Signal Transduction by the Receptors for Thrombopoietin (c-mpl) and Interleukin-3 in Hematopoietic and Nonhematopoietic Cells


MEMBERS OF THE hematopoietin receptor gene family are characterized by sequence-similar motifs in their extracellular and, for several of the signal transducing subunits, also in their intracellular domains. The family can be divided into groups based on the identity of shared signal transducing subunits. The groups consist of receptors that use (1) gp130 (for the interleukin-6 [IL-6]-type cytokines IL-6, IL-11, leukemia inhibitory factor [LIF], oncostatin M [OSM], and ciliary neutrophil factor [CNTF]), (2) IL-2 receptor γ (IL-2Rγ for IL-2, IL-4, IL-7, IL-9, IL-13, and IL-15), and (3) IL-3Rβ (for IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor [GM-CSF]). The receptors for granulocyte-CSF (G-CSF), erythropoietin (EPO), prolactin (PRL), and growth hormone (GH) are considered as a homodimeric complex.

Based on its sequence, c-mpl has been recognized as a member of the hematopoietin receptor family before its ligand thrombopoietin (TPO) was identified. The signaling capacity of c-mpl was recognized in several chimeric receptor contexts, ie, its viral counterpart, v-mpl, which was constitutively active and counter, for the quantitative difference in IL-3 and TPO response, two structural motifs (box 1 and box 2) in the cytoplasmic domain of c-mpl was recombined with the extracellular domain of the IL-4Rα or G-CSFR. Antisense oligonucleotides to c-mpl were found to block its expression and subsequent effects on megakaryocyte progenitor cells.

The function of most hematopoietin receptors has been defined by the ability of the receptors to control proliferation of hematopoietic cells. To achieve a proliferative cell response, two structural motifs (box 1 and box 2) in the cytoplasmic domain of the signaling subunits are required. It is likely that these motifs are involved in binding and activation of accessory proteins, in particular the protein tyrosine kinases, JAK1 and JAK2, and signal transducing proteins (STATs). Several hematopoietin receptors have also been noted to control the expression of immediate growth response genes and the transcriptional activity of differentiated genes.

We established hematopoietic cells as experimental systems to characterize the signaling mechanisms of hematopoietin receptors involved in the stimulation of gene transcription via cytokine-responsive elements of the acute-phase plasma protein genes. These systems proved to be useful not only for dissecting the function/structure relationship of receptor forms intrinsic to liver, such as those for IL-6-type cytokines, but also nonhepatic receptors, because ectopically expressed receptors could effectively recruit the signal transducing system of the host cells. By comparing the specificity of gene regulation, we recently observed that hematopoietin receptors exert several signals that are distinguishable by the activation of specific gene elements. Every tested hematopoietin receptor elicited a “hematopoietin receptor signal” that required minimally the presence of the box 1 motif. The signal derived from box 1 and 2 was detectable by the activation of the “cytokine response element (RE)” of the rat α2-acid glycoprotein gene and the signal from box 1, 2, and 3 was defined by the activation of “IL-6 RE” present in several rat APP genes, such as β fibrinogen, haptoglobin, and hemopexin. Before its ligand thrombopoietin was known, the specificity of c-mpl

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signaling had been identified by incorporating its transmembrane and cytoplasmic domain into the chimeric receptor G-CSFR-mpl. G-CSFR-mpl was responsive to G-CSF in liver cells and displayed the same specificity as the receptors for the IL-6-type cytokines in which the box 3 cytoplasmic motif and carboxy terminal portion had been truncated, i.e., G-CSFR(56), G-CSFR-gp130(109), and G-CSFR-LIFR-(140). With the cloning of the thrombopoietin, the gene-regulatory action of the native c-mpl could now be assessed. Given the structural similarity of c-mpl with the IL-3R-chain and the known, albeit weak, synergistic activity of IL-3 on megakaryocyte progenitors, we hypothesized that c-mpl might form part of a novel IL-3-responsive receptor complex. Using heterologous receptor reconstitution assay systems, we show that c-mpl mediates gene induction in hepatic cells and activation of STAT proteins in fibroblasts with a specificity similar to the IL-3R and that this action is independent of coexpressed IL-3R subunits. Moreover, a cell line-specific difference in the cytokine responsiveness of c-mpl, but not the IL-3R in transfected human and rat hepatoma cells, suggests that these two receptor types use in part distinct signal transduction mechanisms for mediating the gene-regulatory effects.

