Rel Induces Interferon Regulatory Factor 4 (IRF-4) Expression in Lymphocytes: Modulation of Interferon-regulated Gene Expression by Rel/Nuclear Factor κB

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Abstract

In lymphocytes, the Rel transcription factor is essential in establishing a pattern of gene expression that promotes cell proliferation, survival, and differentiation. Here we show that mitogen-induced expression of interferon (IFN) regulatory factor 4 (IRF-4), a lymphoid-specific member of the IFN family of transcription factors, is Rel dependent. Consistent with IRF-4 functioning as a repressor of IFN-induced gene expression, the absence of IRF-4 expression in c-rel−/− B cells coincided with a greater sensitivity of these cells to the antiproliferative activity of IFNs. In turn, enforced expression of an IRF-4 transgene restored IFN modulated c-rel−/− B cell proliferation to that of wild-type cells. This cross-regulation between two different signaling pathways represents a novel mechanism that Rel/nuclear factor κB can repress the transcription of IFN-regulated genes in a cell type-specific manner.

Key words: Rel/NF-κB • lymphocytes • IRF-4 • interferon • transcription

Introduction

During antigen and mitogen stimulation of lymphocytes, specific genetic programs are established by the coordinated activation of various signal transduction pathways, which in turn, lead to morphologic changes, cell division, differentiation, and the acquisition of immunological function. The temporal patterns of gene expression that underlie these processes in lymphocytes collectively span a time frame that extends from minutes to days, with the induction of immediate early and early response genes coinciding with that period of mitogenic stimulation required to commit a cell to a program of activation (1).

One group of transcription factors essential for lymphocyte activation and immune function is the Rel/nuclear factor (NF)-κB proteins (2), homodimers and heterodimers composed of related subunits that are encoded by a small multigene family. Rel/ NF-κB proteins regulate gene expression by binding to specific decameric sequences (κB elements) located within the transcriptional regulatory regions of cellular genes, particularly those encoding proteins involved in immune, acute phase, and inflammatory responses (3, 4). The five mammalian Rel/NF-κB subunits share a conserved NH2-terminal domain (Rel homology domain [RHD]) that encompasses sequences essential for DNA binding, dimerization, and nuclear localization (3). The 50- and 52-kD forms of NF-κB1 and NF-κB2, respectively, comprise the RHD and lack intrinsic transcriptional transactivating properties, whereas Rel, RelA, and RelB all possess transactivation domains within their divergent COOH termini (3). In most cell types, the majority of Rel/NF-κB is sequestered as an inactive cytoplasmic complex with inhibitor or IκB proteins (5, 6). Diverse stimuli promote the nuclear translocation of cytoplasmic Rel/NF-κB by activating an IκB kinase complex (for a review, see reference 2) that phosphorylates conserved serine residues on the IκB proteins, targeting them for ubiquitin-dependent proteasome-mediated degradation (5, 6).

Rel is dispensable for normal embryonic development and hemopoiesis, but is essential for the division, survival, differentiation, and immune function of mature lymphocytes and macrophages (7–11). To date, most of the genes known to be direct transcriptional targets of Rel encode...
cytokines, cytokine receptors, and immune-regulatory molecules such as IL-2, IL-3, GM-CSF, IL-6, TNF-α, IL-2Rα, and inducible nitric oxide synthase (iNOS) [2, 3]). However, impaired expression of these proteins only partly explains the immune defects exhibited by c-Rel−/− mice. For example, the B cell proliferative defect resulting from the absence of Rel is due to a cell cycle block and enhanced apoptosis [10]. This impaired mitogenic response is a result of the combined failure to induce the expression of prosurvival genes such as A1 [11], and Rel-regulated genes necessary for G1 progression, the latter of which remain to be identified.

As part of a strategy aimed at identifying genes regulated by Rel, the expression of genes normally induced early in lymphocyte activation were examined in c-Rel−/− B and T cells. Among those surveyed, IFN regulatory factor 4 (IRF-4), a lymphoid-restricted member of the IFN family of transcription factors, emerged as a promising candidate. IRF-4 expression is rapidly induced in resting lymphocytes by mitogens [12] with kinetics that closely follow the nuclear induction of Rel [13]. Like Rel, it also appears to be critical in lymphocyte proliferation, as IRF-4−/− B and T cells respond poorly to mitogens [14] and deregulated overexpression of IRF-4 due to the translocation of the gene into the Ig CH locus occurs in a subset of human multiple myelomas [15]. Proteins comprising the IRF family, of which there are at least 10 members, function either as transcriptional activators or repressors [16]. All share a conserved NH2-terminal domain required for the recognition and binding of a specific DNA consensus sequence, the IFN stimulation response element (ISRE) found in the promoters of IFN-regulated genes [17, 18]. Although originally identified as mediating the diverse biological activities of type I and type II IFN s [19, 20], subsequent studies have revealed roles for IRFs that are independent of IFN signaling. These include controlling susceptibility to oncogenic transformation, promoting cell cycle progression, and the induction of growth arrest and programmed cell death [18]. Here we show that IRF-4, a repressor of ISRE-mediated transcription, while normally rapidly upregulated in mitogen-stimulated B and T cells is not induced in activated c-Rel−/− lymphocytes. This impaired expression of IRF-4, which coincides with a hyperresponsiveness of c-Rel−/− lymphocytes to type I and type II IFN s and enhanced expression of certain IFN-induced genes, establishes a novel mechanism that Rel/NF-κB can regulate IFN-induced gene expression in a cell type-specific fashion.

