A New Isoform of Interleukin-3 Receptor \( \alpha \) with Novel Differentiation Activity and High Affinity Binding Mode*†‡

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Interleukin-3 (IL-3) promotes both self-renewal and differentiation of early multipotential progenitors and is involved in inducible hematopoiesis in response to infections. Here we report new insights into these processes with the identification of a new isoform (SP2) of IL-3 receptor \( \alpha \) (IL-3R\( \alpha \)), present in mouse and human hematopoietic cells, which lacks domain 1 of the full-length receptor (SP1). Binding assays with \( \beta_{IL\_3} \) mutants showed that mouse SP2 uses a different high affinity binding mode to SP1, although both mouse and human SP2 and SP1 can stimulate IL-3-dependent growth. In IL-3-dependent differentiation models, human SP2 and SP1 gave differential effects on lineage commitment or self-renewal dependent on the cellular context, suggesting that different modes of ectodomain binding may modulate intracellular signaling. In a multipotential factor dependent cell-Paterson mix, the transcription factors C/EBP\( \alpha \) and PU.1 and microRNAs miRNA-15a, -223, and -181a were up-regulated in cells undergoing SP2-supported differentiation compared with SP1-supported self-renewal. Similarly in M1 cells, SP2 promoted differentiation compared with SP1 and gave up-regulation of PU.1 and miRNA-155 and -223. These findings suggest that IL-3-promoted lineage commitment uses similar mechanisms to those of steady-state hematopoiesis. Both the SP1 and SP2 isoforms activated the Jak2/STAT5, Akt, and Erk1/2 signaling pathways in M1 cells, although the activation was more prolonged for the SP2 isoform.

IL-3\(^2\) is a multipotent cytokine affecting all steps in myeloid differentiation (1–4). It promotes the self-renewal of early multipotential cells, the proliferation and differentiation of myeloid progenitors, and the survival and activation of mature cells. IL-3 is mainly produced by activated T lymphocytes and mast cells suggesting a role in inflammation and responses to infections. It appears to have a unique role in relation to plasmacytoid dendritic cells (5) and basophils (6), which have important roles in both innate and adaptive immunity. Studies of IL-3-deficient mice demonstrate that IL-3 is required for the elevation of mast cells and basophils in response to parasite infections (7). The IL-3R\( \alpha \) is overexpressed on leukemic stem cells and blasts suggesting a role in leukemia pathogenesis (8–10).

IL-3 signaling in humans is mediated by a receptor system (IL-3R) composed of an IL-3-specific \( \alpha \) receptor and a \( \beta \) receptor (h\( \beta \)c) that is shared with two related cytokines, IL-5 and GM-CSF (11–14). In mice, there is a shared \( \beta \)c (m\( \beta \)c) receptor but also an IL-3-specific \( \beta \) receptor (\( \beta_{IL\_3} \)) (15). Both \( \beta \) receptors require the mouse IL-3R\( \alpha \) (mIL-3\( \alpha \)) for activation (16). Upon receptor activation, the cytoplasmic portions of the \( \beta \) receptors, which lack any intrinsic kinase activity, initiate a number of signaling pathways, including the Janus kinase 2/signal transducers and activators of transcription, phosphatidylinositol 3-kinase, and Ras/mitogen-activated protein kinase pathways (17). Previous studies of IL-3 signaling have mainly concentrated on growth and reversal of apoptosis, whereas the mechanisms regulating differentiation and self-renewal remain poorly understood. It remains controversial whether cytokine receptors in general can play an instructive role in lineage specification in hematopoiesis, and steady-state blood cell formation appears to largely involve stochastic processes (18). However, there is evidence that the GM-CSF receptor (GM-CSFR) can play an instructive role in the differentiation of multipotential cells (19).

The IL-3R is a member of the cytokine class I receptor family (20). One of the best studied members of this family is the growth hormone receptor, which illustrates the basic principles of receptor activation. Ligand binding with the growth hormone receptor occurs at the elbow region formed between the two domains of the cytokine receptor homology module, and activation involves homodimerization. In contrast, we have shown the ectodomain of the h\( \beta \)c receptor to be a novel intertwined homodimer (21); an unexpected departure from other structures of the family. However, the h\( \beta \)c receptor has a ligand-binding elbow region analogous to that of the growth hormone receptor, although it is composed of domains 1 and 4 of the two different protein chains (22–24). The homologous mouse \( \beta_{IL\_3} \) receptor is also a homodimer, and its elbow region forms the direct IL-3 binding site (22). Surprisingly, however, high affinity IL-3 binding by the \( \beta_{IL\_3} \)–IL-3R\( \alpha \) complex does not involve the residues of the direct IL-3 binding site (22). The \( \beta_{IL\_3} \) receptor therefore represents an interesting departure from the canonical activation mechanisms.
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established for receptors like the growth hormone and erythropoietin receptors.

