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Ikaros Isoform X Is Selectively Expressed in Myeloid Differentiation

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The Ikaros gene is alternately spliced to generate multiple DNA-binding and nonbinding isoforms that have been implicated as regulators of hematopoiesis, particularly in the lymphoid lineages. Although early reports of Ikaros mutant mice focused on lymphoid defects, these mice also show significant myeloid, erythroid, and stem cell defects. However, the specific Ikaros proteins expressed in these cells have not been determined. We recently described Ikaros-x (Ikx), a new Ikaros isoform that is the predominant Ikaros protein in normal human hematopoietic cells. In this study, we report that the Ikx protein is selectively expressed in human myeloid lineage cells, while Ik1 predominates in the lymphoid and erythroid lineages. Both Ik1 and Ikx proteins are expressed in early human hematopoietic cells (Lin CD34+). Under culture conditions that promote specific lineage differentiation, Ikx is up-regulated during myeloid differentiation but down-regulated during lymphoid differentiation from human Lin CD34+ cells. We show that Ikx and other novel Ikaros splice variants identified in human studies are also expressed in murine bone marrow. In mice, as in humans, the Ikx protein is selectively expressed in the myeloid lineage. Our studies suggest that Ikaros proteins function in myeloid, as well as lymphoid, differentiation and that specific Ikaros isoforms may play a role in regulating lineage commitment decisions in mice and humans. The Journal of Immunology, 2003, 170: 3091–3098.

T he Ikaros protein (also designated Lyf-1) (1) and gene (2) were first identified using strategies designed to detect transcription factors that regulate lymphoid-specific genes, leading to the conclusion that Ikaros was a lymphoid-specific transcription factor. Subsequent studies provided evidence that the role of Ikaros proteins in normal hematopoiesis extended beyond the lymphoid lineages (3–6). In addition, Ikaros proteins were shown to act as both positive (5, 7–12) and negative (5, 13–21) regulators of transcription.

The murine Ikaros gene was initially reported to contain seven exons (7) that were alternately spliced to generate transcripts for multiple DNA-binding and nonbinding Ikaros isoforms (Fig. 1A) (3, 7, 22, 23). Human and murine Ikaros were shown to be highly homologous, and isoforms corresponding to murine Ik1 through Ik6 (Fig. 1A) were detected in human cells (23). This suggested that Ikaros mRNA splicing patterns might be identical in mice and humans. However, subsequent studies of human Ikaros expression in leukemia samples (24–26) and in normal hematopoietic cells (27–29) identified transcripts for multiple Ikaros splice variants that had not been reported in murine studies. Our studies showed that Ikaros splice variants with a 60-base insertion following exon 2 (Fig. 1B) were expressed at the protein level in normal human hematopoietic cells (29). We also detected transcripts for Ikaros-x (Ikx; Fig. 1C), a new Ikaros isoform not previously described in mice or humans. We found Ikx to be the predominant Ikaros protein in normal human umbilical cord blood (CB) and bone marrow (BM) cells, although we observed little Ikx in T and B lymphoid cell lines (29). Murine hematopoietic cells have not been examined for expression of Ikx and the other novel Ikaros isoforms identified in human studies.

Studies of mutant mice implicated Ikaros proteins as regulators of critical events in hematopoietic differentiation and proliferation, particularly in the lymphoid lineages. Ikaros-null mice fail to produce B cells and generate T cells only postnatally (30). Mice with a defect in the Ikaros DNA-binding domain show more severe lymphoid defects, including a complete loss of T, B, and NK cells (31). In addition, mice with reduced Ikaros DNA-binding activity show abnormal T cell proliferation and differentiation (32–34).

Although the initial reports of Ikaros mutant mice focused on lymphoid defects, (30, 31), these mice display multiple hematopoietic abnormalities (4, 35, 36), including myeloid and hematopoietic stem cell (HSC) defects (4, 30, 31, 36) and aberrant erythropoiesis (6, 30, 31). Ikaros proteins have now been shown to act as the DNA binding component of the PYR chromatin remodeling complex that functions in globin switching (5, 6). A recent study shows that Ikaros is important at early stages in granulocyte differentiation (37). These findings are consistent with erythroid and myeloid defects exhibited by Ikaros mutant mice (4, 30, 31).