Materials and Methods

Mice. The generation of the c-Rel−/− [21] and nfkb1−/− [21] mice has been described previously. All mutant mouse strains have been backcrossed for greater than nine generations with C57BL/6 mice.

Genomic Clones, Plasmid Constructs, and Retroviral Expression Vector. Phage genomic clones encompassing the murine IRF-4 gene [12] were a gift of Prof. T. Mak (Ontario Cancer Institute, Toronto, Canada). Plasmids IRF-4 PstI-chloramphenicol acetyltransferase (CAT), IRF-4 H3-CAT, and IRF-4 Sph1-CAT consisted of the 2644-bp PstI-Avr1, 1195-bp Hind3-Avr1, and 498-bp Sph1-Avr1 genomic fragments, respectively, from the murine IRF-4 5′ flanking sequence inserted upstream of CAT in the promoterless reporter plasmid pBCAT3 [22]. IRF-4 xB2−/− CAT and IRF-4 xB2−/−CAT are derivatives of IRF-4 Pspl-CAT, in which NF-κB binding sites xB1 (5′-GGGATCCACC-3′ at −1733 to −1724) and xB2 (5′-GGGATCCCCC-3′ at −686 to −657) were altered by in vitro mutagenesis [23] to 5′-GGT CATATAAC-3′ and 5′-GTCACTAACCC-3′, respectively, or in the case of IRF-4 xB1/xB2−/−CAT, where both sites have been mutated. The reporter plasmid p3Pluc-ISRE comprises four copies of the ISRE consensus sequence 5′-GTAC CAGTTCG-GTTTCCCTTG-3′ inserted upstream of the minimal mouse H2Dβ promoter in the reporter plasmid pGem-luc (Promega) provided by H. Thomas (Walter and Eliza Hall Institute for Medical Research). The expression plasmid pCM DIRF-4 was generated by inserting a 2-kb XbaI-Hind3 murine IRF-4 cDNA encompassing the entire IRF-4 coding region into pCM D-B. A cDNA encompassing the murine IRF-4 coding region fused in frame with an NH2-terminal FLAG tag was cloned as a BgII-EcoR1 insert into the retroviral expression vector pMSCV-IR ES-green fluorescent protein (GFP) (a gift of Dr. L. Van Parijs, California Institute of Technology, Pasadena, CA).

Purification of Primary B and T Lymphocytes. Small resting B and T lymphocytes were purified from the spleens of 6- to 8-week-old wild-type, nfkb1−/−, and c-Rel−/− mice by negative sorting [10] on a FACS® or FACSStar® cell sorter (Becton Dickinson). The purity of all sorted B and T lymphocytes was verified by staining a portion of the cells with PE-labeled anti-B220 or anti-Thy1 antibodies, respectively (Caltag Labs) and ranged between 95 and 99%.

Cell Culture and Lymphocyte Activation in Tissue Culture. The Jurkat T cell line was maintained in RPMI 1640 supplemented with 8% FCS, and 293T fibroblasts were grown in DMEM/8% FCS. The B cell lines 404.1 and B1.1 have been described previously [11] and were cultured in DMEM/10% FCS/50 μM BM. Primary B and T lymphocytes were cultured in the high glucose version of DMEM supplemented with 13 mM folic acid, 250 mM l-asparagine, 50 μM 2-ME, and 10% FCS. Spleen cells were cultured at an initial concentration of 106 cells/ml, and FACs®-purified B or T lymphocytes were cultured at an input concentration of 3 × 105 cells/ml. All primary B or T cells were cultured in vitro with LPS (Difco), affinity-purified goat anti-mouse IgM (Fab′) fragment (Jackson Immunoresearch Laboratories), rat anti-mouse radioprotective (RP) mAbs [24], Con A (Amersham Pharmacia Biotech), PMA and ionomycin (Sigma Chemical Co.), or plate-coated mouse anti-C3D and anti-C3D2 mAbs as described previously [11]. In proliferation assays for which the effect of IFN s were assessed, either recombinant IFN-α or IFN-γ (gifts of Drs. P. Hertzog, Monash University, Melbourne, Australia, and T. Kay, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) were added at increasing 10-fold concentrations (1–103 U/ml) with the mitogen combinations anti-R/P/LPS or Con A/IL-2 (for B and T cells, respectively) for 72 h. Cellular proliferation was measured by adding 0.5 μCi of [3H]thymidine for 6 h. Afterwards, cells were harvested onto glass fiber filters, and incorporated radioactivity was quantitated by scintillation counting.