MATERIALS AND METHODS

Cells. Rat hepatoma H-35 cells (clone T-7-18) and human hepatoma HepG2 cells were cultured as described. Mouse L-fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, COS-1 cells in minimal essential medium containing 5% fetal calf serum, and human erythroleukemia (HEL) cells and Mo7e cells (provided by Dr M. Caligiuri, Department of Medicine, RPCI, Buffalo, NY) in RPMI 1640 containing 10% fetal calf serum and IL-3 for the latter cell type. All tissue culture media used in this study contained penicillin, streptomycin, and gentamycin.

Megakaryocyte colony assays. Adult human low-density bone marrow cells were selected for CD34 using the O-sialoglycoprotease method of Sutherland et al. The effects of antisense oligonucleotides AS3 (GGCCCAGGAGGGCATCTT) and AS6 (TGCTGTTACTGCTGCTGATCA-3') and gentamycin. Culture media used in this study contained penicillin, streptomycin, and gentamycin.

Receptor expression vectors. The expression vector used in this study are listed in Table 1. Several vectors have been described before. Human c-mpl cDNA was cloned from HEL cell cDNA by the polymerase chain reaction (PCR) using oligonucleotides based on the published sequence and subcloned into the expression vector pME18S (a gift of Drs A. Miyajima and K. Maruyama, DNAX, Palo Alto, CA). pGMR138 was used to express the human GM-CSF receptor a-subunit. A clone encoding the IL-3Ra-subunit was derived by PCR from TF-1 cell cDNA using oligonucleotides based on the published sequence and was subcloned into pME18S. An expression construct encoding the IL-3R-3/2-subunit was kindly provided by Dr A. Lopez (IMVS, Adelaide, Australia). The chimeric human G-CSFR-murine mpl construct was derived from env-mpl. Briefly, the cDNA fragment encoding the transmembrane and cytoplasmic domain of murine c-mpl were synthesized by PCR and ligated into the expression vector pDC-302 containing the cDNA fragment encoding the 598 amino acid extracellular domain of human G-CSFR. From this construct we prepared the internal deletions (Δ1, Δ2, Δ3) and carboxy terminal truncations (Δ4, SS, and SC) according to Bénit et al. The expression vectors for cDNA to murine JAK2 and the dominant-negative kinase mutant JAK2ΔVIII in pEF-BOS have been described.

CAT reporter gene constructs. To identify the signaling of transdifferentiated and endogenous receptors, the reporter gene constructs listed in Table 1 were used. Plasmid p(8XHRRE)-CAT contains eight tandem copies of the 27-bp HRRE sequence (5'-GATCCA-TCTCTGGAATCTGTGACA-3') with the APRE core nucleotides. In the Bgl II site of pCAT promoter (Promega, Madison, WI), pHX(5XIL-6RE)-CAT contains 5 tandem copies of the 23-bp IL-6RE of the rat hemopexin gene, and p(4XCyR)-CAT contains 4 tandem copies of the high-affinity sis-inducible element S1Em67. Plasmid AGP(4XCyR)-CAT contains 4 tandem copies of the 62-bp AB region of the distal regulatory element of the rat a1-acid glycoprotein in the enhancerless pSV-CAT.