Reports of Ikaros isoform expression in primary cells outside the lymphoid lineages are limited to a few studies that examined Ikaros mRNA expression by RT-PCR (3, 8, 27). However, our studies have shown a poor correlation between the expression of Ikaros proteins and Ikaros mRNA, as assessed by RT-PCR (29). The expression of Ikaros isoforms, at the protein level, has not...
been isolated in primary cells outside the lymphoid lineages, and little is known of the role of Ikaros proteins in the myeloid lineage.

The lack of Ikar in human lymphoid cell lines led us to ask what cells might be responsible for the abundance of Ikar proteins in the myeloid lineage. The predominance of Ikar that we observed in normal human hematopoietic cells also raised the question of whether Ikar and other novel Ikaros splice variants identified in human studies might be expressed in the mouse. This is particularly important because experiments to determine the mechanisms of Ikaros function have relied almost exclusively on the murine model. Neither murine nor human studies have examined differential expression of Ikaros proteins between normal primary cells of the various hematopoietic lineages or at different points in differentiation. Consequently, questions concerning potential roles for specific Ikaros proteins in hematopoietic lineage commitment have not been addressed.

Materials and Methods

Cell sources and preparation

Human CB and BM were collected according to guidelines approved by the Childrens Hospital Los Angeles Committee on Clinical Investigation (Investigational Review Board) and mononuclear cells were isolated as previously described (38).

Nucleated erythroid lineage cells were isolated from mononuclear cells that had been treated with PharmLyse ammonium chloride lysis solution (BD Pharmingen, San Diego, CA) to lyse any remaining erythrocytes. CD66B+ granulocytes were isolated (within 6 h of delivery) from fresh CB cells located below the buffy layer after Ficoll-Hypaque separation. The remaining human hematopoietic cells were isolated from total CB or BM mononuclear cells.

Murine BM, spleen, and thymus were obtained from C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), aged 6–12 wk, and rested for at least 5 days after arrival before organs were harvested.

The murine S17 cell line was a generous gift from Dr. K. Dorshkind (University of Southern California, Los Angeles, CA). The Jurkat E6-1 cell line was obtained from American Type Tissue Culture Collection (Manassas, VA).

Isolation of hematopoietic populations

Murine and human hematopoietic cells were incubated with Abs (see below) for 20–30 min on ice, washed, and then cell populations were isolated by FACS sorting or magnetic separation based on surface Ag expression.

For RT-PCR, cells were isolated by FACS (FACS Vantage; BD Biosciences, San Jose, CA) based on forward scatter and side scatter gates characteristic of living mononuclear cells and surface Ag staining as indicated below (typical purity of 99% or greater).

All HSC and progenitor populations were FACS sorted from CD34+–enriched mononuclear cells. CD34+ enrichment was performed using the MiniMacs CD34 progenitor isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions.

For immunoblot analysis, mature cells of the hematopoietic lineages were isolated using the MiniMACS magnetic separation system (Miltenyi Biotec). Murine and human B lineage cells were isolated using respective, species-specific, CD19 MicroBeads (Miltenyi Biotec) to a purity of ~99%. The remaining lineages were isolated by positive selection (typical purity of 95% or greater) using anti-FITC or anti-PE MicroBeads (Miltenyi Biotec). cDNA was prepared using oligo(dT) primers (Pharmacia Biotech, Upsalla, Sweden) and the Omniscript kit (Qiagen, Valencia, CA) as per manufacturer’s directions. cDNA from equivalent cell numbers was used for RT-PCR analysis of human Ikaros expression in hematopoietic lineages and in progenitor populations. Using primers and conditions described previously (40), integrity of cDNA was assessed by RT-PCR amplification of β2-microglobulin from the same cDNA master mix used to assess Ikaros expression.

RT-PCR analysis

cDNA was subjected to PCR using HotStarTag (Qiagen) as per manufacturer’s directions. Homologous primer pairs were designed to amplify selected murine and human Ikaros transcripts (29), including splice forms with the 60-base insertion following exon 2.

