Interferon regulatory factor-2 drives megakaryocytic differentiation

Emilia STELLACCI*[1], Ugo TESTA†[1], Eleonora PETRUCCI†, Eleonora BENEDETTI*, Roberto ORSATTI*, Tiziana FECCIA†, Marit STAFSNES†, Eliana M. COCCIA‡, Giovanna MARZIALI† and Angela BATTISTINI*[2]

*Laboratory of Virology, Istituto Superiore di Sanità, Viale Regina Elena, 299-00161 Rome, Italy, †Laboratory of Hematology and Oncology, Istituto Superiore di Sanità, Viale Regina Elena, 299-00161 Rome, Italy, and ‡Laboratory of Immunology, Istituto Superiore di Sanità, Viale Regina Elena, 299-00161 Rome, Italy

IRFs [IFN (interferon) regulatory factors] constitute a family of transcription factors involved in IFN signalling and in the development and differentiation of the immune system. IRF-2 has generally been described as an antagonist of IRF-1-mediated transcription of IFN and IFN-inducible genes; however, it has been recently identified as a transcriptional activator of some genes, such as those encoding histone H4, VCAM-1 (vascular cell adhesion molecule-1) and Fas ligand. Biologically, IRF-2 plays an important role in cell growth regulation and has been shown to be a potential oncogene. Studies in knock-out mice have also implicated IRF-2 in the differentiation and functionality of haematopoietic cells. Here we show that IRF-2 expression in a myeloid progenitor cell line leads to reprogramming of these cells towards the megakaryocytic lineage and enables them to respond to thrombopoietin, as assessed by cell morphology and expression of specific differentiation markers. Up-regulation of transcription factors involved in the development of the megakaryocytic lineage, such as GATA-1, GATA-2, FOG-1 (friend of GATA-1) and NF-E2 (nuclear factor-erythroid-2), and transcriptional stimulation of the thrombopoietin receptor were also demonstrated. Our results provide evidence for a key role for IRF-2 in the induction of a programme of megakaryocytic differentiation, and reveal a remarkable functional diversity of this transcription factor in the regulation of cellular responses.

Key words: gene expression, gene transcription, haematopoietic differentiation, interferon regulatory factor, transcription factor.

INTRODUCTION

The IRFs [IFN (interferon) regulatory factors] constitute a family of transcription factors that play a key role in gene regulation by IFNs and viral infections, and in several immunological and growth-related cellular functions, as well as in the development of the haematopoietic system [1,2]. Nine mammalian members of this family have been identified so far, based on a conserved DNA-binding domain located at the N-terminus. By binding to the common target DNA, called the ISREs (IFN-stimulated response elements), IRFs regulate the transcription of target genes. The less well conserved C-terminal region acts as a regulatory domain and classifies IRFs into three groups: activators (IRF-1, IRF-3, IRF-7, IRF-9), repressors (IRF-2, IRF-8), and those that are able to both activate and repress gene transcription depending on the target gene (IRF-2, IRF-4, IRF-5, IRF-8). IRF-2 has generally been described as a transcriptional repressor, competing with the transcriptional activator IRF-1 [3]. More recent observations, however, indicate that for some ISREs, IRF-2 may act as a functional agonist rather than an antagonist of IRF-1 [4]. In addition, IRF-2 acts as a transcriptional activator for a few genes, such as those encoding histone H4, VCAM-1 (vascular cell adhesion molecule-1), gp91phox and Fas ligand [5–8].

Biologically, IRF-2 has been shown to be a potential oncogene, since its overexpression causes anchorage-independent growth in NIH 3T3 cells and tumour formation in mice [9]. The physiological role of IRF-2 in lymphoid and haematopoietic development has been investigated through the phenotypic analysis of knock-out mice. IRF-2-deficient mice, similar to IRF-1$^{-/-}$ mice [10,11], carry defects in Th1 and natural killer cell development and function [4], but they also show a general bone marrow suppression of haematopoiesis and B lymphopoiesis [12]. In addition to the knock-out studies, ectopic expression studies have similarly highlighted a role of IRFs in haematopoietic differentiation. In this context, we showed previously that IRF-1 exerts a key role in the granulocytic differentiation of the murine myeloid progenitor cell line 32Dcl3 [13], whereas IRF-2 blocks granulocytic maturation induced by G-CSF (granulocytic colony-stimulating factor).

Although an involvement of IRF-2 in haematopoiesis has been clearly defined, the exact role of IRF-2 in specific lineage development has not yet been identified. Here we show that IRF-2 expression in the myeloid progenitor cell line 32Dcl3 results in the stimulation of megakaryocytic differentiation, regulating the expression and the activity of key master genes in Mk (megakaryocyte) development, such as Tal-1, GATA-2, GATA-1, FOG-1 (friend of GATA-1) and NF-E2 (nuclear factor-erythroid-2), and inducing transcription of the TPO-R [TPO (thrombopoietin) receptor].

MATERIALS AND METHODS

Cell culture and treatments

32Dcl3 cells were grown in Iscove's modified Dulbecco's medium supplemented with 15 % (v/v) heat-inactivated fetal bovine

Abbreviations used: AP-1, activator protein-1; EMSA, electrophoretic mobility shift assay; FOG, friend of GATA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G-CSF, granulocytic colony-stimulating factor; IFN, interferon; IL-3, interleukin-3; ISRE, interferon-stimulated response element; IRF, interferon regulatory factor; Mk, megakaryocyte; NF-E2, nuclear factor-erythroid-2; PE, phycoerythrin; PF4, platelet factor 4; RT-PCR, reverse transcription–PCR; SBE, STAT-binding element; STAT, signal transducers and activators of transcription; siRNA, small interfering RNA; TPO, thrombopoietin, TPO-R, thrombopoietin receptor; VCAM-1, vascular cell adhesion molecule-1.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed (e-mail battist@iss.it).

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serum and 2 ng/ml recombinant murine IL-3 (interleukin-3; Peprotech, Inc., Rocky Hill, NY, U.S.A.). For morphological analysis, cells were spread on glass slides, fixed and stained with May/Grunwald/Giemsa (Sigma, St. Louis, MO, U.S.A.). Slides were examined by light microscopy at a magnification of $\times 200$. Morphological differences were determined using standard criteria as described previously [14]. Where indicated, 32Dcl3 cells were grown in the presence of 10 ng/ml recombinant murine TPO (R&D Systems, Minneapolis, MN, U.S.A.). Friend leukaemia cells and K562 cells were grown in RPMI-1640 medium supplemented with 10 % (v/v) fetal bovine serum. In some experiments, K562 cells were incubated for 4 days in the presence of 1 mM sodium butyrate (Sigma). All cell lines were negative for Mycoplasma (Mycotest; Life Technologies, Paisley, Scotland, U.K.).

Human progenitor cell purification and differentiation

Adult peripheral blood was obtained from healthy donors after informed consent was obtained. Adult peripheral blood human progenitor cells were purified as described by Gabbianelli et al. [15]. Human progenitor cells were induced to specific megakaryocytic differentiation with saturating amounts of TPO (50 ng/ml; R&D Systems), as reported previously [16]. Human progenitor cells were induced to erythroid differentiation as described previously [17].

Flow cytometry analysis of membrane differentiation markers

Cells were stained with PE (phycoerythrin)- or FITC-conjugated antibodies against mouse CD41, CD61 and CD62P (Pharmingen, San Diego, CA, U.S.A.; all at a final concentration of 5 μg/ml) on ice for 30 min after blocking Fc receptors with a 100-fold excess (i.e. 500 μg/ml) of mouse IgG, washed twice in PBS without Ca^{2+} and Mg^{2+}, and analysed on a FACS SCAN flow cytometer (Becton Dickinson). PE- or FITC-conjugated rat IgG_1, IgG_{2a} or IgG_{2b} or hamster IgG_1 (isotype-matched negative controls) were used at a 1:20 dilution.