Cytokines. cDNA to full-length TPO was cloned by PCR using cDNA derived from human liver (Clontech, Palo Alto, CA) and oligonucleotides based on the published sequence. The cDNA was inserted into pME18S and transfected into COS-7 cells and day-3-conditioned culture medium was collected. The concentration of TPO was estimated by titration on BAF/c-mpl cells to contain 5 × 10^2 U/mL (in which 50 U correspond to 50% maximal stimulation). Conditioned medium from mock-transfected COS-7 cells did not produce any stimulatory effect in the assay systems used (see, eg, Figs 2 through 4 and 5A). hGM-CSF and hIL-3 were provided by Dr F. Meyer (Sandoz, Basel, Switzerland); human G-CSF, LIF, and IL-1β by Immuneex Corp (Seattle, WA); IL-6 by Genetics Institute (Boston, MA); and mouse and human interferon-γ (IFN-γ) by Genentech (South San Francisco, CA). Unless noted otherwise, the cells

<table>
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<th>Table 1. List of Receptor Expression Vectors and Regulated CAT Gene Constructs Used in This Study</th>
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<tr>
<td>Expression Vectors for</td>
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<tr>
<td>c-mpl</td>
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<tr>
<td>G-CSFR-mpl (full-length cytoplasmic domain from 1-121)</td>
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<tr>
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<tr>
<td>G-CSFR-mpl SS (deletion from 13-121)</td>
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<tr>
<td>G-CSFR-mpl SC (deletion of entire cytoplasmic domain)</td>
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<tr>
<td>IL-3Rβ</td>
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<tr>
<td>GM-CSFRα</td>
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<tr>
<td>JAK2</td>
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<td>JAK2ΔVIII</td>
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Receptor-Responsive CAT Gene Constructs

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<tr>
<td>p(8XHRRE)-CAT</td>
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<td>pHX(5XIL-6RE)-CAT</td>
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<tr>
<td>pAGP(4XCyR)-CAT</td>
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<td>pH4SIE)-CAT</td>
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were treated with 100 ng/mL cytokines, with the exception of IL-1β, which was used at 0.5 ng/mL, and TPO, which was used at 5,000 U/mL.

**Cell transfection and analysis.** Plasmid DNA was transfected into H-35 cells, L cells, and COS-1 cells by the diethylaminoethyl (DEAE) dextran method. HepG2 cells were treated with calcium phosphate mixture. Established conditions for optimal reconstitution of receptor function were used. Expression of receptor mRNA in transiently transfected hepatoma cell cultures were verified by Northern blot analysis, as described. However, the low transfection efficiency precluded an immunoochemical quantitation of the receptor proteins in these cultures. Receptor protein could be detected by Western blotting in transiently transfected fibroblasts (see Fig 7).

**CAT gene regulation was determined in cells transfected with a plasmid mixture containing a total of 10 μg DNA/mL and consisting of 6 μg CAT reporter gene construct, 1 μg receptor expression vector, 1.3 μg pIE-MUP as an internal marker, and, where necessary, empty expression vector.** After 16 hours of recovery, the transfected cell culture was subdivided into 6-well cluster plates. After 24 hours, the subcultures were treated with serum-free medium containing 1 μmol/L dexamethasone and cytokines for an additional 24 hours. Cell extracts were prepared and the CAT activities were determined by using 10-fold serially diluted extract to ensure measurement in the linear range of the enzyme assay system. The CAT activity was calculated for the entire cell extract preparation and then normalized to the amount of the major urinary proteins derived from the internal standard plasmid pE-MUP. The values were expressed relative to the untreated control cultures (defined as 1.0).

**Identification of the STAT proteins was performed for 15 minutes in serum-free medium following by analysis of activated STAT proteins.**

**Transfection into COS-1 cells occurred in 10-cm diameter culture dishes using 1 μL of DNA-DEAE dextran mixture composed of 10 μg receptor expression vector, 2 μg of p(8XRRE)-CAT, and 2 μg pIE-MUP.** The transfected cultures were divided into subcultures. After 24 hours, two subcultures in 10 cm² wells of a 6-well cluster plate were treated for 24 hours with serum-free medium alone or containing cytokines and then processed to determine specific CAT activity. The remaining cultures were maintained for 16 hours in serum-free medium. Cytokine treatment was performed for 15 minutes in serum-free medium followed by analysis of active STAT proteins.