Primers and conditions for amplification of human Ikaros cDNAs were as described previously (29). Primers for amplification of mouse Ikaros cDNAs were the following: forward, 5'-TGAAGCCATGCTGCTCCCTGACTG-3', and reverse, 5'-GTCCTCTCTGCATCTGTTGATGA-3'. PCR conditions for mouse primers were as follows: 15-min, 95°C hot start followed by 35 cycles of 1 min and 30 s at 95°C, 30 s at 68°C, and 3 min at 72°C; followed by a final 10-min, 72°C extension and cooling to 4°C. To prevent heteroduplex formation between PCR products generated from different Ikaros isoforms as primer concentrations decreased, 20 additional picomoles each of forward and reverse primers were added two cycles before completion of the PCR.

Sequence of RT-PCR products

Individual PCR products for sequencing were obtained by cutting individual bands from gel and purifying using UltraClean GelSpin DNA purification kit (MO BIO Laboratories, Solana Beach, CA) or by generating single PCR products in a second PCR (identical with above, except run for 30 cycles) performed on samples aspirated by syringe for individual bands. Sequencing was performed by the University of Southern California/Norris Comprehensive Cancer Center Microchemical Core Facility at the Keck School of Medicine (Los Angeles, CA) or the Core Facility at the Center for Molecular Biology and Gene Therapy (Loma Linda University, Loma Linda, CA).

ImmunobLOTS

For preparation of cell lysates, washed cells were frozen at −80°C and thawed, on ice, in cold universal immunoprecipitation buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 25 mM β-glycerophosphate, and 0.3% Igepal in water), brought up to 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin immediately before use. Suspended cells were sonicated on ice for ~10 s, checked for complete lysis by microscopy and then centrifuged at 14,000 rpm for 10 min at 4°C to pellet membranes. Supernatants were diluted in NuPage sample buffer (Invitrogen) and immediately run on gel or stored at −80°C. Lysates were run along with SeeBlue m.w. markers on a NuPage 10% Bis-Tris gel with MOPS buffer using the XCell SureLock system (Invitrogen) and transferred to Immobilon-P membrane (Millipore, Bedford, MA) using the XCell II Blot Module (Invitrogen), all performed as per manufacturer’s instructions.
Ikaros was detected using Ikaros M-20 and Ikaros E-20 (polyclonal goat Abs specific to the N and C termini, respectively, common to all Ikaros isoforms (Santa Cruz Biotechnology, Santa Cruz, CA)), anti-IkH (polyclonal rabbit Ab specific to residues encoded by the 60-base insert following Ikaros exon 2), or the mouse mAb 2A9 (specific to exon 3 of the Ikaros protein) (generous gifts kindly provided by the laboratory of S. Smale (Howard Hughes Medical Institute, University of California, Los Angeles, School of Medicine, Los Angeles CA)). Blots were developed using the ECL+Plus Western blotting detection system and accompanying Abs (Amersham, Arlington Heights, IL) or anti-goat-HRP (Santa Cruz Biotechnology) as per manufacturer’s protocol, with the exception that blocking reagent was present continually during Ab incubation.

**Results**

**Ikx proteins, but not transcripts, are selectively expressed in human myeloid lineage cells**

Our previous studies showed that Ikx (Fig. 1C) is the predominant Ikaros protein in human CB and BM, but not in human lymphoid cell lines (29). One explanation for these observations is that Ikx is selectively expressed in nonlymphoid lineages. To determine whether this explanation is correct, we examined expression of Ikaros mRNA and protein in human lymphoid, myeloid, and nucleated erythroid lineage cells.

Multilineage RT-PCR analysis of Ikaros mRNA expression in human CB and BM showed a similar pattern of products for all of the hematopoietic lineages (Fig. 2A). PCR primers (Fig. 1B) generated products representing multiple DNA binding and nonbinding Ikaros isoforms, including many with the 60-base insertion, in FACS-sorted cells from the T, B, NK, erythroid, and myeloid lineages (Fig. 2A). Isoforms with the 60-base insertion (insertion forms) were designated as Ik1**, Ikx**, Ik2**, etc. The pattern of RT-PCR products generated in the individual lineages matched the pattern that we had previously observed in total human BM and CB (29).