Transfections. CAT A says, and Lucifere A says. Jurkat T cells and 293T fibroblasts were transiently transfected using Superfect (Qiagen) as described previously [11]. Equimolar amounts (1–2 μg) of IRF-4-CAT plasmids or the pluc-ISRE reporter plas-
mid (1 μg) were transfected alone or with a threefold molar excess of pDAM56, PDAM56c-rel (25), pCTax (26), pCDM1×B-κBm (27), pCDMIRF-1 (19), pCDMIRF-2 (20), or pCDMIRF-4. After 48 h, Jurkat and 293T cells were harvested and CAT or luciferase assays were performed on cell extracts that had been standardized for protein content. Transfections were performed five times, with a maximum variance of ~15% observed between replicate experiments.

To determine if Rel directly regulates IRF-4 transcription, a nested set of murine IRF-4 promoter truncations was used.

**Results**

Rel induction of M urine IRF-4 T transcription is mediated by κB elements in the Promoter. In an attempt to identify novel Rel-regulated genes, mRNA hybridization studies were performed on c-rel−/− lymphocytes to examine the expression of genes normally induced during mitogen stimulation with kinetics that parallel Rel translocation into the nucleus. One gene, IRF-4 (also referred to as Pip [29], IC-SAT [IFN consensus binding protein in activated T cells; 33], or LSIRF [lymphoid IFN stimulatory response factor; 12]), encodes a lymphoid-specific member of the IFN family of transcription factors that is strongly upregulated by various mitogens in normal and nfkb1−/− B and T cells within 2 h, but is barely induced in stimulated c-rel−/− lymphocytes (Fig. 1).

To determine if Rel directly regulates IRF-4 transcription, a nested set of murine IRF-4 promoter truncations was used.
extending upstream of the 5′ untranslated region (+177), inserted 5′ of CAT in a promoterless reporter plasmid (Fig. 2 A; 22) were transiently transfected into Jurkat T cells with or without a c-rel expression vector. Basal activity of the full-length promoter, IRF4 Pst1-CAT (−2465 to +177) was equivalent to that of the truncated clones, IRF4 H3-CAT (−1010 to +177) and IRF4 Sp1-CAT (−320 to +177) (Fig. 2 B, lanes 3, 5, and 7), indicating that the sequences necessary for basal transcription appear to reside within 320 nucleotides upstream of the transcription start site. In contrast, whereas IRF4 Pst1-CAT was induced 3.5-fold by Rel, the Rel-dependent expression of IRF4 H3-CAT and IRF4 Sp1-CAT was significantly reduced or completely ablated (Fig. 2 B, compare lanes 4, 6, and 8).

These findings indicated that at least two sequences residing between −2465 and −320 were mediating Rel-dependent transcription. As this region of the murine IRF-4 promoter encompassed two putative Rel/NF-kB binding sites at −1733 (κB1: 5′-GGGGATCCAC-3′) and −686 (κB2: 5′-GGGATCCACC-3′) (12), the requirement of these elements for Rel-mediated transcription was tested directly by mutating either or both of these sequences. Promoter constructs, IRF4 κB1m-CAT, IRF4 κB2m-CAT, and IRF4 κB1m/κB2m-CAT, that contain either or both mutant κB sites within the context of the full-length promoter retained normal basal promoter activity (Fig. 2 B, lanes 3, 9, 11, and 13). However, mutations in κB1 (lane 10) or κB2 (lane 12) diminished the Rel-dependent induction of IRF4 Pst1-CAT by twofold, while the disruption of both sites (lane 14) eliminated Rel-induced transcription. These results demonstrate that these two κB elements are both necessary and sufficient for Rel-dependent IRF-4 transcription.

Mitogen-induced Nuclear Rel Complexes Bind to Both κB Elements in the IRF-4 Promoter. The ability of the two IRF-4 κB sites to bind nuclear Rel complexes was examined by electrophoretic mobility shift assays (Fig. 3). Single major complexes expressed in quiescent wild-type and c-rel−/− T cells (Fig. 3 A, lanes 1 and 3) bound κB1 (denoted C2) and κB2 (denoted C4) probes. Within 2 h of anti-CD3/anti-CD28 stimulation, novel complexes (C1 and C3 for κB1 and κB2 probes) were detected in wild-type (Fig. 3 A, lane 2) but not c-rel−/− T cells (lane 4). The binding specificity of all complexes was demonstrated in normal (Fig. 3 A, lanes 5, 6, 9, and 10) and c-rel−/− (lanes 7, 8, 11, and 12) T cells by competition with excess unlabelled IRF-4 κB1, IRF-4 κB2 (lanes 9–12), or the corresponding mutant probes (lanes 5–8). Equivalent results were obtained for