In the present work we have identified a new ectodomain isoform of IL-3Rα, designated SP2, which is present in mouse and human hematopoietic cells. Unlike the previously characterized (SP1) isoform, mIL-3Rα SP2 utilizes the direct IL-3 binding region of β3 for high affinity binding and signaling showing that the β3R receptor has two alternative modes of receptor activation via the SP1 and SP2 isoforms. Thus the IL-3 receptor is similar to the fibroblast growth factor (FGF) receptor, in that it utilizes ectodomain isoforms which modulate receptor function (25).

Herein, we show that IL-3 receptor activation via the mouse and human SP1 or SP2 IL-3Rα isoforms gives growth signaling in the presence of the appropriate β receptor. However, the two isoforms show differential effects on self-renewal and lineage commitment dependent on the cellular context. However, the mode of ectodomain engagement can modulate intracellular signaling by identical cytoplasmic receptor chains as has been suggested recently for the activation of IL-4Rα/IL-13 receptor α1 heterodimers by IL-4 or IL-13 (26). The elevation in expression of particular transcription factors and microRNAs (miRNAs) in cells undergoing IL-3Rα SP2-supported differentiation, compared with cells undergoing SP1-supported growth/self-renewal, indicates that the mechanisms involved are similar to those found in steady-state blood cell formation. The demonstration of the existence and activity of the SP2 isoform reveals additional complexity in IL-3R signaling and provides valuable insights into how an individual growth factor can initiate many distinct cellular outcomes.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—Unless otherwise stated the cytokines used for cell culture were produced in Hi5 insect cells grown in serum-free medium. For differentiation assays with cells expressing the hIL-3R, hIL-3 was used at 220 units/ml and, where added, mIL-3 at 0.4 units/ml. For cells expressing hGM-CSFR, hGM-CSF was used at 290 units/ml. For differentiation of parental FDCP-mix cells, mGM-CSF was included at 0.1% v/v. Details of the media used are given in the supplemental materials. Mouse eosinophils (99% purity) were isolated from white blood leukocytes prepared from spleens of IL-5 transgenic mice using a BD FACSVantage SE cytometer and gating on forward scatter and side scatter. Mouse mast cells and B lymphocyte blasts were gifts from S. Gustin (University of Western Australia) and C. Goodnow (Australian National University), respectively.

**DNA Constructs and Stable Transfection**—Mouse and human IL-3Rα SP2 cDNAs and DNA constructs of mouse β3R were derived from their respective SP1 forms or mouse β3R using the QuikChange protocol (Stratagene) with Pfu Turbo DNA polymerase and verified by DNA sequencing. cDNAs encoding mouse and human IL-3Rα SP1 and SP2 and hGM-CSFRα were cloned into the pEFIREs-N (G418) or pEFIREs-H (hygromycin B) expression vector and cDNAs for mβc, hβc or mβ3R cloned into pEFIREs-P (puromycin). cDNAs were cloned into the pcEX-3 vector (23) for COS7-based binding assays. Transfection details are given in the supplemental materials. As many as possible stable transfectants were recovered, yielding polyclonal populations of cells.

**miRNA Preparation and Quantification of miRNAs and Transcription Factors**—miRNA was prepared from M1 and FDCP-mix cells expressing human IL-3Rα SP1 or SP2 plus h3c and grown under conditions permitting differentiation using an miNeasy mini kit (Qiagen). The detection and quantification of miRNAs was carried out by quantitative real-time RT-PCR with the miScript system (Qiagen) and 7500 Real time PCR system (Applied Biosystems). Quantitative real-time PCR was also used to detect and quantify mRNA encoding transcription factors from the same miRNA preparation in M1 and FDCP-mix cells (see supplemental materials).