Contrary to what was observed in RT-PCR analysis of mRNA expression, immunoblots showed that Ikaros isoforms are differentially expressed at the protein level in the hematopoietic lineages. Ik1 proteins are predominant in B, NK, and nucleated erythroid lineage cells (Fig. 2B). Human T cells isolated from CB showed only very faint bands representing multiple DNA-binding and nonbinding Ikaros proteins (Fig. 2B). Ikx proteins are selectively expressed in myeloid lineage cells (Fig. 2B). The CD15+ cells isolated from CB include monocytic and granulocytic subsets (41); however, the CD15 Ag is a differentiation and tumor-associated Ag that has been detected on nonhematopoietic cells (42, 43). To more precisely define Ikaros protein expression within the myeloid lineages, we isolated CD14+ (primarily monocytes and macrophages (44)) and CD66B+ (granulocytes (45, 46)) cells from CB. Ikx predominates in both the CD14+ and CD66B+ myeloid populations (Fig. 2B).

Ik1 predominated in the B, NK, and nucleated erythroid lineage cells we examined. However, cells from these three lineages combined generally comprised <20% (and in some cases <10%) of mononuclear cells in CB and only about a third of those in BM (data not shown). This explains the predominance of Ikx that we observed in our previous studies of total human CB and BM mononuclear cells (29).

Immunoblots specific to the amino acids encoded by the 60-base insertion show that Ikaros insertion forms follow a pattern of lineage-specific expression that mirrors that observed for parent Ikaros isoforms: Ikx** is selectively expressed in myeloid cells while Ik1** predominates in B, NK, and erythroid lineage cells (Fig. 2C).

These data show that the Ikx and Ikx** proteins, previously reported to predominate in human BM and CB cells (29), are selectively expressed in myeloid lineage cells. This pattern of protein expression was not predicted based on RT-PCR assessments of Ikaros mRNA.

**Murine hematopoietic cells express transcripts for Ikx**

Given the predominance of Ikx in human hematopoietic cells, we wanted to know whether murine hematopoietic cells express Ikx and the novel Ikx insertion forms identified in human studies. Murine and human primer pairs were designed to amplify selected Ikaros transcripts with the 60-base insertion following exon 2 (Fig. 1B). DNA sequencing confirmed the identity of murine RT-PCR products consistent in size with Ik1, Ikx, Ik4, Ik8, and the new Ikaros isoform, Ikx. (Fig. 3A). RT-PCR products consistent in size with Ikx** and, in some cases, Ik1** were also detected (Fig. 3A). However, we were unable to isolate RT-PCR products from murine insertion forms for DNA sequencing.

**Ikx proteins predominate in mouse BM**

Immunoblots specific to both murine and human Ikaros proteins show a similar pattern of Ikaros expression in mouse and human BM (Fig. 3B). The predominant Ikaros protein is detectable as a broad dense band consistent in size with Ikx forms (Fig. 3B). A fainter band representing Ik1 forms is also detectable in both murine and human cells (Fig. 3B). Although we were unable to identify RT-PCR products for murine Ikaros insertion forms by DNA sequencing, Abs specific to the insert show that the Ikaros insertion forms are expressed at the protein level in mouse BM cells (Fig. 3C). These data show that mice, like humans, express proteins representing the new Ikaros isoform, Ikx, and Ikaros splice forms with the 60-base insertion. Furthermore, Ikx forms are the predominant Ikaros proteins in mouse, as well as human, BM.

**Murine Ikx proteins are differentially expressed in lymphoid and myeloid lineage cells**

Previous studies of Ikaros protein expression in the mouse have focused on the lymphoid lineage and failed to identify Ikx. To
determine whether murine Ikx is differentially expressed in lymphoid and myeloid cells, we examined expression of murine Ikaros proteins in BM myeloid cells and in T and B lymphocytes from multiple organs.

A dense band consistent in size with Ikx forms was expressed in murine myeloid cells. In contrast, Ikx was only faintly detectable in T and B lymphocytes in some immunoblots (Fig. 4). Ik1 forms were the predominant Ikaros proteins in murine B and T cells but represented a smaller fraction of the Ikaros proteins in myeloid lineage cells. (Fig. 4). Surprisingly, total Ikaros proteins in general appeared more abundant in myeloid than lymphoid lineage cells.

A comparison of immunoblots that detected all Ikaros isoforms (Fig. 4A) with immunoblots that were visualized with Abs specific to the insertion sequence (Fig. 4B) shows that both parent and insertion forms of Ik1 and Ikx are expressed in murine cells.