### Figure 2

**Functional analysis of the murine IRF-4 promoter.** 
(A) Schematic representation of the IRF-4 5′ flanking region and the CAT reporter plasmids. The numbers in parenthesis indicate the position of restriction enzyme sites in the murine IRF-4 5′ flanking sequence according to the sequence of Matsuyama et al. (reference 12). The filled boxes represent the two putative NF-κB binding sites κB1 (5′-GGGGATCCAC-3′: −1733 to −1724) and κB2 (5′-GGGGATCCACC-3′: −686 to −677), whereas the corresponding symbol with a cross represents the mutated motifs κB1m (5′-GGTCTAAAC-3′) and κB2m (5′-GGTCTACACC-3′). The arrow denotes the transcription initiation site and the CAT gene is depicted as an open box. Plasmid nomenclature is indicated to the right of each construct. (B) Both IRF-4 NF-κB motifs contribute to Rel-dependent transcription in T cells. Jurkat cells were transiently transfected with 2 μg of the reporter plasmids pCAT (lanes 1 and 2), IRF4 Pst1-CAT (lanes 3 and 4), IRF4 H3-CAT (lanes 5 and 6), IRF4 Sp1-CAT (lanes 7 and 8), IRF4 κB1−/−CAT (lanes 9 and 10), IRF4 κB2−/−CAT (lanes 11 and 12), or IRF4 κB1m/κB2m-CAT (lanes 13 and 14) plus 10 μg of the expression plasmid DAM P56 containing no insert (lanes 1, 3, 5, 7, 9, 11, and 13) or c-rel (lanes 2, 4, 6, 8, 10, 12, and 14). Chloramphenicol acetylation for transfections with pDAM P56 or pDAM P56c-rel is indicated by white and black bars, respectively. Results represent the mean percentage of chloramphenicol acetylation ± SD obtained from six separate sets of transient transfections.
normal and c-rel−/− B cells activated with anti-IgM antibodies (results not shown).

The composition of complexes binding IR F-4 κB1 and κB2 in normal resting and mitogen-stimulated T lymphocytes was examined by supershift analysis using antibodies specific for the different Rel/NF-κB subunits (Fig. 3 B).

Mitogen-induced complexes C1 and C3 were supershifted with antibodies specific for NF-κB1 (lane 6) and Rel (lane 7), but not RelA (lane 8), demonstrating that both complexes predominantly comprise NF-κB1/Rel heterodimers. Although C2 and C4 have similar mobilities to NF-κB1 homodimers, neither complex was supershifted...
with various NF-κB1-specific antibodies or antibodies directed to other Rel/NF-κB proteins. The identity of protein(s) in C2 and C4 remains to be determined.

Although mitogen-induced IRF-4 expression coincided with the binding of NF-κB1/R el to IRF-4 κB1 and κB2, the normal induction of IRF-4 mRNA in mitogen-stimulated nfkbl−/− lymphocytes prompted us to examine the basis of NF-κB1-independent IRF-4 transcription. DNA binding assays performed with nuclear extracts from resting or anti-CD3/anti-CD28 antibody-stimulated nfkbl−/− T lymphocytes are shown in Fig. 3 C. IRF-4 κB1 and κB2 bound complexes in resting cells (lane 1, denoted C6 and C8) and novel complexes of slower mobility (lane 2, denoted C5 and C7) induced in mitogen-activated cells. Supershift studies (Fig. 3 D) indicated that the induced complexes C5 and C7 appear to be R el homodimers, whereas C6 and C8 were not recognized by R el/NF-κB-specific antisera (lanes 2, 3, 4, and 5). While the roles of C6 and C8 in IRF-4 transcription are unknown, ablation of C6 binding by competition with unlabeled IRF-4 κB1 mutant probe (Fig. 3 C, lane 4) indicates that this protein(s) does not bind the IRF-4 κB1 probe via the consensus κB site. Collectively, these data establish that mitogen-induced IRF-4 expression coincides with the binding of R el complexes to both κB elements in the IRF-4 promoter.