Immunofluorescence analysis of transcription factors

The expression of transcription factors was explored by indirect immunofluorescence. Briefly, cytospin spots of 32Dcl3 cells centrifuged for 3 min at 100 ***g*** were fixed for 15 min at room temperature in 2 % (v/v) paraformaldehyde, permeabilized for 5 min in 0.1 % Triton X-100 and then labelled for 30 min at room temperature with rabbit anti-Tal-1 [18] (1:200 dilution), anti-(mouse FOG-1) (sc-10754; Santa Cruz Biotechnology; 1:20 dilution), anti-(mouse GATA-1) (sc-266; Santa Cruz Biotechnology; 1:20 dilution), rabbit anti-(human/mouse GATA-2) (1:200 dilution; kindly provided by Dr S. H. Orkin, Dana-Faber Cancer Institute, Boston, MA, U.S.A.) or appropriate negative controls, and with appropriate secondary antibodies [i.e. TRITC (tetramethylrhodamine β-isothiocyanate)-labelled goat anti-rabbit IgG or goat anti-rat IgG; both from Dako Cytomation, Copenhagen, Denmark; diluted 1:40]. After extensive washing, the cells were mounted in PBS/glycerol containing anti-fading reagent (Molecular Probes, Eugene, OR, U.S.A.). Immunofluorescence analysis of cell slides was carried out using an inverted fluorescence microscope equipped for confocal microscopy (Olympus Flowview FV500).

RT-PCR (reverse transcription–PCR) analysis

Total RNA was prepared from human primary cells and normalized as described [19]. The normalized RNA was reverse transcribed according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD, U.S.A.). RT-PCR was normalized for β_2-microglobulin expression [20]. To evaluate the expression of the IRF-2 gene in these cells, an aliquot of the reverse-transcribed RNA (corresponding to 2 ng of RNA) was amplified within the linear range by 40 PCR cycles.

Total RNA was prepared from 32Dcl3 cells using the RNeasy Extraction kit from Qiagen (Valencia, CA, U.S.A.) and reverse transcribed according to the manufacturer's instructions (Gibco BRL). RT-PCR was normalized for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression. An aliquot of reverse-transcribed RNA (corresponding to 2 ng of RNA) was amplified within the linear range by 30 PCR cycles.

Primers used were: 5′-GCAACATTGAGAATCAAGAGATTGTCAC-3′ and 5′-CAGCTCTTGACGCGGGCCTGGGTG-3′ for IRF-2; 5′-CAGCGCAAGGTGCCGTTCACAGC-3′ and 5′-GGTGTAGGTCTGGAAGCGAGGG-3′ for TPO-R; 5′-AGTCCTGAGCTGCTGCTTCT-3′ and 5′-GGCAAATTTTCCTCCCATTC-3′ for PF4 (platelet factor 4); 5′-TGGATTTTCCTGGTCTAGGG-3′ and 5′-AGTAGGCCAGTGCTGATGCT-3′ for GATA-1; 5′-ACGCGGACATGTACCCAGTGG-3′ and 5′-GCCACCTTGTTCTTCCCCGT-3′ for NF-E2; 5′-TGCTATATGTGCGCCTTGTC-3′ and 5′-TTGATGACTGCGGTAGCAAG-3′ for FOG-1; and 5′-AGGCATCTGAGGGCCCACTG-3′ and 5′-CTCTTGCTCAGTGTCCTTGC-3′ for GAPDH. Internal probes were TCCAGGTCACCATCAAAGAGGAGAGC for IRF-2, GCTCAAGAGACCTGCTACCAGCTCCGG for TPO-R, CGAAGAAAGCGATGGAGATC for PF4, CATCTTCTTCCACTTCCCCA for GATA-1, TGGCCATGAAGATTCCTTTC for NF-E2, ACCCTGTGCAGGAACCAGTA for FOG-1 and GACAATGAATACGGCTACAGCAAC for GAPDH.

PCR conditions were as follows: 94 °C/30 s, 58 °C/30 s, 72 °C/45 s for IRF-2; 94 °C/30 s, 61 °C/30 s, 72 °C/30 s for TPO-R; 94 °C/30 s, 53 °C/45 s, 72 °C/45 s for both PF4 and GATA-1; 94 °C/30 s, 59 °C/45 s, 72 °C/60 s for NF-E2; 94 °C/30 s, 52 °C/45 s, 72 °C/60 s for FOG-1; 94 °C/30 s, 60 °C/30 s, 72 °C/30 s for GAPDH.

Transfection experiments and enzymic assays

32Dcl3 cells were transfected by electroporation as described in [21]. The CMV-IRF-2 construct has been described previously [13], and the CMV-ΔIRF-2 construct was obtained from the CMVbl-IRF-2 (160 amino acids) construct [22], digested with *Xba*I and cloned in the same site of RcCMV (Invitrogen Corp. Life Technologies). After transfection, cells were selected for 2 weeks with 1 mg/ml G-418 geneticin sulphate (Gibco BRL), and bulk populations were selected to avoid clonal variability. Bulk populations were frozen after identification and aliquots were thawed periodically (every 6–8 weeks).

Transient transfection experiments were performed with a construct encoding a portion of the TPO-R promoter upstream of the luciferase reporter gene. The sequence between nucleotides − 595 and − 1 was amplified by PCR from the full-length TPO-R cDNA (a gift from W. Alexander, Walter and Eliza Hall Institute, Royal Melbourne Hospital, Melbourne, Victoria, Australia) with a sense primer (5′-GGCCGGTACCGTCACTCCAGGAGCGTGGC-3′) that introduced a unique *Kpn*I site and an antisense primer (5′-GGCCGAGCTCCTTCTCCGGCACTGTGTGCC-3′) that introduced a unique SacI site. After digestion with *Kpn*I and *Sac*I, the PCR product was purified on an agarose gel and ligated into

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the pGL3Basic vector (Promega Corp., Madison, WI, U.S.A.) cut previously with the same enzymes.

The plasmid carrying the mutated IRF site was prepared using the QuickChange site-directed mutagenesis kit (Stratagene). The sequence of the primer used to introduce the specific mutation was 5′-CCAACAGAAGGCTCATGGCTTCTCCCCCGAATTCCTCTCACCC-3′ (nucleotides −231 to −188). The nucleotide sequences of both the wild-type and mutated plasmids were confirmed by a dideoxy DNA sequencing procedure using the Sequenase kit (Amersham Pharmacia Biotech).

The Tk-Renilla plasmid was co-transfected and used as a control for transfection efficiency. Reagents from Promega were used to assay extracts for luciferase activity in a Lumat LB9501 luminometer (E&G Berthold, Bad Wildbad, Germany).

Transfection of siRNA (small interfering RNA)

The sequence of the sense strand of the siRNA oligonucleotide was 5′-UCCGCCAGACAUUUGCCAA-3′ for the wild type and 5′-UGGGGGAGACAUUUGCCAA-3′ for the mutant (mutated bases underlined). These RNA oligonucleotides and the corresponding antisense RNAs were synthesized at Darmarcon Research Inc. (Lafayette, CO, U.S.A.). An overhang of two thymidine residues (dTdT) was included at the 3′ end of all RNA oligonucleotides. Double-stranded siRNA molecules were dissolved in sterile water as 20 μM solutions, divided into aliquots and stored at −20 °C. The siRNAs were transfected to a final concentration of 25 or 50 nM using Oligofectamine Reagent (Invitrogen Corp., Life Technologies) according to the manufacturer's recommendations. At the indicated times, cells were split and analysed by Western blot or treated with TPO, to evaluate their response to this cytokine in terms of cell proliferation and differentiation.

Western blot assay

Portions of 20–50 μg of nuclear cell extracts were separated on SDS/10 %-PAGE. Blots were incubated with polyclonal anti-IRF-2 antibody (sc-498; Santa Cruz Biotechnology Inc.; 1:200 dilution) or with a polyclonal anti-IRF-2 antibody (1:1000 dilution) directed toward the N-terminus of the protein [22] [a gift from Dr J. Hiscott (Lady Davis Institute for Medial Research, Jewish General Hospital, Montreal, Quebec, Canada)], and then with anti-rabbit horseradish peroxidase-coupled secondary antibody (Amersham Pharmacia Biotech; 1:2000 dilution) using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

DNA EMSA (electrophoretic mobility shift assay)

EMSA experiments were performed as described previously [21] on nuclear cell extracts [23]. Where indicated, 1 μg of anti-Jun, anti-c-Fos or anti-NF-E2 antibodies (sc-44X, sc-447X and sc291X respectively; Santa Cruz Biotechnology) was added to 20 μg of cell extract in the reaction mixture.