**RT-PCR, Deglycosylation, and Western Blot Analysis**—Detection of mouse and human IL-3Rα SP1 and SP2 in mouse or human cells was conducted by RT-PCR and/or Western blot analysis. Deglycosylation of mouse IL-3Rα SP1 and SP2 was performed by treatment with peptide N-glycosidase F. Western blot analysis was also performed to examine the expression of transcription factors in M1 cells. For details see the supplemental materials.

**Proliferation Assays**—Growth responses of CTLL-2, M1, and FDCP-mix cells expressing IL-3Rα and/or relevant β subunit receptors to hIL-3 or mIL-3 were measured in triplicate by [3H]thymidine incorporation as described previously (23). For CTLL-2 cells, proliferation was measured after 2 days of incubation and for M1 and FDCP-mix after 4–5 days. Growth assays for WEHI 3B D+ cells were conducted by counting the viable cell number with trypan blue exclusion under the light microscope.

**Flow Cytometry**—M1 and WEHI 3B D+ cells expressing the appropriate human receptors were incubated with or without hIL-3 (220 units/ml) or hGM-CSF (290 units/ml) for 4 days in growth medium. Similarly, FDCP-mix cells were incubated with hIL-3 (220 units/ml) with or without low mIL-3 (0.4 unit/ml), or incubated with low mIL-3 or 0.1% v/v mouse GM-CSF for 6 days. The expression of surface markers and IL-3R subunits was examined by flow cytometry (see supplemental materials).

**Binding Assays**—COS7 cells were transiently transfected with mouse IL-3Rα SP1 or SP2 with wild-type or mutant mouse β3R constructs. Two days after transfection, the COS7 cells were harvested and binding assays were done as described previously (22).

**Morphological Analysis**—Details of light and electron microscopy are given in the supplemental materials.

**Analysis of Signal Transduction Pathways**—Phosphorylation of Jak2, STAT5, Akt, and Erk1/2 was investigated in M1 cells expressing hIL-3Rα and h3c. Details of phosphorylation analysis and Western blot are shown in the supplemental materials.

**Statistical Analysis**—A Student’s two-tailed non-paired t test was used to determine the statistical significance.

**RESULTS**

**A New Isoform IL-3Rα**

We identified previously unrecognized alternatively spliced isoforms of the IL-3Rα among the mRNA sequences in the
NCBI expressed sequence tag databases in mouse (GI:5599968; from a placental library) and human (GI:19018027; from a retinal pigment epithelium and choroid library), which we designated SP2. The new SP2 isoforms lack domain 1 of the extracellular region due to precise deletion of exons 3 and 4 of the gene (Fig. 1, A and B). The additional sequence available from the trace archive indicated that apart from deletion of domain 1, the SP2 isoforms were otherwise identical with the previously characterized SP1 isoform (16).

Measurement of expression of the SP2 isoforms in mouse hematopoietic cells by RT-PCR showed that mRNA for the SP2 isoform was readily detectable in a variety of hematopoietic cell lines and in eosinophils, mast cells, and B lymphocyte blasts (Fig. 2A), although it was expressed at a lower level than the SP1 form. Clones encoding the SP2 isoforms were isolated and sequenced from mouse FDCP-1, FDCP-mix, WEHI-3B, and GB2 cells and shown to conform to the structure in Fig. 1. We also examined IL-3Rα isoform protein levels in mouse cells by Western blotting. The sizes of the receptor bands indicated extensive glycosylation (five potential N-glycosylation sites in SP1 and four in SP2). Deglycosylation was carried out on the samples to simplify analysis, and bands corresponding to the predicted molecular masses of the mature SP1 and SP2 proteins (41 and 31 kDa, respectively) were demonstrated in a variety of mouse cell lines (Fig. 2B). The mIL-3Rα SP1 protein was the major form detected in FDCP-1, WEHI-3B, and GB2 cells, although in multipotential FDCP-mix cells the levels of SP2 and SP1 proteins were more comparable. Although low levels of the SP2 form were detected by RT-PCR in the M1 myeloblastic leukemia cell line, no SP2 protein was detected by Western blotting. Similarly in FDCP-mix, the marked differences between relative RNA and protein levels suggested the existence of translational controls affecting SP1 and SP2 levels.

Without deglycosylation, the majority of samples showed receptor bands of ~54 and 41 kDa consistent with glycosylated SP1 and SP2 isoforms. In WEHI-3B cells the receptor was of much higher molecular mass, but deglycosylated samples gave similar receptor sizes to the other cell lines (Fig. 2B).