R el-deficient B Cells Exhibit Increased Responsiveness to Type I and Type II IFNs. IFN-α-induced gene expression is controlled by the coordinated interplay of transcriptional activators and repressors (18). IFN-α-regulated transactivators IRF-1 and ISGF3 induce transcription by binding to ISREs found in the promoters and enhancers of IFN-regulated genes (18), while a negative regulator such as IRF-2 is thought to repress transcription by competitive binding to these motifs (17). Since IRF-4 functions as a repressor of IFN-α-induced gene expression (33, 34), one consequence of these motifs (17). Since IRF-4 functions as a repressor of IFN-α-induced gene expression (33, 34), one consequence of the inability to upregulate IFN-induced gene expression during the mitogenic activation of c-rel−/− lymphocytes may be increased responsiveness of these cells to the action of IFN-αs. Inhibition of normal lymphocyte proliferation by high concentrations of type I and type II IFNs (35, 36) prompted us to compare the proliferative response of normal and c-rel−/− B cells over a wide range of IFN-α and IFN-γ concentrations (Fig. 4). The stimulus anti-R el plus LPS was chosen for these experiments because in contrast to individual mitogens which fail to induce c-rel−/− B cell proliferation (7), certain mitogen or mitogen plus cytokine combinations function synergistically to promote the proliferation of R el-deficient cells (7, 10). Inhibition of B cell proliferation over a wide range of IFN-α and IFN-γ concentrations (Figs. 4, A and B) was significantly greater in c-rel−/− cultures. This heightened sensitivity of c-rel−/− B cells to the antiproliferative activity of both cytokines was concentration-dependent. While inhibition of c-rel−/− B cell proliferation was more pronounced with increasing concentrations of IFN-γ (Fig. 4 C), the difference in the inhibition of normal and c-rel−/− B cells by IFN-α was maximal at 100 IU/ml (Fig. 4 B). Consistent with the specificity of IRF-4 induction by R el in lymphocytes and its proposed role in modulating IFN responsiveness, proliferating c-rel−/− T cells but not c-rel−/− fibroblasts display a heightened sensitivity to the antiproliferative activity of IFN-αs (Grumont, R., unpublished results). Enforced IRF-4 E Expression in c-rel−/− B Cells Restores Normal IFN Responsiveness. We have previously shown that surface IgM+ c-rel−/− B cell lines, like primary c-rel−/− B cells undergo apoptosis upon B cell receptor engagement due in part to an absence of R el-induced A1 expression (11). To ascertain if these cell lines could also be useful models in determining whether the R el-dependent induction of IRF-4 in B cells has a direct role in modulating the responsiveness of proliferating B cells to IFN-α, we first examined the expression of IRF-4 in control and c-rel−/− B cell lines. In contrast to the constitutive expression of IRF-4 in the control B cell line W404.1, IRF-4 mRNA was not detected in the c-rel−/− B cell line B1.1 (Fig. 5 A). To determine if an absence of IRF-4 expression in B1.1 was associated with an increased sensitivity to the antiproliferative action of IFN-αs, both W404.1 and B1.1 were treated with a range of IFN-α and IFN-γ concentrations for 72 h and proliferation assessed by tritiated thymidine incorporation (Fig. 5 B). Consistent with the trends observed for primary B cells after 72 h, proliferation of the c-rel−/− B cell line B1.1 was significantly less over a range of IFN-α and IFN-γ concentrations compared with that of W404.1. The role of IRF-4 in reducing the antiproliferative action of IFN-αs was examined by infecting the W404.1 and B1.1 cell lines with a retrovirus expressing R el-terminally Flag-tagged IRF-4. Clones of each cell line expressing comparable levels of exogenous IRF-4 (Fig. 5 C) were then treated with IFN-α or IFN-γ for 72 h and proliferation assessed (Fig. 5 D). These experiments, which showed that enforced expression of IRF-4 restored c-rel−/− B cell proliferation, established that IRF-4 can modulate IFN-α-induced inhibition of lymphocyte proliferation to that of c-rel−/− B cells in the presence of IFN-α.
Northern blot analysis of transiently transfected Jurkat and 293T cells revealed that IRF-1–dependent transcription in a lymphoid-specific manner, press IRF-1–induced gene expression (sixfold, lane 5) in both cell types by cotransfection with an equimolar concentration of IRF-1. The results obtained with these two cell lines were representative of other independent c-rel−/− and c-rel+/+ cell lines. (B) C-rel−/− B cell lines are more sensitive to the antiproliferative activity of IFN-α. The W404.1 (c-rel+/+; open symbols) and B1.1 (c-rel−/−; filled symbols) B cell lines were either untreated or stimulated with 1, 10, 100, or 1,000 U/ml of IFN-α (lanes 1 and 3) or IFN-γ (lanes 2 and 4) for 48 h. Cellular proliferation was measured at 24-h intervals over 72 h by [3H]thymidine incorporation. Proliferation of all cell lines in the presence of a particular concentration of IFN at the 72-h time point is represented in this figure as a percentage of [3H]thymidine incorporated by normal or c-rel−/− cells, respectively, in the absence of IFN. All results represent the mean ± SD from four experiments. (C) Expression of exogenous IRF-4. Whole cell extracts from W404.1 (c-rel+/+; lanes 1 and 2) and B1.1 (c-rel−/−; lanes 3 and 4) cells that had been infected with a control retrovirus (lanes 1 and 3) or a retrovirus expressing NH2-terminally FLAG-tagged IRF-4 (lanes 2 and 4) were resolved by SDS-PAGE and subjected to Western blot analysis using mAbs directed to the FLAG epitope. (D) Enforced IRF-4 expression overcomes the heightened anti-proliferative action of IFNs that result from the loss of R el. The cell lines W404.1 (circles) and B1.1 (triangles) that were infected with a control retrovirus (open symbols) or a virus expressing FLAG-tagged IRF-4 (filled symbols) were either untreated or stimulated with 1, 10, 100, or 1,000 U/ml of IFN-α or IFN-γ. The data shown represents the 72-h time point and are expressed as a percentage of [3H]thymidine incorporated by normal or c-rel−/− cells, respectively, in the absence of IFN. All results represent the mean ± SD from four experiments.