**Transfection into COS-1 cells occurred in 10-cm diameter culture dishes using 1 μL of DNA-DEAE dextran mixture composed of 10 μg receptor expression vector and 2 μg pIE-MUP.** After an overnight recovery, the transfected cell culture was divided into two. Twenty-four hours later, the subcultures were maintained for 16 hours in medium containing 10 mmol/L sodium vanadate, 10 mmol/L NaCl, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). In the case of COS-1 cells, one quarter of the recovered cells was used for Western blot analysis of receptor. Whole cell extracts were prepared according to the procedure of Sadowski et al. The STAT proteins were then analyzed by electrophoresis on 6\% SDS-PAGE gels, transblotted to nitrocellulose membranes, and probed with either rabbit antihuman JAK1 or rabbit antimouse JAK2 (Upstate Biotechnology, Inc. Lake Placid, NY) and antimouse JAK2 (Upstate Biotechnology, Inc. Lake Placid, NY). The membranes were then briefly incubated with hydrogen peroxide, washed three times with phosphate-buffered saline (PBS) and then treated with rabbit antigoat IgG followed by 125I-labeled goat antirabbit Ig and exposed for 3 days to X-ray film. For analyzing expression of JAK1 and JAK2, equal amounts of Triton-X-100 was loaded onto a 6% SDS-PAGE gel. After 24 hours, the subcultures were treated with serum-free medium as well as the medium used for treatment were measured for TPO activity by bioassay on BAF/c-mpl cells. TPO expression by CD34+ bone marrow stimulated by IL-3 plus aplastic anemia serum (AAS) as a source of TPO (Fig 1). These results are consistent with the known action of c-mpl as the receptor for TPO. Intriguingly, CD34+ bone marrow stimulated by IL-3 plus AAS was cultured for 1, 2, and 3 days. The results suggested that IL-3 might act on CD34+ via three alternative pathways: (1) IL-3 stimulated the production of TPO; (2) TPO was produced by accessory cells in the cell culture, or was introduced for the assay medium, and synergized with IL-3; or (3) IL-3 signaled via a c-mpl-containing receptor complex. To test the first two possibilities, cultures of CD34+ cells were stimulated with IL-3 and the resulting conditioned medium as well as the medium used for treatment were measured for TPO activity by bioassay on BF/c-mpl cells. TPO expression by CD34+ cells was also determined by RTPCR for TPO mRNA. In neither assay system was TPO production detected (data not shown). We, therefore, assessed whether AS3 has lowered expression of IL-3Ra or IL-3Rβ by mRNA analysis of the treated cells. However, we were unable to detect any effect of AS-3 on IL-3Ra or IL-3Rβ mRNA expression in TF-1 cells (data not shown).
but comparable levels of polyadenylated mRNA of the predicted sizes (data not shown). The CAT activities measured in the transfected cells indicated that c-mpl mediated a TPO-specific increase of the reporter gene expression. However, the untreated cells showed an elevated basal expression that ranged from 7- to 30-fold, depending on the final density of the test cultures. A minor autocrine stimulation appeared to occur in H-35 cells. Indeed, when adding concentrated conditioned medium of untransfected and untreated H-35 cells to c-mpl-transfected HepG2 cells (which do not show signs of autocrine stimulation; see below) a c-mpl-dependent CAT gene activity above the control levels was observed. No autocrine action nor TPO response was detectable when either IL-3R, GM-CSFR, or G-CSFR-mpl were transfected in H-35 cells. The assay data also illustrate that the COS-cell–derived TPO preparations did not contain detectable stimulatory activities other than TPO. The maximal level of CAT gene regulation achieved with TPO-activated c-mpl was comparable to that observed with the endogenous IL-6R or with the other transfected hematopoietin receptors.

The specificity of the c-mpl signal for gene-regulatory elements was assessed by cotransfecting c-mpl and CAT reporter gene constructs containing various characteristic response elements into H-35 cells (Fig 3). Despite a low autocrine response, a TPO-mediated activation of the CytRE of the rat α1-acid glycoprotein gene was obtained. No regulatory effect was recorded with the high-affinity sis-inducible element of the c-fos gene nor with the IL-6RE of rat hemopexin gene. The responsiveness of each reporter gene used was defined in the same cell cultures by the stimulation via the appropriate endogenous cytokine receptor.