The oligonucleotides used as probes or competitors were the following: GGCAGTGCCTTATCTCTGCGGCG for GATA, GGCAGTGCCACCTCTCTCCGGCG for GATAm, TGGGGAACCTGTGCTGAGTCACTGGAG for NF-E2, TGGGGAACCTGTGCTTAGTCACTGGAG for NF-E2m1, and TGGGGAACCTGTGCTGAGTAACTGGAG for NF-E2m2.

DNA affinity purification assay

DNA affinity purification assays were performed as described previously [24]. Biotinylated oligonucleotides corresponding to the TPO-IRF-E probe [5′-CATGGCTTCTCTTTCGAATT-3′ (nucleotides −218 to −198) and 5′-TGAGCCTTTCCTCTTTCTGGG-3′ (nucleotides −438 to −418)] or two different mutant oligonucleotides (5′-CATGGCTTCTCCCCCGAATT-3′ and 5′-CATGGCTACTCTTTCGAATT-3′) or the SBE [STAT (signal transducers and activators of transcription)-binding element] sequence present on the β-casein gene promoter (5′-GATTTCTAGGAATTCAATCC-3′) were mixed with 200 μg of nuclear extract, and the complexes formed were pulled down with magnetic beads (Streptavidin MagneSphere Paramagnetic Particles; Promega). Bound material eluted by boiling in sample buffer was analysed by Western blot with antibody against IRF-2 or against tyrosine-phosphorylated STAT-5 (p-STAT-5; UBI, Lake Placid, NY, U.S.A.; 1:500 dilution).

Immunoprecipitation and immunoblot analysis

Whole-cell extracts (300 μg), prepared as described in [21], were incubated overnight with 1 μg of polyclonal anti-GATA-1 antibodies (Geneka Biotechnology Inc., Montreal, Quebec, Canada; 1:1000 dilution) or 1 μg of monoclonal anti-GATA-2 antibodies (sc-267; Santa Cruz Biotechnology Inc.; 1:200 dilution). Cell extracts were then incubated with Ultralink immobilized protein A/G Sepharose (Pierce Biotechnology, Rockford, IL, U.S.A.) for 2 h at room temperature. After extensive washing, immunoprecipitates were eluted by boiling the beads for 5 min in 1×SDS sample buffer. Eluted proteins were separated by SDS/PAGE and subjected to Western blot with goat polyclonal anti-FOG-1 antibodies (sc-9362; Santa Cruz Biotechnologies Inc.; 1:200 dilution).

RESULTS

IRF-2-expressing 32Dcl3 cells undergo megakaryocytic differentiation

To investigate whether IRF-2 could exert a specific role in myeloid differentiation, 32Dcl3 cells constitutively expressing IRF-2 or a truncated version of the protein containing only the DNA-binding domain (32D/ΔIRF-2) were generated. The expression of the transgene was assessed by Western blot with an antibody directed towards the N-terminal domain of the protein. Bulk populations of transfected cells were chosen to avoid clonal variability. No IRF-2 expression was detectable in parental cells (results not shown) or in cells transfected with the empty vector (32D/RcCMV), whereas clear expression of the protein was present in IRF-2-expressing cells (32D/IRF-2) (Figure 1, upper panel). As expected, an IRF-2 protein of a lower molecular mass was detected in 32D/ΔIRF-2 cells.

The morphology of 32D/RcCMV, 32D/IRF-2 and 32D/ΔIRF-2 cells grown under standard conditions is illustrated in Figure 1 (lower panels). 32D/RcCMV cells maintained in IL-3 were composed of a homogeneous blast cell population with < 5 % myeloperoxidase-positive cells. In contrast, IRF-2-expressing cells showed the appearance of cells exhibiting bilobated nuclei (approx. 20 %), a finding consistent with the possible Mk nature of these cells. The morphological features of 32D/ΔIRF-2 cells closely resembled those of 32D/RcCMV cells, suggesting that the stimulatory effect of IRF-2 on Mk differentiation may be mediated by the regulatory domain of the protein.

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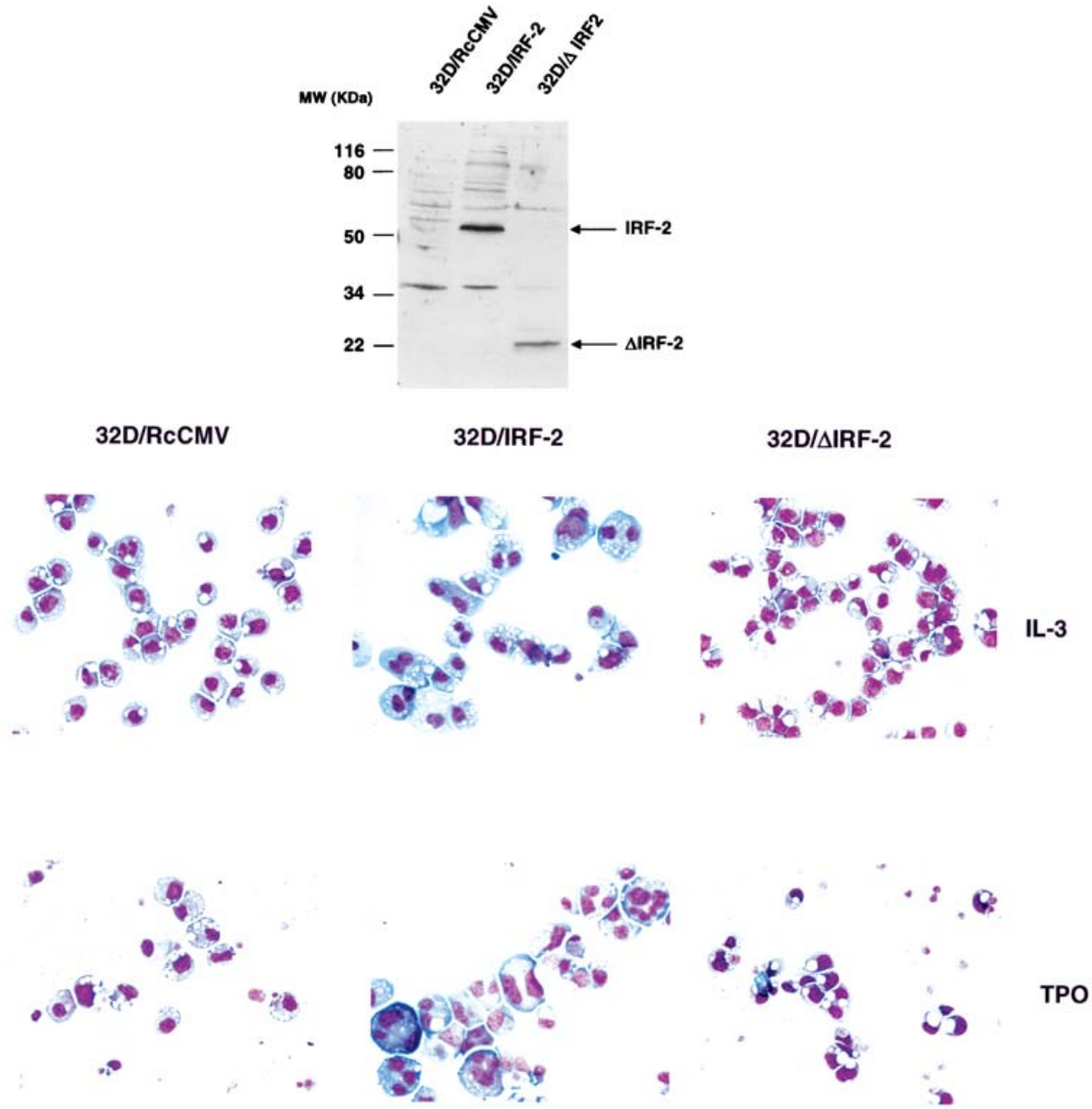

Figure 1 IRF-2 expression in 32Dcl3 cells correlates with a differentiated phenotype

Upper panel: constitutive expression of the IRF-2 and ΔIRF-2 transgenes was assessed by Western blot with whole-cell extracts (50 μg) prepared from 32Dcl3 cells transfected with the empty vector (32D/RcCMV), the IRF-2-expressing vector (32D/IRF-2) or the ΔIRF-2-expressing vector (32D/ΔIRF-2). Lower panels: May/Grünwald/Giemsa staining of 32D/RcCMV, 32D/IRF-2 and 32D/ΔIRF-2 cells grown in IL-3 or TPO for 4 days. Representative photomicrographs (original magnification × 200) are shown.