**Figure 5.** Constitutive IRF-4 expression promotes c-rel−/− B cell proliferation in the presence of IFNs. (A) IRF-4 is not expressed in c-rel−/− B cell lines. 5 μg samples of total RNA isolated from the immortalized B cell lines W404.1 (c-rel+/+; lane 1) and B1.1 (c-rel−/−; lane 2) were analyzed by Northern blot hybridization. Filters were sequentially hybridized with murine IRF-4 and rat GAPDH cDNA probes and exposed for autoradiography for 24 h. (B) The results obtained with these two cell lines were representative of other independent c-rel−/− and c-rel+/+ cell lines. (B) C-rel−/− B cell lines are more sensitive to the antiproliferative activity of IFNs. The W404.1 (c-rel+/+; open symbols) and B1.1 (c-rel−/−; filled symbols) B cell lines were either untreated or stimulated with 72 h with 1, 10, 100, or 1,000 U/ml of IFN-α (circles) or IFN-γ (squares). Cellular proliferation was measured at 24-h intervals over 72 h by [3H]thymidine incorporation. Proliferation of all cell lines in the presence of a particular concentration of IFN at the 72-h time point is represented in this figure as a percentage of [3H]thymidine incorporated by normal or c-rel−/− cells, respectively, in the absence of IFN. All results represent the mean ± SD from four experiments. (C) Expression of exogenous IRF-4. Whole cell extracts from W404.1 (c-rel+/+; lanes 1 and 2) and B1.1 (c-rel−/−; lanes 3 and 4) cells that had been infected with a control retrovirus (lanes 1 and 3) or a retrovirus expressing NH2-terminally FLAG-tagged IRF-4 (lanes 2 and 4) were resolved by SDS-PAGE and subjected to Western blot analysis using mAbs directed to the FLAG epitope. (D) Enforced IRF-4 expression overcomes the heightened anti-proliferative action of IFNs that result from the loss of R el. The cell lines W404.1 (circles) and B1.1 (triangles) that were infected with a control retrovirus (open symbols) or a virus expressing FLAG-tagged IRF-4 (filled symbols) were either untreated or stimulated with 72 h with 1, 10, 100, or 1,000 U/ml of IFN-α or IFN-γ. The data shown represents the 72-h time point and are expressed as a percentage of [3H]thymidine incorporated by normal or c-rel−/− cells, respectively, in the absence of IFN. All results represent the mean ± SD from four experiments.

Rel Induction of IRF-4 Coincides with a Repression of IFN-Regulated Gene Expression. The heightened responsiveness of c-rel−/− lymphocytes to the action of IFN led us to determine if R el could be shown to modulate ISR E-dependent transcription. This was determined by comparing ISR E-dependent transcription in Jurkat T cells or 293T embryonic kidney epithelial cells transiently transfected with an ISR E-regulated reporter plasmid and various combinations of expression vectors encoding IRF-1, IRF-2, IRF-4, and R el (Fig. 6 A). IRF-1–induced ISR E-dependent transcription was reduced significantly in both cell types by cotransfection with an equimolar concentration of IRF-2 or IRF-4 plasmids (compare lanes 2 and 7 with lanes 3, 4, 8, and 9). However, R el was only able to repress IRF-1–induced gene expression (sixfold, lane 5) in Jurkat T cells. Consistent with R el repressing IRF-1–dependent transcription in a lymphoid-specific manner, Northern blot analysis of transiently transfected Jurkat and 293T cells (Fig. 6 B) revealed that IRF-4 mRNA levels were only upregulated in Jurkat T cells transfected with c-rel (lane 4). Collectively, these findings are consistent with R el repressing ISR E-dependent transcription in a lymphoid-restricted manner by upregulating IRF-4.

IFN-regulated endogenous gene expression was also examined in c-rel−/− cells. We focused on the gene encoding the 1.7-kb transcript for 2′-5′ OAS because the molecular basis of its IFN-α or IFN-1–induced transcription has been well studied (30, 36–38) and transcription of the human gene is repressed by IRF-4 (33). The IFN induction of 2′-5′ OAS mRNA was examined in normal and c-rel−/− fibroblasts and B cells stimulated with IFN-α or IFN-α and rat GAPDH cDNA probes were exposed to autoradiography for 24 h.

**Figure 6.** Rel regulation of IRF-1–dependent transcription. (A) Rel repression of IRF-1–dependent transcription is lymphoid specific. Jurkat T cells (lanes 1–5) and 293T fibroblasts (lanes 6–10) were transiently transfected with 2 μg of the ISR E-regulated reporter plasmid plus ISRE in the absence (lanes 1 and 6) or presence of an equimolar concentration of expression vectors encoding IRF-1 (lanes 2 and 7) IFN-α plus IRF-2 (lanes 3 and 8), IRF-1 plus IRF-4 (lanes 4 and 9), or IRF-1 plus c-rel (lanes 5 and 10). Luciferase activity for transfections with vector control (white bars), IRF-1 (black bars), IRF-1 plus IRF-2 (hatched bars), IRF-1 plus IRF-4 (stippled bars), or IRF-1 plus c-rel (cross-hatched bars) represent the mean activity ± SD obtained from four separate sets of transient transfections. (B) Rel repression of IRF-1–regulated transcription in T cells coincides with IRF-4 induction. 10-μg samples of total RNA isolated from Jurkat and 293T cells transiently transfected with 15 μg of pDAM56 (vector control) or pDAM56 c-rel after 48 h were analyzed by Northern blot hybridization with murine IRF-4 and rat GAPDH cDNA probes. Filters were probed with 2′-5′ OAS because the molecular basis of its IFN-α or IFN-1–induced transcription has been well studied (30, 36–38) and transcription of the human gene is repressed by IRF-4 (33). The IFN induction of 2′-5′ OAS mRNA was examined in normal and c-rel−/− fibroblasts and B cells stimulated with IFN-α or IFN-α and rat GAPDH cDNA probes were exposed to autoradiography for 24 h.
plus LPS. A representative example of these experiments is shown in Fig. 7. Consistent with previous findings for various cell types (37), 2'-5'OAS expression is absent or low in unstimulated normal and mutant fibroblasts or lymphocytes (lanes 1, 2, 7, and 8). While the induction of 2'-5'OAS mRNA by IFN-α or LPS plus IFN-α is equivalent in wild-type and c-rel−/− fibroblasts (lanes 3-6), mRNA levels are between four- and fivefold higher than normal in c-rel−/− B cellsactivated with IFN-α and LPS (lanes 11 and 12). This is in contrast to stimulation with IFN-α only, where 2'-5'OAS mRNA levels are equivalent in wild-type and mutant B cells (lanes 9 and 10). Since IRF-4 is upregulated by mitogens but not IFNs (12), reduced expression of 2'-5'OAS in normal but not c-rel−/− B cells treated with LPS and IFN-α is consistent with repression of 2'-5'OAS transcription arising from the R el-dependent induction of IRF-4.