Expression of the human IL-3Rα (hIL-3Rα) SP2 isoform was demonstrated in a variety of human leukemia lines by RT-PCR. hIL-3Rα SP2 isoform expression was lower than the SP1 form, a finding analogous to the results from mouse (Fig. 2C). Clones encoding the SP2 isoform were isolated and sequenced from human TF-1 cells and shown to conform to the structure in Fig. 1.

**Growth Signaling by the Mouse and Human IL-3Rα SP2 Isoforms**

To test the ability of the SP2 isoform to function in growth signaling, the mIL-3Rα SP2 isoform was expressed in CTLL-2 cells with and without βIL-3 or mβc. Similarly, the hIL-3Rα SP2 isoform was expressed in human TF-1 cells with and without βIL-3 or mβc.
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We found that mIL-3 stimulated growth signaling of CTLL-2 cell lines expressing mIL-3Rα SP2/βIL-3, but that higher levels were required than for the receptor system involving the SP1 form to achieve half-maximal stimulation (Fig. 3A). CTLL-2 cells expressing the SP2 form alone were unresponsive to mIL-3, even at concentrations of 480 units/ml. Interestingly, mIL-3Rα SP2 was shown to signal specifically with βIL-3 and could not signal with mβc (Fig. 3B). Similarly, CTLL-2 cells expressing hIL-3Rα SP2/hβc gave full growth signaling in response to hIL-3, but also required higher levels of hIL-3 than the receptor system involving the SP1 form to achieve half-maximal stimulation (Fig. 3C). CTLL-2 cells expressing the hIL-3Rα SP2 form alone were unresponsive to hIL-3, even at doses of 1795 units/ml. In a previous study on hIL-3Rα, where domain 1 was deleted by mutagenesis to yield a receptor similar (but not identical) to the naturally occurring SP2 isoform studied here, a requirement for higher levels of IL-3 to give full growth was also observed (27).

The Canonical IL-3 Binding Site on βIL-3 Is Utilized by mIL-3Rα SP2 but Not SP1

The mIL-3Rα SP1 or SP2 isoform was co-expressed with βIL-3 in monkey COS7 cells, and radiiodinated IL-3 was used to determine the binding constants for high affinity binding. Consistent with previous studies, the Kd for mIL-3Rα SP1/βIL-3 high affinity IL-3 binding was ~200 pM (22, 23). In contrast, the Kd for mIL-3Rα SP2/βIL-3 was found to be 750 pM (Fig. 4A). Table 1. These findings are broadly consistent with the relative levels of mIL-3 required for growth signaling by the SP1 and SP2 isoforms in CTLL-2 cells described above.

βIL-3 Receptors carrying individual mutations in the residues comprising the direct IL-3 binding site were also tested. Our previous, detailed studies have shown that mIL-3 high affinity binding was abolished by the Y21A mutation (Fig. 4B). We show in the present work that mIL-3 high affinity binding with βIL-3 Y21A (TYr21 → Ala) was similar to that of the wild-type βIL-3 Receptor (Table 1). In contrast, mIL-3Rα SP2 high affinity binding was abolished by the Y21A mutation within βIL-3. Indeed, each of the βIL-3 residues that mediate direct IL-3 binding (TYr21, Phe85, Asn87, Ile320, Tyr348, and Tyr401) were critical for high affinity binding with mIL-3Rα SP2 (Table 1), indicating that the SP1 and SP2 isoforms have different mechanisms of high affinity binding. To verify this finding isoform was expressed in CTLL-2 with and without hβc. CTLL-2 is an IL-2-dependent mouse cytotoxic T cell line that does not express the mIL-3Rα, βIL-3 or mβc receptors. Expression of the relevant receptor proteins was verified by FACS (supplemental Figs. S1 and S2A). In the case of the mIL-3Rα SP2 isoform, we were unable to find a suitable antibody for detection by FACS, and Western blotting was used to verify expression (supplemental Fig. S2B).