When 32D/IRF-2 cells were grown in the presence of TPO for 4 days, a clear increase (48.5% compared with 18.5%) in the number of cells exhibiting megakaryocytic morphology was observed (Figure 1, lower panels), while 32D/RcCMV and 32D/ΔIRF-2 cells grown under the same conditions were, in large part, dead or apoptotic.

Overexpression of IRF-2 in 32Dcl3 cells induces expression of megakaryocytic lineage-specific markers

The expression of Mk-specific membrane markers was then evaluated. The platelet glycoprotein IIB (CD41), a megakaryocytic antigen expressed early during differentiation, was expressed at low levels in 32D/RcCMV (Figure 2A) and 32D/ΔIRF-2 (results not shown) cells, whereas it was clearly detected in the majority of 32D/IRF-2 cells. Its expression was further and substantially increased after TPO treatment (Figure 2A). A similar pattern was observed for the CD61 antigen, with the only exception being that it was expressed in 32D/RcCMV cells at higher levels than the CD41 antigen. Similarly, the CD62P antigen, another Mk-specific membrane marker, was undetectable in 32D/RcCMV cells and in 32D/ΔIRF-2 cells (results not shown), but was expressed at significant levels in 32D/IRF-2 cells (Figure 2A). Double-labelling experiments demonstrated that the highest levels of CD41 antigen were correlated with the highest levels of CD61 antigen (Figure 2B).

To exclude any effect related to the procedure of gene transfer or selection, the specificity of the observed effect of IRF-2 on

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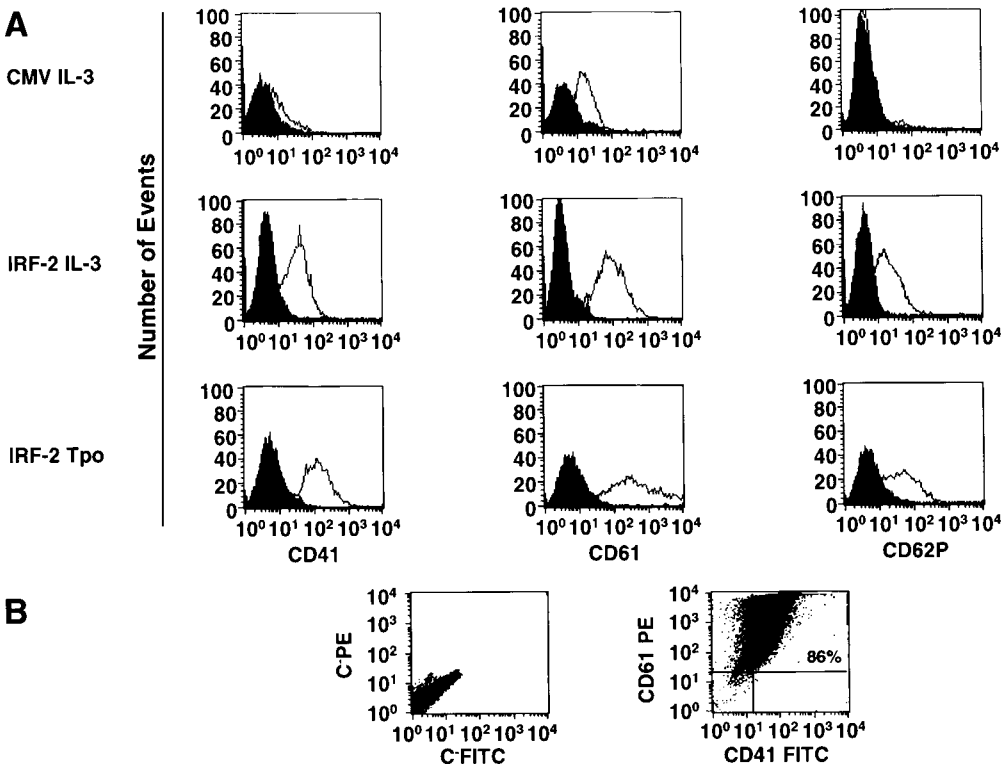

Figure 2 Flow cytometric analysis of 32D/RcCMV and 32D/IRF-2 cells grown for 4 days in the presence of either IL-3 or TPO

(**A**) Representative flow cytometric scans of single staining of 32D/RcCMV cells or 32D/IRF-2 cells grown for 7 days in the presence of IL-3 or TPO and incubated with anti-CD41, -CD61 or -CD62P antibodies. For each antibody, the fluorescence of cells labelled with an isotype-matched negative control is shown in black, while the fluorescence of the cells labelled with the specific monoclonal antibody is shown in white. (**B**) Double staining of 32D/IRF-2 cells grown in the presence of TPO with irrelevant rat immunoglobulins (negative control; left panel) or with FITC-labelled anti-CD41 and PE-labelled anti-CD61 (right panel).

megakaryocytic differentiation was demonstrated by using siRNA oligonucleotides. The results reported in Figure 3 show that the inhibition of IRF-2 expression in 32D/IRF-2 cells by siRNA, as assessed by Western blot (Figure 3A), resulted in an inhibition of Mk differentiation, as demonstrated by the decrease in CD61 and CD41 expression (Figure 3B). Similarly, the response to the growth- and survival-promoting activity of TPO induced by IRF-2 (see below) was abrogated in siRNA-treated cells (Figure 3C). These effects were not observed when 32D/IRF-2 cells were incubated with a mutated siRNA not affecting IRF-2 expression (Figures 3A–3C).

Mechanisms underlying the stimulation by IRF-2 of megakaryocytic differentiation

Effects of IRF-2 on cell growth and apoptosis

Alterations in growth rates and/or apoptosis generally occur during cell differentiation. Growth kinetic curves and percentages of apoptotic cells were measured in 32D/RcCMV, 32D/IRF-2 and 32D/ΔIRF-2 cells grown either in the absence or in the presence of TPO. 32D/RcCMV and 32D/ΔIRF-2 cells maintained in the presence of IL-3 grew exponentially, whereas they stopped growing in the presence of TPO or in the absence of growth factors (Figure 4A). In contrast, 32D/IRF-2 cells were able to grow in the presence of TPO, whereas in the presence of IL-3 they grew at a significantly lower rate (10-fold) than 32D/RcCMV cells.

The study of apoptosis showed that, after 4 days of TPO treatment, $\sim 60\,\%$ of parental and 32D/ΔIRF-2 cells had died from apoptosis, whereas only a negligible percentage of apoptotic cells was present in the 32D/IRF-2 cell population. In the absence of growth factors, all cell types died by apoptosis (Figure 4B).

These results indicate that IRF-2 enables 32Dcl3 cells to respond to the proliferative and anti-apoptotic effects of TPO.

IRF-2 induces the expression and activity of Mk-specific transcription factors

Several transcription factors have been identified so far as being key in megakaryocytic development, including Tal-1, GATA-2, GATA-1, FOG-1 and NF-E2 [25]. Indirect immunofluorescence studies showed that FOG-1 and GATA-1 were not expressed in 32D/RcCMV or 32D/ΔIRF-2 cells either before (Figure 5, upper panels) or after (results not shown) TPO treatment, whereas they were clearly and substantially induced in 32D/IRF-2 cells (i.e. $\geqslant 98\,\%$ of the cells were positive), with potentiation of their expression after TPO treatment (Figure 5, upper panels). The early-acting transcription factors GATA-2 and Tal-1 were expressed at very low levels in 32D/RcCMV and 32D/ΔIRF-2 cells, but their expression was markedly higher in 32D/IRF-2 cells, either in the presence of IL-3 or after TPO treatment (Figure 5, upper panels).