Induction of IRF-4 by Tax Is R el/NF-κB Dependent. Since IRF gene expression is often induced in cells in response to viral infection (20, 38), it was noteworthy that IRF-4 is expressed constitutively in HTLV-1-induced T cell leukemias but not in other T cell tumors of varying etiology (33). Consistent with this finding, Tax, a potent HTLV-1-encoded transcriptional activator essential for viral-mediated oncogenesis upregulates IRF-4 expression in Jurkat cells (33). As Tax induces the expression of various cellular genes by activating or enhancing the activity of cellular transcription factors including Rel/NF-κB proteins (39, 40), we examined whether Tax-induced IRF-4 transcription was mediated by Rel/NF-κB (Fig. 8). While Tax upregulated the IRF4 Pst1-CAT reporter plasmid fourfold in Jurkat cells (compare lanes 4 and 5), an IκB-α super repressor reduced the Tax-dependent induction of the IRF4 promoter to near basal levels (lane 6). Cotransfections of Tax and the IRF-4 xB1 promoter mutants established that both IRF-4 xB1 and αB2 are essential for Tax-dependent IRF-4 transcription. Whereas Tax-mediated transactivation of IRF4 xB1m-CAT (lane 8) and IRF4 xB2m-CAT (lane 11) was 70 and 50%, respectively, of wild-type promoter activity, the loss of both xB sites completely abolished transactivation (lane 14). As was the case with IRF4 Pst1-CAT, the diminished Tax-induced activation of IRF-4 reporter plasmids containing xB1 or xB2 mutations was further reduced to basal levels by IκB-α (lanes 9 and 12). These findings establish that the Tax induction of IRF-4 transcription in T cells is mediated by Rel/NF-κB.

Discussion

Previous studies have shown that in lymphocytes, Rel is crucial in promoting cellular activation and the acquisition of immunological function (2). Consistent with the immunological component of Rel function, cytokine genes such as IL-2, IL-3, and GM-CSF are transcriptional targets of Rel (7, 8). Here we show that expression of IRF-4, a lymphoid-specific IFN regulatory factor is directly induced by Rel in activated lymphocytes and that an absence of Rel coincides with a heightened responsiveness of lymphocytes to type I and type II IFNs. The implications of this finding for lymphocyte proliferation, viral replication, and the cross-regulation of IFN signaling by Rel/NF-κB are discussed.

Rel-dependent transcription. Here we show mitogen-induced IRF-4 transcription is Rel-dependent and is regulated by two xB elements located in the IRF-4 promoter that...
bind Rel/NF-κB complexes. Although the major nuclear complex induced in lymphocytes that binds to both IRF-4 κB elements is NF-κB1/Rel, normal upregulation of IRF-4 in nkb1−/− lymphocytes that results from Rel homodimers binding to these sites indicates that NF-κB1 is a nonessential heterodimeric partner of Rel in controlling the transcription of this gene. A similar finding has been made for mitogen-induced regulation of A1 expression, which is also under the transcriptional control of Rel/NF-κB (11). Together, this establishes that under normal physiological conditions, Rel homodimers can function as transcriptional activators.

Rel/NF-κB binding sites 5′-GGG^3/4_g R R N N^3/4_C C-3′ comprise a conserved 5′ half site, GGGA/GR, and a divergent 3′ half site. Both dimer partners contribute to DNA binding, with different subunits binding to the half sites with varying affinity (41, 42). Since NF-κB1 preferentially interacts with the conserved 5′ half site (41), NF-κB1 homodimers bind with strongest affinity to symmetrical motifs (41). By contrast, DNA sequences randomly selected in vitro to bind recombinant Rel homodimers with high affinity (5′-NGGR N^3/4_g T TCC-3′) exhibit considerable sequence variation (43). The IRF-4 and A1 κB sites, all of which bind NF-κB1 homodimers, Rel homodimers, and NF-κB1/Rel heterodimers, comprise the consensus sequence 5′-GGG^3/4_g G G G A T C C ^3/4_g C C-3′. As each of these sites shares the invariant core motif 5′-GGGATCC-3′, we suggest that this may represent a signature sequence for functional Rel/NF-κB sites that bind Rel homodimers. Interestingly, the A1 and IRF-4 κB sites do not conform to the artificial high-affinity Rel consensus sequences, but instead are more closely related to NF-κB1 homodimer binding sites (43). While the reason for this difference is unclear, one plausible explanation may be that physiological κB sites need to bind Rel homodimers with lower affinity than the artificial sites which were selected in vitro to favor high-affinity protein-DNA interactions.