Immunoblots specific to exon 3 (present in Ik1, Ikx, Ik3, and Ik5) provide additional information on the Ikaros proteins expressed in the mouse. Ikaros proteins are more readily detected by anti-Ik exon 3 (Fig. 4C) than by anti-Ik N-terminal sequence (NTS) and C-terminal sequence (CTS) Abs (Fig. 4A) or anti-IkH (Fig. 4B). This is likely due to differences in primary or secondary Ab affinity as the same protein preparations were used for all blots shown and identical results were obtained in several independent experiments. The faint band detected by anti-NTS and anti-CTS Abs just below Ikx forms in total BM and myeloid cells (Fig. 4A, Ik2/3 forms) likely represents Ik2, as it was not detected (Fig. 4C) by Abs specific to exon 3 (present in Ik1, Ikx, Ik3, and Ik5). Anti-exon 3 Abs also identify a DNA-nonbinding isoform in both lymphoid and myeloid cells that contains exon 3.

**Transcripts for multiple DNA-binding and nonbinding Ikaros isoforms are expressed at early points in human hematopoietic differentiation**

The differential expression of Ikaros proteins that we observed in mature murine and human hematopoietic cells of the various lineages raised the question of Ikaros expression at early points in differentiation. To address this question, we first used RT-PCR to determine whether human HSC and progenitor populations express Ikaros mRNA. Total human CD34+/H11001 CB and BM cells, and subsets enriched for primitive HSCs (CD34+/H11001 CD38−) or for multipotential and committed progenitors (CD34+/H11001 CD38+) were FACS sorted as shown in Fig. 5A. RT-PCR analysis of CB and BM subsets showed that transcripts for a range of DNA-binding and nonbinding Ikaros isoforms, including those with the 60-base insert, were expressed in progenitor and in primitive HSC populations in human CB and BM (Fig. 5B). The pattern of RT-PCR products generated by primitive hematopoietic cells (Fig. 5B) was similar to that observed in mature human hematopoietic cells of the various lineages (Fig. 2A).
Ik1 and Ikx protein levels are differentially modulated during myeloid and lymphoid differentiation

Our data demonstrate that the ability to generate transcripts for a range of DNA-binding and nonbinding Ikaros isoforms is present very early in hematopoietic differentiation. However, we have observed a poor correlation between the expression of Ikaros transcripts and proteins. Therefore, to determine which of these transcripts are expressed as proteins, we examined Ikaros protein expression in early hematopoietic cells and the progeny they generate during lymphoid and myeloid differentiation.

FACS-sorted CB cells that lack markers of lineage-committed cells (CD3, CD19, CD56, glycophorin A, CD15, CD14, and CD66B) but express CD34 (Lin−/H11002 CD34+/H11001; Fig. 6A) were used to prepare lysates for immunoblot analysis or placed into culture conditions that selectively support either lymphoid or myeloid lineage differentiation. However, we have observed a poor correlation between the expression of Ikaros transcripts and proteins. Therefore, to determine which of these transcripts are expressed as proteins, we examined Ikaros protein expression in early hematopoietic cells and the progeny they generate during lymphoid and myeloid differentiation.

FACS-sorted CB cells that lack markers of lineage-committed cells (CD3, CD19, CD56, glycophorin A, CD15, CD14, and CD66B) but express CD34 (Lin−/CD34+; Fig. 6A) were used to prepare lysates for immunoblot analysis or placed into culture conditions that selectively support either lymphoid or myeloid lineage differentiation.
Collectively support lymphoid or myeloid differentiation (see frozen for later immunoblot analysis or cultured under conditions that se-

cifice to the NTS and the CTS common to all conditions were assessed by immunoblot analysis for total Ikaros protein and their progeny generated under lymphoid (L) or myeloid (M) culture.

When Lin-H11002 isolated Lin-H11002 myeloid progeny were harvested for immunoblot analysis. After 18 days in culture, lymphoid and myeloid and lymphoid differentiation.

FIGURE 6. Ik1 and Ikx proteins are differentially modulated during myeloid and lymphoid differentiation. A, CD34^+--enriched CB mononuclear cells that fall within a forward scatter and side scatter gate (R1) characteristic of lymphoid cells are shown. Lin^CD34^- cells were FACS sorted based on R1, R2 and R3 gates. B, Sorted Lin^CD34^- cells were frozen for later immunoblot analysis or cultured under conditions that selectively support lymphoid or myeloid differentiation (see Materials and Methods). C, Total CB mononuclear cells, FACS-sorted Lin^CD34^- cells, and their progeny generated under lymphoid (L) or myeloid (M) culture conditions were assessed by immunoblot analysis for total Ikaros protein expression using Abs specific to the NTS and the CTS common to all Ikaros isoforms.

differentiation (Fig. 6B). After 18 days in culture, lymphoid and myeloid progeny were harvested for immunoblot analysis.