The expression of GATA-1, NF-E2, FOG-1 and PF4, a differentiation marker specific for the megakaryocytic lineage, was also measured at the mRNA level by semi-quantitative RT-PCR (Figure 5, lower panel). Consistent with the cytochemical data, transcripts for all of these proteins were significantly

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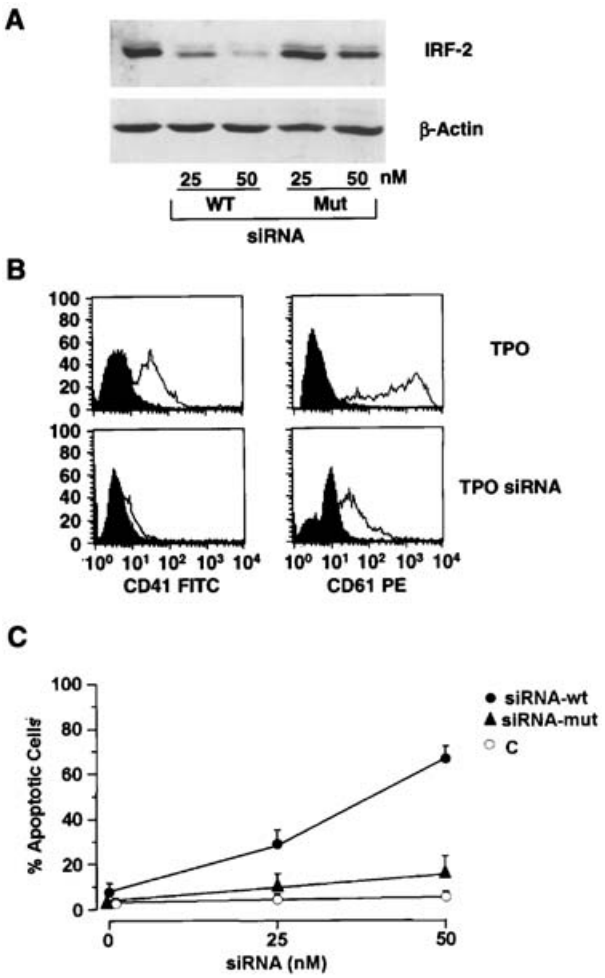

Figure 3 Inhibition of IRF-2 expression in 32D/IRF-2 cells by siRNA

(**A**) Western blot analysis of IRF-2 expression in control cells or cells treated with the indicated amounts of wild-type (WT) or mutated (Mut) siRNA. (**B**) Analysis of cell membrane markers CD41 and CD61 in 32D/IRF-2 cells untreated or treated with siRNA (25 nM) as indicated, and grown in the presence of TPO for 3 days. (**C**) Percentage of apoptotic cells observed in 32D/IRF-2 cells grown without siRNA (○), with wild-type siRNA (wt; ●) or with mutated siRNA (mut; ▲) at two different concentrations (25 and 50 nM) in the presence of TPO for 3 days.

up-regulated or induced only in IRF-2-expressing cells, but not in 32D/ΔIRF-2 or 32D/RcCMV cells. As expected, TPO treatment of 32D/IRF-2 cells further stimulated the expression of all of these genes.

The DNA-binding activities of GATA-1 and NF-E2 were then evaluated by EMSA, using nuclear extracts from 32D/RcCMV and 32D/IRF-2 cells incubated with a radiolabelled GATA probe (Figures 6A and 6B). As reported previously [26] and consistent with data shown in Figure 5, 32Dcl3 cells lack GATA-1 expression and, accordingly, no GATA-1 DNA-binding activity was detected in control and empty vector-transfected cells (Figure 6A, lane 2). Conversely, a clear stimulation of GATA-1 binding activity (lane 3) was present in IRF-2-expressing cells, confirming that the GATA-1 protein induced in 32D/IRF-2 cells is active in binding DNA. Competition experiments with a 200-fold molar excess of self (lane 4) or mutated (lane 5) oligonucleotides demonstrated the specificity of the GATA-1 complex. Accordingly, the labelled mutated oligonucleotide did not induce any shift with the same extracts (lane 6). Cell extracts from Friend leukaemia cells (lane 1) served as positive control of GATA-1 binding activity.

EMSA analysis performed with nuclear extracts prepared from 32D/RcCMV and 32D/IRF-2 cells and NF-E2-binding sites from the human porphobilinogen deaminase gene (Figure 6B) again readily revealed DNA binding of the NF-E2 heterodimer and of AP-1 (activator protein-1) only in IRF-2-expressing cells. The specificity of the complexes was demonstrated by supershift with specific antibodies (Figure 6B, lanes 6–8). Interestingly, the pattern of competition with oligonucleotides that distinguish the partially overlapping NF-E2 and AP-1 site (m2; lane 9) or specific for the AP-1 core sequence (m1; lane 10) gave a binding pattern similar to that obtained with extracts from primary Mks [27,28]. These results indicate that, in IRF-2-expressing 32Dcl3 cells, the same complexes present during the differentiation of primary Mks are stimulated.

FOG-1 co-immunoprecipitates with GATA-1 and GATA-2 in 32D/IRF-2 cells

To assess the presence, in IRF-2-expressing cells, of a protein complex containing FOG-1 and GATA factors, a prerequisite for the biological activity of FOG-1 in megakaryopoiesis [29], co-immunoprecipitation experiments were performed. 32D/RcCMV, 32D/IRF-2 and 32D/ΔIRF-2 cell extracts were immunoprecipitated with anti-GATA-2 or anti-GATA-1 antibodies, followed by immunoblot with anti-FOG-1 antibody. As shown in Figure 6(C), the FOG-1 protein was readily detected in both the anti-GATA-2 and anti-GATA-1 immunocomplexes obtained only from cells expressing IRF-2. This result indicates that, in 32D/IRF-2 cells, physical interactions do indeed occur between FOG-1 and GATA-2/GATA-1. As expected from the data in Figure 5, FOG-1 was not expressed in cells transfected with the empty vector or with the truncated version of IRF-2, as demonstrated by Western blot analysis of the whole-cell extracts.

IRF-2 binds and stimulates TPO-R promoter activity

Based on the responsiveness of 32D/IRF-2 cells to TPO treatment (see Figures 1, 2 and 4), we investigated whether the expression of TPO-R can be modulated by IRF-2. Semi-quantitative RT-PCR analysis of TPO-R expression showed that both TPO-R transcripts, which arise by differential splicing, were expressed at low levels in parental cells, but they were clearly up-regulated in IRF-2-expressing cells (Figure 7A). Interestingly, cells expressing the truncated version of IRF-2 showed marked down-modulation of TPO-R expression. TPO-R harbours a functional GATA-1-binding site on its promoter. To assess whether the effect of IRF-2 was mediated by the increased GATA-1 activity (Figures 5 and 6) or by a direct transcriptional effect of IRF-2, we searched for IRF-binding sites within the TPO-R promoter. By computer-assisted analysis (TPO-R; accession no. S76841), two putative IRF-binding sites (IRF-E) were identified at nucleotides −218 to −198 (5′-CATGGCTTCTCTTTCGAATT-3′) and nucleotides −438 to −418 (5′-TGAGCCTTTCGTCTTTCTGGG-3′) [IRF-2 consensus sequence G(A)AAA(G/C)(T/C)GAAA(G/C)(T/C)]. Specific binding of IRFs to these sites was then determined by DNA affinity purification assays with nuclear extracts from both 32D/RcCMV and 32D/IRF-2 cells and biotinylated TPO-IRF-E probes. The composition of the isolated complexes was then determined by immunoblotting against IRF-2. As shown in Figure 7(B), no proteins from the empty vector-transfected cell extracts were retained by the −218 to −198 probe, whereas IRF-2 was clearly detected when extracts from 32D/IRF-2 cells