TABLE 1

| βIL-3 Location of modification | mIL-3Rα mIL-3Rα SP2 mIL-3Rα SP2/mIL-3Rα SP1 | No. sites | No. experiments |
|--------------------------------|-----------------------------------------------|-----------|----------------|
| Wild type                      | SP1                                           | 1         | 199 ± 16       | 3               |
|                                 | SP2                                           | 2         | 235 ± 15       | 4               |
| Wild type SP1                  |                                               | 1         | 278 ± 53       | 4               |
| Wild type SP2                  |                                               | 1         | 710 ± 20       | 4               |
| Wild type                      |                                               | 1         | 310 ± 7        | 2               |
| Wild type                      |                                               | 1         | 783 ± 4        | 4               |
| Wild type                      |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP1                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP2                  |                                               | 1         | 710 ± 20       | 4               |
| Wild type SP1                  |                                               | 1         | 310 ± 7        | 2               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
| Wild type SP1                  |                                               | 1         | 54 ± 3         | 4               |
| Wild type SP2                  |                                               | 1         | 783 ± 4        | 4               |
in terms of growth signaling, stable transfectants of CTLL-2 expressing β_{IL-3} Y21A and either the mIL-3Rα SP1 or SP2 isoforms were prepared and tested for IL-3 growth stimulation. The Y21A β_{IL-3} mutation blocked growth signaling by the mIL-3Rα SP2 isoform but did not affect that by mIL-3Rα SP1 (Fig. 4C). Receptor expression was verified by FACS and Western blotting (supplemental Fig. S2, A and B). These findings provide strong support for the existence of two different modes of β_{IL-3} receptor activation involving the two ectodomain isoforms of mIL-3Rα.

**Activity of the hIL-3Rα Isoforms in Differentiation**

*In vitro* models of hIL-3-supported granulocyte/macrophage differentiation have not previously been established. However, because hIL-3 does not cross-react with the mIL-3 receptor system, ectopic expression of the human IL-3Rα isoforms in mouse cells allows testing of their role in differentiation. We established hIL-3 differentiation models in FDCP-mix, M1, and WEHI-3B D+ cells.

hIL-3Rα in Differentiation of FDCP-mix Cells—FDCP-mix is a non-leukemic multipotential myeloblastic cell line, which has a normal karyotype and grows continuously in an undifferentiated state in high concentrations of mIL-3 (28). In the presence of low levels of mIL-3, FDCP-mix can differentiate into neutrophils, monocytes, erythrocytes, or dendritic cells depending on the cytokines provided in the medium. FDCP-mix is thus capable of self-renewal or cytokine-supported differentiation and has the properties expected for an immortalized multipotential hematopoietic progenitor. It has previously been shown that, when FDCP-mix is transfected with hGM-CSFRα and hβc, hGM-CSF supports granulocyte/macrophage differentiation comparable to that achieved with mGM-CSF in parental cells (29). Thus hβc can interact effectively with the mouse signaling machinery involved in mGM-CSF supported differentiation.

To verify the capacity of FDCP-mix for cytokine-directed differentiation, we treated parental cells with mGM-CSF and obtained differentiation to neutrophils and monocytes (Fig. 5A) in agreement with previous findings (19). Evaluation of surface markers by FACS showed significant increases in expression of Mac-1 and Gr-1 but little increase in Mar-1 (FceRIα) when compared with cells treated with low levels of mIL-3 (Fig. 5B), consistent with the differentiation observed. With FDCP-mix cells expressing hIL-3Rα SP2 isoform and hβc, in the presence of low levels of mIL-3, hIL-3 induced differentiation, whereas in the absence of mIL-3 the cells died (Fig. 5C). When hIL-3Rα SP2 was expressed alone in FDCP-mix cells, hIL-3 did not induce differentiation. In FDCP-mix cells co-expressing hIL-3Rα SP1 and hβc, hIL-3 stimulated growth but no differentiation, in the presence or absence of low levels of mIL-3 (Fig. 5C). The lack of differentiation activity of the hIL-3Rα SP1 isoform is an unexpected result, but our results agree with a previous detailed study that included clonogenic assays and showed that the hIL-3Rα SP1 isoform only promoted self-renewal in FDCP-mix cells (29). The expression of the introduced receptors was verified by FACS in all cases (supplemental Fig. S1).