Unlike total CB cells which predominantly express Ikx, freshly isolated Lin^-CD34^- cells express both Ik1 and Ikx (Fig. 6C). When Lin^-CD34^- cells were placed in culture conditions that selectively support myeloid differentiation, Ikx expression was up-regulated in their progeny (Fig. 6C). In contrast, progeny generated in culture conditions that promote lymphoid differentiation showed down-regulation of Ikx (Fig. 6C). Thus, both Ik1 and Ikx are expressed in early hematopoietic cells and the expression of Ikx is modulated as cells undergo lineage commitment and differentiation.

Discussion

In this report, we describe a comprehensive analysis of Ikaros mRNA and protein expression during normal human hematopoiesis. Our studies show that Ikaros protein expression follows a lineage- and stage-specific pattern of expression that correlates poorly with Ikaros mRNA expression as assessed by RT-PCR. Both Ik1 and Ikx proteins are expressed early in hematopoiesis. Thereafter, Ikx is selectively expressed in the myeloid lineage, while Ik1 predominates in erythroid and most lymphoid lineage cells. The expression pattern of murine Ikaros proteins, including Ikx, in lymphoid and myeloid cells is similar to that observed for human Ikaros.

In contrast to expression at the protein level, RT-PCR analysis of human Ikaros mRNA shows a similar pattern of expression for DNA-binding and nonbinding Ikaros isoforms in all of the hematopoietic lineages and in HSC and progenitor populations. One possible explanation for the poor correlation between RT-PCR products and Ikaros proteins is that the expression of Ikaros is regulated after transcripts for the various isoforms have been generated by RNA processing. A poor correlation between mRNA and protein expression has been reported in a number of other cases. These include genes expressed in hematopoietic cells (47) and genes that, like Ikaros, are alternately spliced to generate multiple isoforms (48).

Alternatively, simultaneous RT-PCR amplification of transcripts for multiple isoforms may not provide an accurate indicator of the relative levels of each transcript i.e., amplification of some transcripts may be more efficient than others. A difference in ability to compete for primers may seem unlikely given that primers bind an identical sequence in all transcripts. However, particular transcripts may be at a selective disadvantage for primer binding or primer extension due to secondary structures conferred by specific exons. For example, Ikaros isoforms that lack exon 3 (Ik2 and Ik4) are disproportionately overrepresented among RT-PCR products as compared with their representation at the protein level. In contrast, isoforms that contain exon 3 (Ik1 and particularly Ikx) are underrepresented among RT-PCR products as compared with protein expression (Figs. 2, 3, 5, and 6).

Given the predominance of Ikx that we observe in normal human and murine hematopoietic cells, it might seem surprising that Ikx was not identified in early studies. However, the Ikaros gene gives rise to a number of splice forms, many of which were not identified in the initial reports (7, 22). Ikaros expression has most frequently been examined by RT-PCR and products consistent in size with Ikx were detected in a number of murine (3, 23) and human studies (23, 27, 49, 50). However, these products were not identified and, in some cases, were thought to be artifacts. The comparatively low frequency of Ikx among RT-PCR products did not suggest that Ikx proteins might be abundantly expressed. In addition, there was no indication that the unidentified RT-PCR product (consistent in size with Ikx) might correspond to a myeloid-specific Ikaros protein, because the lineage-specific expression of Ikx proteins is not observed for Ikx transcripts (Fig. 2).

Due to the initial link between Ikaros and the lymphoid lineages, most studies of Ikaros protein expression have been performed on lymphoid cells which express little, if any, Ikx. Ikx was not identified in these studies, although a protein product consistent in size with Ikx was detectable in some cases in both murine (22) and human (25–27, 49, 50) cells. Thus, the identity of Ikx and its abundance and almost exclusive expression in the myeloid lineage were not known before our studies.