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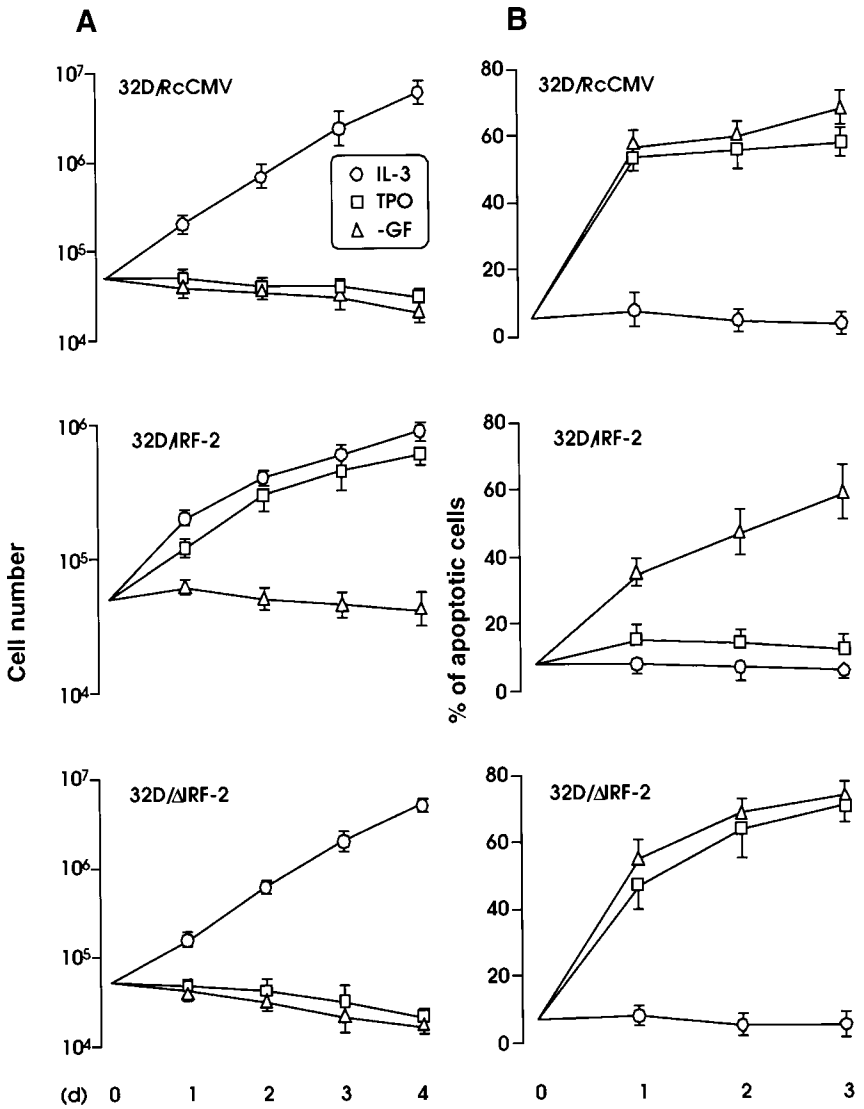

Figure 4 IRF-2 expression affects the growth rate and degree of apoptosis of 32Dcl3 cells and confers sensitivity to the proliferative effects of TPO

(**A**) Growth curves of 32D/RcCMV, 32D/IRF-2 and 32D/ΔIRF-2 cells cultured in the absence of growth factor (GFs; △) or in the presence of either IL-3 (○) or TPO (□). The values are the means for separate cultures from three independent experiments. (**B**) Percentage of apoptotic cells observed in cells grown as in (**A**). Apoptotic cells were determined by annexin-FITC staining, and results are expressed as means ± S.D. observed in three separate experiments.

were used. IRF-2 binding was highly specific, since two mutated oligonucleotides (TPO-IRF-E m1 and m2), or an unrelated oligonucleotide corresponding to the SBE present on the β-casein promoter, did not retain any protein from the same cell extracts. Nevertheless, the SBE oligonucleotide was able to retain the STAT-5 activity present in cell extracts of 32D parental cells pulsed with IL-3 for 15 min. When the TPO-IRF-E −438 to −418 probe was used in similar experiments, comparable results were obtained, but the affinity of IRF-2 for this sequence seemed lower (results not shown).

The biological activity of IRF-2 at the TPO-R promoter was then assessed after transient transfection of a TPO-R promoter–luciferase reporter gene spanning nucleotides −595 to −1. A 6–8-fold induction of luciferase activity (depending on the experiment) was observed in 32D/IRF-2 cells as compared with the activity in cells transfected with the empty vector (32D/RcCMV) (Figure 7C). Consistent with the DNA affinity assays, promoter stimulation was greatly reduced (~80% decrease) when a mutation that impairs IRF-2 binding was introduced in the construct (m1 mutant).

Altogether, these results indicate that IRF-2 specifically binds to and positively transactivates the TPO-R.

IRF-2 is stimulated during normal megakaryocytic differentiation

IRF-2 expression in normal Mks was evaluated by semi-quantitative RT-PCR (Figure 8) in purified human $CD34^+$ progenitor cells induced to differentiate along the megakaryocytic pathway using a cell culture system allowing progressive and selective differentiation along the Mk lineage [16,30]. Purified progenitors, freshly isolated, apparently lacked IRF-2 expression;

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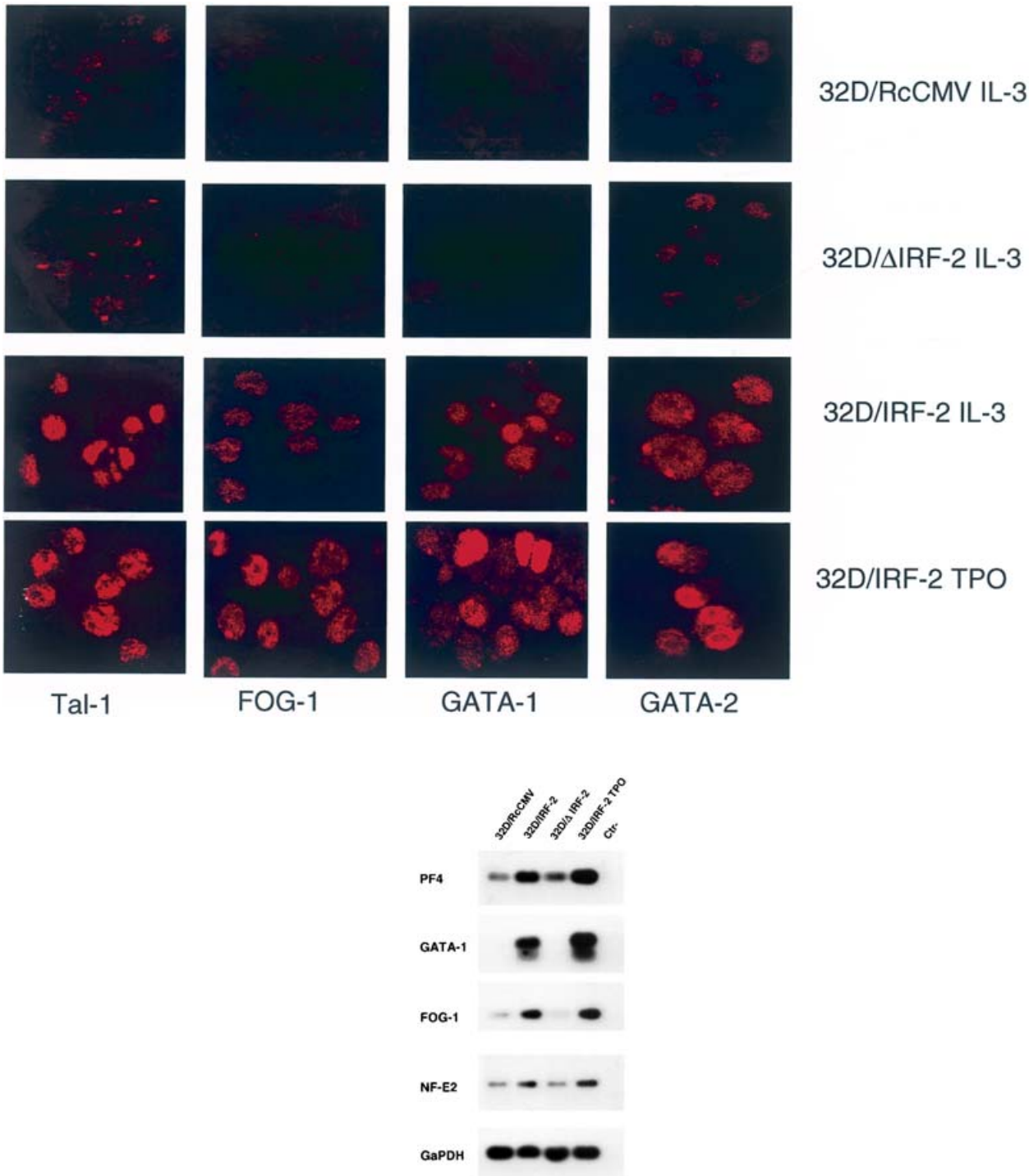

Figure 5 IRF-2 induces expression of Mk-specific transcription factors

Upper panels: IRF-2 induces Tal-1, FOG-1, GATA-1 and GATA-2 expression. Tal-1, FOG-1, GATA-1 and GATA-2 expression in cells transfected with the indicated vectors and grown in the presence of IL-3 (32D/RcCMV, 32D/ΔIRF-2 and 32D/IRF-2 cells) or TPO (32D/IRF-2 cells) for 4 days was assessed by indirect immunofluorescence analysed by confocal microscopy. Negative controls were performed using isotype-matched immunoglobulins and showed no labelling. One representative experiment, out of three performed, is shown. Lower panel: IRF-2 induces PF4, GATA-1, FOG-1 and NF-E2 mRNA. RT-PCR analysis of PF4, GATA-1, FOG-1 and NF-E2 expression in 32Dcl3 cells transfected with the indicated vectors is shown. A representative experiment of three performed is shown. GAPDH was used for the normalization of the quantity of cDNA used for each sample. A negative control (ctr-) of the RT-PCR is included.

however, in the presence of TPO, beginning at day 3 of culture and peaking at day 6, IRF-2 expression was clearly detectable and remained constantly present throughout the differentiation process, including in fully differentiated cells (day 13 of culture). T-lymphocytes were used as a positive control for IRF-2-expressing cells (Figure 8A).