The data indicated that c-mpl generated gene-regulating signals in H-35 cells that were, in part, similar to those generated from the endogenous receptors for IL-6–type cytokines. The specificity of gene regulation by c-mpl coincided with that of G-CSFR–mpl, IL-3R, and GM-CSFR (data not shown). Because c-mpl, IL-3R, and GM-CSFR activated both HRRE and CytRE but not the IL-6RE, we concluded that these receptors are devoid of a box 3-restricted signal capability.

c-mpl shows cell-line specific differences in signaling reaction. H-35 cells, among different hepatic tissue culture cells, have proven to be best suited for defining the signal specificity of hematopoietin receptors. However, because we expressed human c-mpl in rat cells, we repeated the analysis with human HepG2 cells to assess whether a species-restricted signal pathway existed for c-mpl. In the transiently transfected HepG2 culture cells, as in H-35 cell cultures, quantitation of receptor protein was technically not feasible. However, Northern blot analysis indicated that all receptor constructs were expressed and that an approximately 2- to 10-fold higher level of each receptor mRNA was achieved in HepG2 cells than in H-35 cells. The highest expression among the receptor constructs was consistently observed with G-CSFR–mpl (data not shown). In separate experiments, we had verified that the amount of each receptor expression vectors used for transfection of two hepatoma cell lines was greater than what was needed for gaining maximal cell regulation (see also Baumann et al.).

The CAT reporter gene responses (Fig 4) indicated that
c-mpl exerted essentially the same element-specific regulation in HepG2 cells as observed in H-35 cells. The magnitude of regulation appeared lower, in part, because of the substantially higher basal expression of the reporter gene constructs in HepG2 cells. There was no enhanced basal expression observed in untreated c-mpl-transfected HepG2 cells, suggesting the absence of an autocrine stimulatory loop. Under the conditions used in Fig 3, the magnitude of c-mpl-mediated gene regulation was in the range of that of transfected G-CSFR–mpl and IL-3R or the endogenous IL-6R.

However, dose-response analyses of H-35 and HepG2 cells transfected with identical combinations of receptors indicated a marked difference in the sensitivity of c-mpl action. Direct comparison between these cell lines was possible, because the cytokine response of the reporter gene could be internally normalized to the action of the endogenous IL-

### Table 1

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<th>IL-3Rα+</th>
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### Table 2

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### Figure 2

Gene-regulatory action of hematopoietin receptors in H-35 cells. Cultures of H-35 cells were transfected with a mixture of plasmid DNAs containing p(8xHRRE)-CAT, pIE-MUP, and expression vectors for the receptor subunits listed at the top. Subcultures of each transfection were treated with the factors indicated at the bottom. CAT activity was determined in 5 μL out of 100 μL extract from each culture (autoradiogram of the thin-layer pattern is shown). In all those cases in which the CAT activity was outside of the linear range of the enzyme assay (>80% conversion of chloramphenicol substrate to acetylated products), 1/10 and 1/100 dilution of the extracts were tested. The CAT activity was normalized to MUP and expressed relative to the CAT activity of control H-35 cell cultures that had not received a receptor expression vector (defined = 1.0; picture of control cultures not shown). The relative values are given above the autoradiogram of the thin-layer pattern.

### Figure 3

Gene element specificity of c-mpl action. H-35 cells were transfected with an expression vector encoding c-mpl together with the CAT reporter gene constructs listed at the top. The subcultures were treated as indicated at the bottom. The CAT activities were normalized to the control cultures for each CAT reporter gene. The relative values are shown above the autoradiogram.
transfected with p(8xHRRE)-CAT, PIE-MUP, and the expression vectors for the receptor subunits listed at the top. The subcultures were treated as indicated at the bottom and the specific CAT activity determined relative to the controls.

Fig 4. Receptor action in HepG2 cells. HepG2 cells were transfected as H-35 in Fig 1 with mixtures of plasmid DNAs containing p(8xHRRE)-CAT, PIE-MUP, and the expression vectors for the receptor subunits listed at the top. The subcultures were treated as indicated at the bottom and the specific CAT activity determined relative to the controls.