May-Grunwald-Giemsa staining showed that the differentiation achieved by the hIL-3Rα SP2 isoform was novel. The majority of differentiated cells appeared to be basophil-like with lobulated nuclei partially obscured by granules (Fig. 5A) (30, 31). In contrast to the cells resulting from mouse GM-CSF-stimulated differentiation, the expression of Mac-1 and Gr-1 was not increased in the hIL-3 differentiated progeny (Fig. 5B), and they did not express c-Kit or B220 (data not shown). CD49b (DX5) expression was slightly increased, and FceRIα (Mar-1) was very slightly elevated. Although Mar-1 is typical of basophils and mast cells, previous evidence has shown that high levels of expression requires the presence of IgE, which is absent from our *in vitro* differentiation conditions (32, 33). The lobulated nuclei evident by light microscopy and the lack of increase of c-Kit expression determined by FACS suggested the differentiated FDCP-mix cells were basophils (30, 31, 34). To further confirm their identity, the differentiated cells were examined by electron microscopy (Fig. 5D). The lobulated nuclei in the cells were verified, and a number of electron-dense staining granules were evident. The electron microscopy gave further support that the cells were basophil-like (35, 36). Interestingly, the cells appeared to be undergoing extensive degranulation by mechanisms involving individual granules and granules organized into large degranulation chambers (Fig. 5D). Both mechanisms have been described in detail previously (36).

hIL-3Rα in Differentiation of M1 Myeloblastic Leukemia Cells—The ability of the hIL-3Rα SP1 and SP2 isoforms to promote differentiation was also tested using the mouse myeloblastic leukemia line, M1. This cell line proliferates independently of growth factors, has a blast-like morphology, and has been previously used for differentiation assays in response to human GM-CSF (hGM-CSF) (37). For comparison, we used M1 cells expressing hGM-CSFRα and hβc and showed these cells could be differentiated into monocytes/macrophages by hGM-CSF (Fig. 6A). This activity required the presence of the hβc receptor. Differentiation was shown by changes in morphology and increased expression of the surface markers Mac-1 and F4/80 (Fig. 6, A and B). At high concentrations of hGM-CSF, cessation of growth was observed (data not shown). Our findings with hGM-CSF differentiation of M1 cells are in good agreement with a previous study (37). When hIL-3Rα SP2 was co-expressed in M1 with hβc, hIL-3 also promoted differentiation to monocytes/macrophages, and again this activity was dependent of the presence of hβc. At high concentrations of hIL-3, cessation of growth was evident (data not shown). The changes in morphology, increases in surface marker expression, and cessation of growth induced by hIL-3 and hGM-CSF were quite similar (Fig. 6, A and B). In contrast, when analogous M1 cell lines containing the hIL-3Rα SP1 isoform were examined, hIL-3 stimulated none of the changes observed for SP2, although a slight increase in F4/80 expression and a slight reduction of growth was detected. The expression of the introduced receptors was verified by FACS (supplemental Fig. S1). The activity of the hIL-3Rα SP2 isoform in the presence of hSP1 was tested in M1 cells by ectopically expressing both receptors together with hβc. In these circumstances the hSP2 isoform still supported differentiation but there were differences compared with SP2 alone. Although there was a significant increase in F4/80, there was no detectable increase in Mac-1. Also, the
differentiated cells appeared less mature (supplemental Fig. S3).

**hIL-3Ra in Differentiation of WEHI-3B Myelomonocytic Leukemia Cells**—WEHI-3B D+ is a mouse myelomonocytic cell line capable of monocytic differentiation. Previous studies have shown that the differentiation of WEHI-3B D+ cells expressing hGM-CSFR/h9251/h9252 into monocytes is stimulated by hGM-CSF (37), and we verified this finding in our experiments (Fig. 7A). hGM-CSF also promoted elevated expression of Mac-1 and F4/80 (Fig. 7B), consistent with the differentiation into monocytes. Either hIL-3Ra SP1 or SP2 was introduced in combination with hβc, and the expression of the exogenous receptors was verified by FACS (supplemental Fig. S1). In contrast to FDCP-mix and M1 cells, the human SP1 isoform and not the SP2 isoform was the most active in stimulating the differentiation of WEHI-3B D+ cells into monocytes (Fig. 7A). Morphological differentiation was associated with elevated expression of Mac-1 and F4/80 (Fig. 7B) and a reduction in cell numbers (Fig. 7C). Thus in the three different models, the human IL-3Ra SP1 and SP2 isoforms showed differential activity in supporting differentiation dependent on the cellular context and signaling environment.