Murine HSC and progenitor populations have been shown to express Ikaros mRNA (3, 8). As with mature human cells of the
various lineages (Fig. 2), we found that highly purified, FACS-sorted human HSC (CD34+/CD38-) and progenitor (CD34+ CD38-) populations generate transcripts for multiple DNA-binding and nonbinding Ikaros isoforms (Fig. 5). Our data are consistent with a study by Nakayama et al. (27) that detected transcripts for DNA-binding and nonbinding Ikaros isoforms in CD34+ enriched human CB cells and in culture-generated myeloid and erythroid cells.

The rarity of CD34+CD38- cells (~0.05% of CB and BM mononuclear cells (38)) precluded immunoblot analysis of protein expression in this population. Therefore, for protein analysis, we isolated CD34+ CB cells that lacked markers of lineage commitment (~0.75% of mononuclear cells). Both Ik1 and Ikx proteins are detectable in the Lin-CD34+ population. However, this population is heterogeneous, including primitive HSCs, the CB multilineage cells, and potentially a common myeloid progenitor. Thus, our studies do not provide an analysis of differential Ikaros protein expression in subpopulations of HSCs and early progenitors. However, they do provide the first report of Ikaros protein expression in early hematopoietic cells and demonstrate that Ik1 and Ikx proteins are expressed very early in normal human hematopoietic differentiation.

Among the human hematopoietic lineages, Ik1 proteins predominate in BM nucleated erythroid lineage cells and in mature NK and B cells isolated from CB. Surprisingly, CB T cells express only low levels of multiple DNA-binding and nonbinding Ikaros proteins. However, it is possible that the pattern of Ikaros expression we observed is unique to naive human T cells, because CB T cells have not yet encountered Ag. In the murine hematopoietic lineages, as in human, Ikx proteins predominate in BM and are selectively expressed in myeloid lineage cells. In contrast to human CB T cells, murine T cells, isolated from spleen and thymus, express Ik1 similarly to B cells. Surprisingly, total Ikaros proteins appear to be more abundant in myeloid than in lymphoid lineage cells, particularly in the mouse.

Using a mouse erythroid leukemia cell line, O’Neill et al. (5) identified two Ikaros proteins (~65 and 55 kDa in size) that co-purified with the PYR complex that functions in globin switching. Based on size, these proteins were identified as Ik1 and Ik2, respectively. Although the 65-kDa protein does correspond to forms of murine Ik1 identified in our studies, the 55-kDa protein is consistent in size with murine Ikx proteins that we identified using Abs specific to Ikaros exon 3 (Ik2 does not contain exon 3). We did not examine Ikaros expression in murine erythroid lineage cells. Our human studies show abundant Ik1 expression in nucleated erythroid lineage cells; however, Ikx is only faintly detectable. Although there may be variation in Ikx expression between species, it is also possible that Ikx is selectively incorporated into the PYR complex.

In the first study to describe alternate splicing of murine Ikaros transcripts, Haem et al. (22) describe a murine Ikaros sequence that is homologous to the 60-base insert identified in human studies. Using murine cell lines, their study identified transcripts for Ikaros splice forms that represent Ik5- and Ik6+ (designated Ikaros isoform IV and isoform II, respectively) (22). Our studies show that splice variants with the insert (primarily Ik1+ and Ikx+) are expressed at the protein level in normal murine and human hematopoietic cells of multiple lineages. The pattern of expression for Ikaros proteins with the insert mirrors that seen for parent isoforms that do not include the insert.

The selective expression of Ikx proteins in early progenitors and in the myeloid lineage suggests that particular Ikaros isoforms may have specific functions in hematopoiesis. Due to the relatively large numbers of cells required for immunoblot analyses, there have been few reports of Ikaros isoform expression, at the protein level, in primary murine or human hematopoietic cells of any lineage. The poor correlation between Ikaros RT-PCR products and Ikaros protein expression underscores the importance of examining Ikaros expression at the protein level. Given the tumor suppressor activity ascribed to Ikaros (30, 49–53), aberrant Ikaros expression in leukemia and other cell lines seems likely. Therefore, an understanding of the role of Ikaros in normal hematopoiesis may depend on knowledge of the specific Ikaros proteins expressed in primary hematopoietic cells.

The studies described in this report suggest that the expression of Ik1 and Ikx could play a role in myeloid vs lymphoid and erythroid lineage commitment. Current studies in our laboratory are aimed at examining the effects of overexpression of Ik1 and Ikx on lineage commitment and hematopoietic differentiation in human HSCs.

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References