6R and transfected IL-3R (Fig 5A). The IL-3R produced a similar dose response to IL-3 in both cell types with half-maximal activation of the reporter gene at 0.03 to 0.1 ng/mL. TPO dose response in c-mpl-transfected H-35 cells yielded a maximal stimulation with 1/100 diluted COS-cell–derived TPO preparation (5,000 U/mL) with a half-maximal stimulation at 1 × 10^{-4} dilution (~5 U/mL). By contrast, in c-mpl–transfected HepG2 cells, no true maximal stimulation of the reporter construct was achieved under the conditions used in Fig 5A. When compared with the H-35 cell response, c-mpl in HepG2 cells was approximately 100 times less sensitive to TPO.

Two potential mechanisms could explain the cell-type difference in c-mpl action: (1) an additional receptor subunit is needed for optimal c-mpl action, and this subunit is more prevalent in H-35 cells than in HepG2 cells; and/or (2) the c-mpl–mediated cytoplasmic signaling reaction requires specific mediators and HepG2 cells, but not H-35 cells, are deficient in those. Although additional subunits have been described for many other receptors of this family, the latter explanation appeared more likely to apply because transfected G-CSFR–mpl showed a similarly low sensitivity to ligand as c-mpl in HepG2 cells (Fig 5A). We assume that the G-CSFR does not require additional subunits for optimal dose responsiveness, although this remains to be proven. Because we could not rule out the participation of an additional receptor subunit in c-mpl function, we tested combinations of c-mpl with other cloned members of hematopoietin receptors for enhanced TPO response in HepG2 cells. We were unable to achieve any functional cooperativity with any subunits known to participate in heterologimerization, i.e., IL-2Rβ or -γ, IL-3Rα or -β, GM-CSFRα, LIFR, IL-4R, IL-7R, and gp130 (Fig 5B, and data not shown) and conclude that none of the tested receptor subunits are part of the functional TPO receptor. We hypothesize that c-mpl acts as a homodimer or homo-oligomer and that c-mpl and IL-3R, although having a comparable specificity of gene regulation, do not use identical signal transduction pathways accounting for the cell-line–specific difference.

c-mpl mediates STAT protein activation. Signal initiation by hematopoietin receptors is accompanied by the activation of STAT proteins that are generally recognized by their binding to the high-affinity SIE sequence, forming large molecular size complexes migrating with mobilities such as observed for SIF-A, -B, and -C on polyacrylamide gel.

STAT proteins have been suggested to partake in stimulating transcription of specific genes, yet a causal role in regulating genes in cells remains to be proven in most instances. Although c-mpl was unable to stimulate gene expression via SIE or IL-6RE (Fig 3), a process that has been suggested to involve SIF-A complex containing a dimer of STAT3 (or APRF), we attempted to determine whether the action of c-mpl involved an activation of any SIFs. Therefore, we transfected c-mpl into L and COS-1 cells that yielded a higher transfection efficiency and receptor expression levels than did hepatoma cells and thus enabled a characterization of the receptor-initiated signaling event. Moreover, in L cells, the function of the transfected receptor could also be monitored by the stimulation of p(8xHRRE)-CAT reporter construct as in hepatic cells, albeit at much lower magnitude (Fig 6). Both c-mpl and G-CSFR-mpl reconstituted not only a HRRE-CAT gene stimulation (Fig 6A), but also an induction of SIFs (Fig 6B).

The SIF complex that was activated by c-mpl, comigrated with the IL-6–induced SIF-A and each of these complexes was recognized by anti-STAT3 but not anti-STAT1 antibodies, as evidenced by supershift assay (Fig 6E and data not shown). This finding suggests that the c-mpl was able to activate STAT3 containing DNA-binding complex similar to what has been defined for IL-6R.