**Mechanisms of hIL-3-supported Differentiation**

It was of interest to determine whether IL-3 signaling promoted differentiation by similar mechanisms to those established for steady-state blood cell formation. A variety of studies of normal hematopoiesis has shown that lineage determination is the result of the combinatorial action of several transcription factors (38). Additionally, there is growing evidence of the important role of miRNAs in regulating hematopoietic differentiation (39, 40). We studied the changes in the expression of relevant transcription factors and miRNAs in FDCP-mix, because this is a non-leukemic multipotenti-
in M1 cells directed along the monocyte differentiation pathway by hIL-3 signaling via the SP2 isoform. In contrast, the expression of GATA-2 was not significantly changed (Fig. 8B).

**Induced Changes in Expression of miRNAs**

The levels of eight miRNAs known to be involved in the regulation of differentiation and hematopoiesis were measured to determine if miRNAs were likely to be involved in regulating the IL-3-supported differentiation of FDCP-mix (Fig. 8C) and M1 (Fig. 8D). In differentiating FDCP-mix cells miR-15a, miR-181a, and miR-223 were noticeably elevated. Human miR-15a has been shown to suppress Bcl-2 and to be down-regulated in B-lineage chronic lymphoid leukemia (47). In mouse, miR-181a is up-regulated in myoblast differentiation and is involved in lymphopoiesis such as B-cell differentiation (48), whereas miR-223 behaves like a myeloid gene and is up-regulated in myeloid differentiation associated with an increase in the levels of PU.1 and C/EBPa (49).

In differentiating M1 cells, miR-223 was also elevated, but the other major increase was in miR-155, an miRNA previously reported to be oncogenic, correlated with granulocyte/monocyte expansion and induced during the macrophage inflammatory response (50).

**Downstream Signaling Pathways Activated by the SP1 and SP2 Receptors**

Preliminary studies of the signaling pathways activated by the two IL-3Rα isoforms were carried out in M1 cells. Both receptors activated the Jak2/STAT5, Akt, and Erk1/2 signaling pathways. Interestingly, the SP2 receptor gave more prolonged activation of Jak2, Akt, and Erk1/2 (Fig. 8E). More detailed studies are in progress to try and understand the differences in signaling from the two receptor isoforms.

**DISCUSSION**

The importance of alternative RNA splicing in the generation of genetic diversity is now widely accepted. Different receptor isoforms can have unique properties, and abnormal overexpression of particular isoforms has been linked to disease pathogenesis. The alternatively spliced isoforms of the FGF receptor (51) and of CD44 (52) are well studied examples. In the present work we have identified a new ectodomain isoform of IL-3Rα, which is expressed in mouse and human hematopoietic cells and which lacks the first domain of the receptor. We have shown that IL-3Rα SP2 can signal for IL-3-dependent growth in CTLL-2 cells in the presence of an
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Figure 7. Effect of hIL-3R SP2 and SP1 isoforms on the differentiation of WEHI 3B D+ cells in response to hIL-3. Responses to hIL-3 of cells expressing the hSP1 or hSP2 isoforms with and without hβc after 4 days incubation were compared with responses to hGM-CSF of cells expressing hGM-CSFRα and hβc. A, morphological examination by light microscopy after staining with May-Grunwald Giemsa. Scale bar = 10 μm. B, representative FACS analysis of expression of the surface markers, F4/80 and Mac-1. C, suppression of growth on WEHI 3B D+ cells by hIL-3Rα SP1 and hβc in response to hIL-3. Growth assay was performed by counting the cell numbers following trypan blue staining at the end of the differentiation assay. Data are mean from three independent experiments; error bars are standard deviations, *, p = 0.03; **, p = 0.0002 by Student’s t test.

appropriate β receptor. mIL-3Rα SP2 cannot signal with mβc, but only with β3 upon mIL-3 stimulation, raising the possibility that the β3 receptor may have a unique function not fulfilled by mβc. Previous investigations have not found any unique role for this receptor (53), although more detailed studies on IL-3 functions in immune responses and in inflammation are required. In the case of the hIL-3R, both the hIL-3Rα SP1 and SP2 isoforms interact with hβc. The question arises as to whether SP2 isoforms also exist for IL-5Rα and GM-CSFRα. With hIL-5Rα, no high affinity binding remains when domain 1 is deleted (54), and all three domains contribute to IL-5 binding (55). The role of domain 1 in GM-CSFRα has not been investigated. We were unable to find evidence of analogous SP2 isoforms for hIL-5Rα or GM-CSFRα in the mouse and human expressed sequence tag databases.

