnucleofection with Pin1 shRNA or a scrambled shRNA control using primers specific for the Rel/NF-κB–regulated genes *cyclin D1, VEGF*, or *Bfl-1*. Actin mRNA was amplified as a control.

Discussion

Accumulating evidence shows that Pin1 is up-regulated in many human cancers including breast, prostate, lung, hepatic, cervical, and colon cancers, and that increased expression of Pin1 is correlated with poor prognosis in prostate cancer (13, 40–42). Because ablation of Pin1 could prevent development of mammary carcinoma induced by oncogenic Neu or Ras in mice, this suggests an important role for Pin1 in cancer (43). Others suggested that it plays a tumor suppressor role (25). Indeed, loss of Pin1 can reportedly lead to deregulation of cyclin E and c-Myc, thereby increasing genomic instability, and is believed to sensitize cells to oncogenic transformation (44, 45). Here we provide evidence that Pin1 functionally associates with the oncogenic Rel subunits of NF-κB and that this interaction plays an important role in promoting the nuclear localization and the transcriptional, pro-proliferative, and transforming properties of Rel proteins. We show that Pin1 markedly enhances the weak transforming activity of hc-Rel in primary lymphocytes. We also show that inhibition of Pin1 severely compromises proliferation of Rel/NF-κB–dependent human lymphoma cells and is accompanied by suppression of Rel/NF-κB–dependent gene expression. These findings are consistent with accumulating evidence supporting an important role for Pin1 deregulation during tumorigenesis and the pro-proliferative capacity of tumor cells (46), and emphasize an important role in Rel oncogenic activity. This may be particularly relevant because up-regulation of Pin1 is seen in many human leukemia and lymphoma specimens in which Rel/NF-κB is known to be constitutively activated (Supplementary Fig. S1).

Whereas Pin1 failed to transform primary lymphocytes on its own, it significantly enhanced Rel transforming activity dependent on its ability to alter the dynamics of Rel protein nuclear import/export to tip the equilibrium in favor of increased nuclear accumulation. This most likely results from the ability of Pin1 to induce proline isomerization, thereby preventing Rel inhibition by IκBα (12). In this regard, it is not surprising that Pin1 had a more dramatic effect in enhancing the transforming activity of the weakly transforming hc-Rel protein compared with the potently oncogenic v-Rel because v-Rel is known to be significantly more resistant to inhibition by IκBα than c-Rel (47). Up-regulation of Pin1 may thus emerge as a novel means to enhance the contribution of hc-Rel in human cancer by helping to dampen its negative feedback inhibition. Additionally, because the 14-3-3 proteins can facilitate efficient nuclear export of IκBα-p65 complexes by binding to both RelA/p65 and IκBα, and 14-3-3 binds to RelA (amino acids 278–283) in close proximity of the Thr254-Pro recognition motif for Pin1 (48), this raises the possibility that Pin1 might also preclude export of NF-κB/IκB complexes by interfering with NF-κB regulation by 14-3-3. In both scenarios, altered nucleo-cytoplasmic shuttling of Rel/NF-κB following up-regulation of Pin1 would contribute to sustained activation of Rel/NF-κB signaling and oncogenesis.

The NH_2-terminal sequences that flank the RelA Thr254-Pro motif are highly conserved among Rel/NF-κB family members, but greater variability is seen in those that flank its COOH terminus. Although the kinase responsible for phosphorylation of Thr254 in the RelA Thr-Pro motif has yet to be identified, efficient association of RelA with Pin1 is dependent on cell stimulation with cytokine TNFα (ref. 12; Fig. 1*C, right*). In contrast, TNF had little effect on the interaction of v-Rel or hc-Rel with Pin1 (Fig. 1*C*). This, together with the sequence divergence at the COOH terminus of the Pin1 recognition motif in Rel/NF-κB proteins, suggests that different regulatory mechanisms may dictate interaction of Pin-1 with Rel and RelA proteins. Further studies will be needed to address this issue.

Our finding that Pin1 knockdown compromised the growth of Rel-dependent lymphoma cells and the expression Rel-dependent target genes points to Pin1 as a possible therapeutic target in these and other Rel-dependent tumors. However, because pharmacologic inhibition of Pin1 with juglone compromised both the growth and the survival of lymphoma cells, it seems that juglone shows some off-target effects. Indeed, juglone has been shown to affect the activity of RNA polymerase II through Pin1 and to also prevent postmitotic protein dephosphorylation (49, 50), although its effects on the growth and survival of lymphoma cells were observed at a concentration 10- to 75-fold lower than those reported to inhibit the activity of RNA polymerase II (49) or block dephosphorylation of mitotic phosphoproteins (50). Nevertheless, our results with Pin1 shRNA revealed off-target effects for juglone and suggest that more specific inhibitors of Pin1 will need to be identified for possible therapeutic application. Overall, our studies show that Pin1 plays a crucial role in promoting Rel-mediated transcription and transformation, and that inhibition of Pin1 can significantly compromise the proliferation of Rel-dependent lymphoma cells. Thus, Pin1 may be an attractive therapeutic target in Rel/NF-κB–dependent leukemia/lymphomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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