IRF-4, a transcriptional target of Rel: Implications for the immune response and cell division. Our findings establish that Rel/NF-κB transcription factors are dichotomous regulators of IFN signaling. While NF-κB has been shown to be important in viral-induced human IFN-β transcription (44), we show here that Rel can modulate IFN-regulated gene expression in lymphocytes by inducing IRF-4, a repressor of ISRE-regulated transcription. While higher than normal 2′,5′ OAS expression in mitogen plus IFN-activated c-rel−/− lymphocytes is consistent with a role for IRF-4 in the transcriptional repression of this gene, the expression of certain other IFN-induced genes is not altered in c-rel−/− B and T cells (Grumont, R., unpublished results). The reason for the selective modulation of IFN-regulated gene expression in c-rel−/− lymphocytes remains to be determined. For example, it may reflect the asymmetry by which IRF-4 blocks IFN-induced transcription, which is thought to occur either by direct competition with activators for binding to ISREs (33, 34) or through protein-protein interactions with activators such as IRF-1 or ISGF3, as has been proposed for IFN consensus sequence binding protein (ICSBP)-mediated repression (45).

In contrast to other IRF genes, the expression of IRF-4 is not regulated by type I or type II IFNs but instead is induced by Rel/NF-κB in response to mitogenic stimulation. As IRF-4 is required for lymphocyte proliferation (14) and inhibits IFN-induced transcription (33), it had been proposed that this novel mode of IRF regulation represents a mechanism that permits lymphocyte activation under conditions normally refractory to cellular proliferations (29, 34). Particularly as the growth inhibitory activity of IFN-γ (35) would be detrimental to lymphocyte function during an immune response, attenuating the effects of IFN by upregulating IRF-4 could be envisaged as a means of maximizing the antiviral immune response of B and T cells in a microenvironment with high local concentrations of these cytokines. Certainly, such a dual role for IRF-4 in regulating lymphocyte proliferation and IFN gene expression is compatible with the finding that IRF-4 is a cellular gene induced by HTLV-1 Tax. As retroviral replication is dependent on the division of target cells (46), Tax induction of IRF-4 would contribute in maintaining an activated cellular state, while aiding HTLV-1-infected cells to evade the anti-viral response by repressing IFN-regulated gene expression.

Although IRF-4 is a transcriptional target of Rel that regulates lymphocyte responsiveness to IFNs during cellular activation, the finding that mature B and T cells from young IRF-4−/− mice, like c-rel−/− lymphocytes, proliferate poorly when treated with diverse mitogens (14) indicates that IRF-4 is also important in controlling IFN-independent lymphocyte proliferation. Although these observations support a model in which c-rel−/− B cell proliferative defects are in part due to an inability to upregulate IRF-4, this appears to be at odds with the finding that lymphoproliferative disorders develop in older IRF-4−/− mice (14), but not c-rel−/− animals (7). The apparent paradox that an absence of IRF-4 can both reduce and increase lymphocyte proliferation supports an emerging idea that normal cellular proliferation requires the balanced expression of IRF transcriptional activators and repressors, and that altering this balance perturbs cell growth (18). Such a model would also account for the observation that dysregulated overexpression of IRF-4, resulting from a translocation of the gene into the Ig CH locus contributes to the development of multiple myeloma in humans (15). The finding that IRF-4 is a transcriptional target of Rel may also have important implications for v-Rel-mediated transformation. Because v-R-Rel lacks part of the Rel transactivation domain, the transcriptional activity of the viral oncprotein differs from that of Rel (47, 48). Consequently, a v-Rel-induced change in the normal pattern of IRF-4 expression could perturb cell growth and may help to explain the basis of lymphoid-specific transformation by v-R el.

Although it remains to be determined which IRF-4–regulated genes are involved in the control of lymphocyte proliferation, 2′,5′ OAS represents an interesting candidate. 2′,5′ OAS functions to convert ATP into 2′-5′ oligoadenylate, which in turn activates the latent endonuclease RNase L (49). In addition to the 2′,5′ OAS/RNase L pathway be-
ing a key mechanism for inhibiting viral replication (50),
activation of this enzyme is also likely to be important in
degrading cellular RNA and as a consequence modulating
normal cellular functions. Since overexpression of 2′-5′ OAS
can inhibit cellular proliferation (51), the enhanced expres-
sion of 2′-5′ OAS in mitogen-activated c-rel−/− lymphocytes
treated with IFN-α may account in part for the increased
sensitivity of these cells to the antiproliferative action of this
cytokine. Ultimately, the identification of direct targets of
Rel and genes further along the transcriptional cascade will
offer important insights into understanding how Rel regu-
lates cellular processes in response to a variety of extracellu-
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