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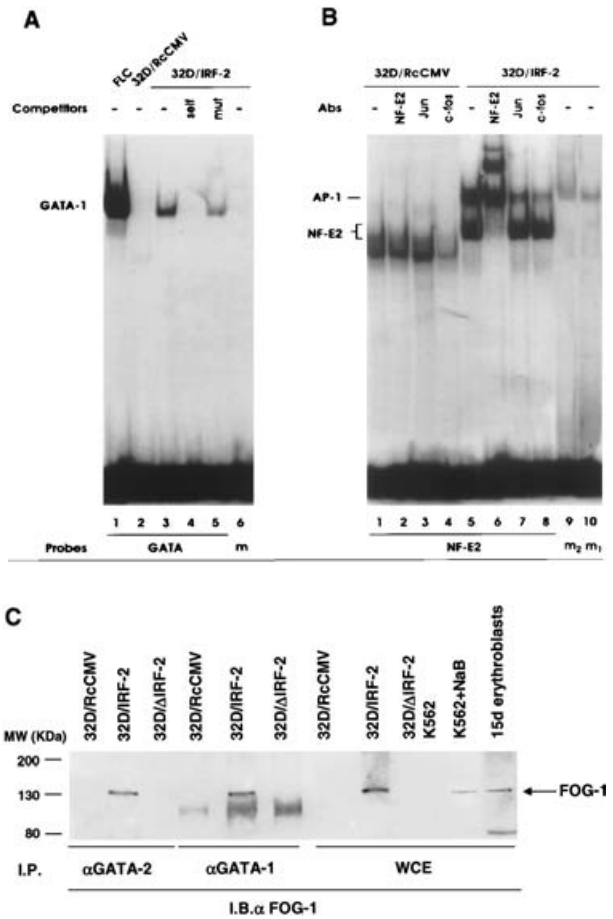

Figure 6 IRF-2 induces activity of Mk-specific transcription factors

(**A**, **B**) GATA-1 and NF-E2 binding activity is stimulated in IRF-2-expressing cells. Nuclear extracts (20 μg) from 32D/RcCMV and 32D/IRF-2 cells were incubated with labelled oligonucleotides corresponding to GATA and NF-E2 consensus or mutated (m) sites (sequences are given in the Materials and methods section). Nuclear extracts from Friend leukaemia cells (FLC) served as positive control of GATA-1 binding activity. Competition experiments were performed with a 200-fold molar excess of the indicated unlabelled oligonucleotides. Supershift assays were performed in the presence of specific anti-NF-E2, anti-Jun or anti-c-Fos antibodies (Abs), as indicated. (**C**) FOG-1 associates intracellularly with GATA-1 and GATA-2 in IRF-2-expressing cells. Whole-cell extracts (300 μg) from 32D/RcCMV, 32D/IRF-2 and 32D/ΔIRF-2 cells were immunoprecipitated with anti-GATA-2 or anti-GATA-1 antibodies. Immunoprecipitates were analysed by PAGE and subsequently probed with anti-FOG-1 antibody. Whole-cell extracts (WCE; 10 μg) were separated by PAGE and probed with anti-FOG-1 antibody as described in the Materials and methods section. K562 cells served as negative control, and sodium butyrate (NaB)-induced K562 cells and human primary erythroblasts as positive controls, of FOG-1-expressing cells.

The expression of the Mk markers CD41 and CD61 in human progenitor cells grown under conditions allowing the selective proliferation and differentiation of Mk cells [16], expressed as positive cells at different time points, is shown in Figure 8(B).

DISCUSSION

The differentiation of haematopoietic stem cells is characterized by the progressive restriction of differentiation potential and the acquisition of a mature phenotype through transcription factor-induced activation and repression of specific programmes of gene expression [31,32]. Restricted expression of lineage-specific genes is achieved through the action of both lineage-restricted and widely expressed transcriptional regulators. Accordingly, deregulated activity of specific transcription factors is associated with several haematopoietic disorders and leukaemia [31].

In the present study, we have characterized IRF-2 as a transcription factor that plays an important role in megakaryocytic differentiation. Several lines of evidence support this conclusion: (i) the ectopic expression of IRF-2 leads to a shift in the differentiation programme of 32Dcl3 cells from the granulocytic to the megakaryocytic lineage; (ii) the expression of Mk-specific genes and of master transcription factors, such as Tal-1, GATA-2, FOG-1, GATA-1 and NF-E2, is induced by IRF-2; (iii) IRF-2 directly transactivates the TPO-R, and enables 32Dcl3 cells to respond to TPO; (iv) inhibition of IRF-2 expression by specific siRNA inhibits both responsiveness to TPO and Mk differentiation; (v) a truncated form of IRF-2 lacking the regulatory domain of the protein impairs the differentiation process; and (vi) IRF-2 is induced early during the megakaryocytic differentiation of normal human progenitors, and expression occurs throughout the differentiation process.

32Dcl3 cells are a myeloid progenitor cell line that can be induced to granulocytic differentiation by G-CSF. We have shown previously that IRF-1 exerts a pivotal role in granulocytic differentiation, and that its induction by G-CSF represents a limiting step in the early events of differentiation. Interestingly, IRF-2 expression in these cells induces a switch from the granulocytic to the megakaryocytic lineage. This switch is characterized by induction of or an increase in the expression of megakaryocytic membrane markers, such as CD41, CD61 and CD62P (Figure 2A), associated with a marked decline ($\sim$80% of control cells not treated with TPO) in CD11b and CD18 expression (results not shown).The finding that 32D/IRF-2 cells continue to express low levels of CD11b and CD18 antigens probably reflects the intrinsic limitation of the cell line model as compared with normal progenitors. It has to be noted that 32Dcl3 cells express low, but significant, levels of CD41 and CD61, a finding in line with recent studies in mice showing that both antigens are expressed at low levels in the haematopoietic progenitor cell compartment [33].We cannot, therefore, exclude the possibility that the stimulatory effect of IRF-2 on Mk differentiation of 32D cells could be also related to the selective survival of cells that are already committed to the Mk lineage.

A series of transcription factors orchestrate Mk differentiation, acting in a co-ordinated manner and according to the differentiation-related stage from the early committed progenitors to the end stages of megakaryocytic maturation [25]. In this context, Tal-1 is strictly required for the correct differentiation of megakaryocytic progenitors [33] and acts on megakaryocytopoiesis at an earlier stage than GATA-1 and NF-E2 [32]. GATA-1 is also required for megakaryocytopoiesis, as indicated by the observation that knock-out mice exhibit severe anaemia [34] and a defect in megakaryocytic maturation [35,36]. NF-E2-deficient mice, on the other hand, totally lack normal circulating platelets [37]. More recently, FOG-1, a protein that interacts physically with GATA-1, has also been implicated in the control of megakaryocytopoiesis. FOG-1$^{-/-}$ mice exhibit a complete impairment of Mk production [38]. Here we show that, in 32Dcl3 cells, IRF-2 induces the expression of all of these transcription factors. In particular, the induction of Tal-1 and GATA-2, which act early in haematopoietic differentiation, suggests that IRF-2 could play a role in the initial steps of haematopoietic differentiation. This conclusion is supported by the multiple defects observed at the level of several haematopoietic lineages in IRF-2 knock-out mice [12]. Late-acting transcription factors, such as NF-E2, GATA-1 and FOG-1, are also induced in cells expressing IRF-2. It remains to be established whether these transcription factors are induced directly by IRF-2 through a transcriptional