Mutation of the β3 residues involved in direct IL-3 binding enabled us to show that the mIL-3Rα SP1 and SP2 isoforms have different mechanisms for receptor activation. High affinity binding with mouse IL-3Rα SP2 requires each of the residues of β3 involved in direct IL-3 binding. In contrast, activation of the receptor by mIL-3Rα SP1 does not involve the direct binding site. This difference is exemplified by the Y21A mutant of β3, which gives normal growth signaling with mIL-3Rα SP1, but is unable to signal with mIL-3Rα SP2 upon mIL-3 stimulation. The mIL-3Rα SP2 isoform provides a rationale for the existence of the direct IL-3 binding site on β3, which was previously a puzzle because it appeared to play no part in IL-3 signaling with the SP1 isoform.

The ectodomain isoforms of the IL-3 receptor share some similarities with those of the FGF receptor. The FGF receptor is composed of three Ig-like domains, with domains 2 and 3 forming an FGF binding region. Through alternative splicing affecting the C-terminal half of domain 3, each of the Fgr-1→3 genes generates two ectodomain isoforms with distinct FGF binding specificity, tissue-specific expression, and biological response (51). In the case of IL-3Rα, domain 1 is an Ig-like domain and domains 2 and 3 constitute the classic cytokine receptor homology module, which typically binds ligand at the elbow region. Very recently, structures of the IL-13/IL-4 receptor complexes have shown the nature of the cytokine-receptor interactions involving both the Ig domain and domains 2 and 3 of IL-13Rα1 (26). An important concept arising from the work with the IL-13/IL-4 receptor system is that different ectodomain interactions (via IL-13 and IL-4) with the same receptor system can give different signaling outcomes (26). As discussed below, we have shown that the SP1 and SP2 ectodomain isoforms of hIL-3Rα interacting with IL-3 and hβc can also give different signals raising interesting questions as to the mechanisms involved.

The lack of cross-species activity of hIL-3 in mouse cells enabled us to investigate the signaling properties of the hIL-3Rα isoforms in three in vitro models of cellular differentiation.
It is significant that both the hIL-3Rα SP1 and SP2 isoforms could signal for growth in CTLL-2 cells co-expressing hβc, but there were clear differences in the signaling properties of the two isoforms in the differentiation models. In FDCP-mix and M1 cells, the hIL-3Rα SP2 isoform was active in supporting growth, but did not support differentiation of either cell line but supported growth with retention of blast morphology in FDCP-mix, consistent with previous detailed studies showing promotion of self-renewal by this isoform (29). The differentiation of FDCP-mix to basophil-like cells in the present work is consistent with the marked effects of IL-3 on the basophil lineage in vivo. In WEHI-3B cells, the hIL-3Rα SP1 and SP2 isoforms also had different signaling properties, but in this case SP1 was active in supporting differentiation, whereas SP2 was not. Thus, the signaling properties of the two isoforms in these assays are dependent on the cellular context.

In FDCP-mix cells undergoing hIL-3Rα SP2-supported differentiation, the major up-regulation of C/EBPα and the increase in PU.1 are analogous to the changes in levels of these transcription factors during steady-state granulopoiesis. C/EBPα plays a primary role in the fate decision of mouse basophil/mast cell progenitors (30), and PU.1 stimulates monocyte/macrophage development (42). Id1, NFYA, and c-Myb, which have been reported to be involved in proliferation, were down-regulated in SP2-supported differentiation of FDCP-mix cells. Recently, miRNAs, a class of ~22-nucleotide non-coding RNAs were discovered to play important regulatory roles in mammalian hematopoiesis (40). The observed up-regulation of miR-223 in this study has also been reported to be associated with up-regulation of PU.1 and C/EBPα in steady-state blood cell formation (49). It is believed that C/EBPα has a specific function in granulopoiesis: replacing nuclear factor I-A on the miR-223 promoter and up-regulating miR-223 transcription (39). The increase in miR-223 levels results in down-regulation of the nuclear factor I-A protein and stimulation of granulocytic differentiation. Thus, the differentiation promoted by IL-3R signaling, which may normally be utilized for inducible hematopoiesis in response to infections, may employ similar mechanisms to those involved in normal blood cell formation. The intracellular signaling links from ligand binding to the transcriptional responses involved in IL-3-dependent differentia-
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