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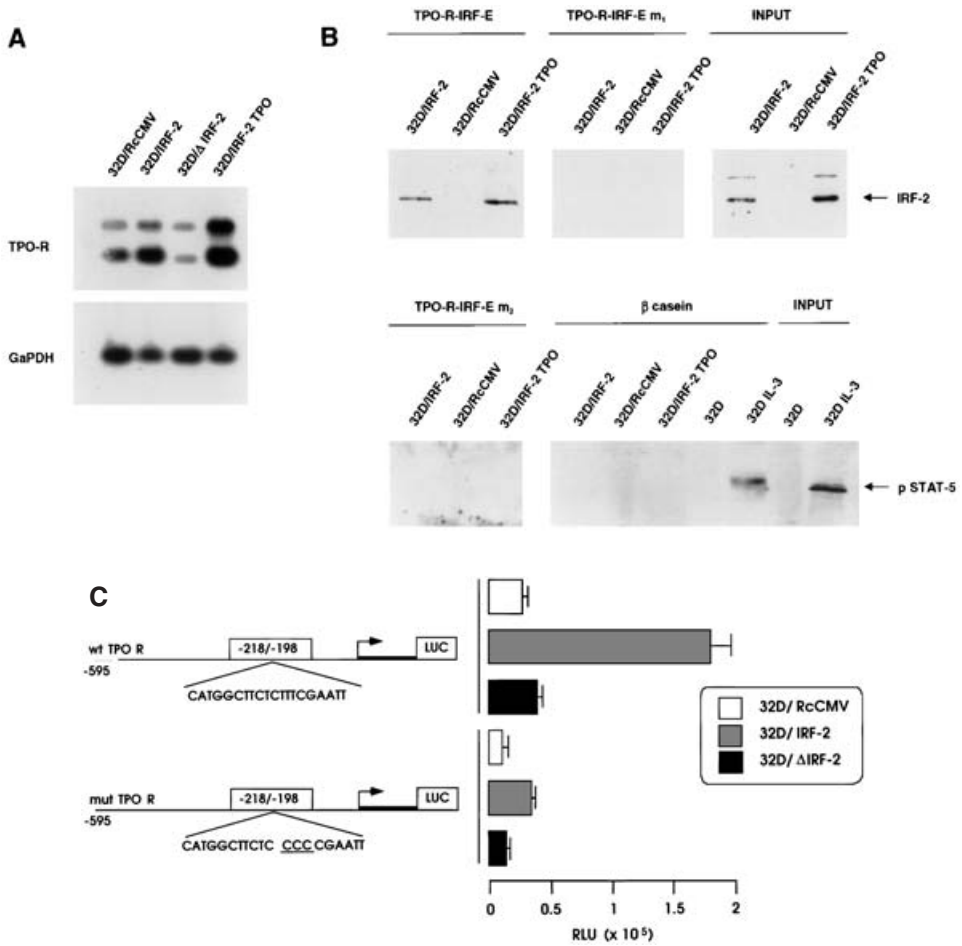

Figure 7 IRF-2 binds to the TPO-R promoter and stimulates transcription

(**A**) TPO-R mRNA expression was evaluated by semi-quantitative RT-PCR in 32D/RcCMV, 32D/IRF-2 and 32D/ΔIRF-2 cells grown in IL-3 or in TPO for 4 days, as indicated. A representative experiment, out of three performed, is shown. GAPDH was used for normalization. (**B**) DNA pull-down assays. Biotinylated oligodeoxynucleotides containing the wild-type or mutated (m) versions of the IRF-E site within the TPO-R gene promoter or the SBE sequence present within the β-casein gene promoter (sequences given in the Materials and methods section) were incubated with nuclear extracts from cells transfected with the indicated vectors and grown in IL-3 or in the presence of TPO for 4 days as indicated. 32D parental cells were starved for 4 h and then pulsed for 15 min with IL-3 where indicated. Streptavidin MagneSphere-bound material was eluted and analysed by Western blot using antibodies against IRF-2 or p-STAT-5. INPUT indicates the level of IRF-2 and p-STAT-5 in cell extracts (20 μg) before the DNA pull-down assay. (**C**) Schematic diagram of the 595 bp fragment of the proximal TPO-R promoter and its mutated version in the IRF-E site. The wild-type (wt) or mutated (mut) TPO-R promoter linked to the luciferase reporter gene was transiently transfected into 32D/RcCMV, 32D/IRF-2 and 32D/ΔIRF-2 cells, and total cell extracts were processed for luciferase activity after 48 h (RLU, relative luciferase units). Means $\pm$ S.D. from three separate experiments were calculated after normalization with TK Renilla activity.

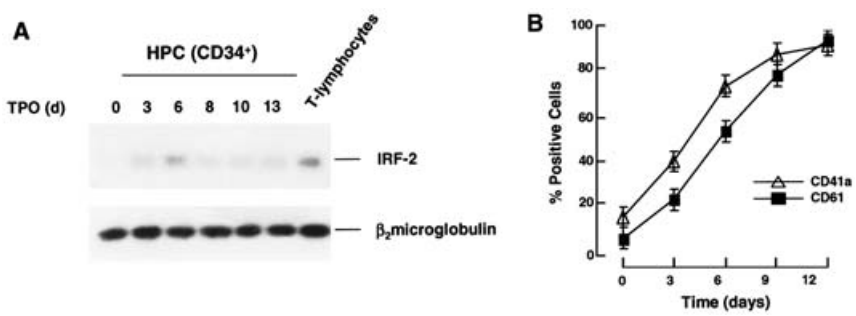

Figure 8 IRF-2 is induced during megakaryopoiesis of normal human CD34$^+$ progenitor cells

(**A**) Purified human haematopoietic progenitor cells (HPC) were induced to specific megakaryocytic differentiation in the presence of TPO. At the indicated time points, total RNA was extracted and IRF-2 expression was evaluated by semi-quantitative RT-PCR. A representative RT-PCR experiment from two independent sets of RNA is shown. β_2-Microglobulin was used for normalization. (**B**) Kinetics of CD41a and CD61 expression in human progenitor cells induced to specific megakaryocytic differentiation in the presence of TPO. CD41a and CD61 expression was evaluated by flow cytometry, and the results are expressed as percentage of positive cells. Mean values from five separate experiments are reported.

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mechanism, as occurs for the TPO-R gene (Figure 7), or whether their induction reflects the development of a genetic programme induced, upstream, by GATA-2 and Tal-1.

Direct transcriptional activation of the TPO-R gene by IRF-2 is demonstrated by our data. TPO-R is present primarily on Mks and on a subpopulation of haematopoietic progenitors, and its ligand, TPO, increases the size, ploidy and number of Mks, stimulating the expression of platelet-specific markers [39]. We demonstrated that IRF-2 stimulates the transcription of the TPO-R gene through binding to an IRF-E site present on its promoter. Interestingly, although IRF-1 and IRF-2 share high identity in DNA-binding sequences, no IRF-1 binding could be detected to the TPO-R promoter, further stressing the specificity of the IRF-2 activity on selected key target genes in Mk differentiation. This is the first reported example of a gene directly involved in the control of cell differentiation that is transcriptionally and specifically induced by IRF-2. Previous studies have shown a positive transcriptional activity of IRF-2 on the genes encoding histone H4, VCAM-1, gp91phox and Fas ligand, none of which are directly involved in the control of cell differentiation. In fact, IRF-2 has been regarded as a transcription factor involved in the stimulation of cell growth and/or apoptosis, and the correct balance of its physiological levels has been considered to be critical for normal regulation of cell growth.

Finally, our data are consistent with a physiological role for IRF-2 in normal Mk differentiation. IRF-2 expression is, in fact, clearly stimulated in normal haematopoietic $CD34^+$ progenitors allowed to differentiate into Mks, starting from day 3 and remaining constantly expressed until day 13 of culture, at which point Mks are fully differentiated. This sustained IRF-2 expression is suggestive of a requirement for IRF-2 in gene transcription during all phases of the Mk differentiation process.

In summary, our results indicate that IRF-2 in myeloid cells activates a developmentally determined pattern of gene expression in a sequentially ordered fashion leading to Mk differentiation, thus providing new evidence for a role for IRF proteins in haematopoietic differentiation. Furthermore, as shown for IRF-1 [2], our results point to a remarkable functional diversity of IRF-2 in the regulation of cellular responses that is mediated by its ability to differentially regulate gene expression depending on the cell type, the state of the cell and the nature of the stimulus.

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