The relative activation of SIFs by G-CSFR–mpl was somewhat higher than by c-mpl. This quantitative difference was even more prominent in COS-1 cells (Fig 6C), which have an approximately 10-fold higher expression level of transfected plasmids than L cells. Of note is that, although c-mpl induced in L cells primarily the SIF-A (Fig 6B), in COS-1 cells, c-mpl, like other transfected hematopoietin receptors, such as IL-3R, stimulated an SIF complex that migrated with SIF-C (Fig 6C), the SIF form that predominates in IFN-γ–treated cells and probably is composed of STAT1 homodimer. By antibody reaction, we verified that most of the c-mpl–induced SIF in COS-1 cells appeared to contain STAT1 but not STAT3 (Fig 6E and data not shown).

Because we have determined the SIF-inducing action of c-mpl in transfected cells of nonhematopoietic lineages, we corroborated this receptor activity in HEL and Mo7e cells that normally express c-mpl (Fig 6D). As observed for L cells, TPO treatment of the two leukemic cell lines produced
Fig 5. Dose response of receptor-transfected hepatoma cells. (A) H-35 and HepG2 cells were transfected with p(8xHRRE)-CAT, pIE-MUP, and expression vectors for either c-mpl, G-CSFR-mpl, or the combination of IL-3Rα and β. The subcultures were treated with 10-fold serially diluted cytokine preparations. The stock solutions of cytokines (10⁻⁸ dilution) were conditioned medium from mock-transfected COS cells (COS CM) and from COS cells expressing TPO (both applied to c-mpl-transfected cells), 1 μg/ml G-CSF (applied to G-CSFR-mpl transfected cells), and 1 μg/ml IL-3 (applied to IL-3Rα- and β-transfected cells). The relative change in CAT activities for each experimental series was determined. The mean value of the IL-6-mediated stimulation of CAT expression in the various series constituting this experiment is indicated. (B) Signaling activity of combinations of c-mpl and IL-3R subunits. HepG2 cells were transfected with expression vectors encoding the receptor subunits listed. The transfected cultures were treated with 10-fold serially diluted TPO or IL-3 (as in A) or with IL-6. The relative change in CAT activity in each experimental series was determined. Mean value and SD of the IL-6 treatments were calculated for all series and are indicated.

an increase of the SIF form comigrating with SIF-A and immunoreacting with anti-STAT3 (Fig 6E). Unlike hepatoma cells and fibroblasts (Fig 6B and C), HEL cells displayed a high basal activity for SIF activity comigrating with SIF-B and -C.

Taken together, these results indicate that the signaling functions of c-mpl or the chimeric G-CSFR-mpl construct include STAT protein activation and stimulation of gene transcription via HRRE and CytRE. Yet to be determined is whether these two processes are causally connected.
Identification of functionally relevant subregion in the cytoplasmic domain of c-mpl. To define the cytoplasmic domain regions that are required for signaling function, a series of deletions were introduced into G-CSFR–mpl constructs (Fig 7, top). The deletions were patterned after the series used by Bénit et al.12 for identification of the regions controlling pathogenicity of v-mpl. The mutations selectively removed either box 1 motif (Δ1), box 2 motif (Δ2), or the carboxy terminal part containing the regions with tyrosine residues in a sequence context potentially involved in signaling functions (Δ3 and Δ4). The reasons for using G-CSFR–mpl rather than c-mpl were (1) this receptor form was highly active in hepatic and fibroblastic cells; (2) the gene-regulatory action could be determined in H-35 cells without autocrine contribution; and (3) the receptor expression could be assessed by using the available anti-G-CSFR antibodies.

The vectors for the various receptor mutants directed a similar level of receptor protein expression in transiently transfected COS-1 cells as determined by Western blot analysis (Fig 7, top). The same expression vectors, together with HRRE-CAT gene introduced into H-35 cells, indicated that Δ3 mutant was equally active as the full-length c-mpl. No gene-stimulatory activity was detectable for Δ1 and the truncated forms, SS and SC. Approximately 10% of the activity of the full-length c-mpl form was measured for Δ2 and Δ4. SIF activation profile correlated with gene regulation with the exception of mutant Δ2, which had low gene regulatory activity but no detectable action on STAT proteins. Transfection of pairwise combinations of Δ1 and Δ2, Δ1 and Δ4, or Δ2 and Δ4 failed to produce a complementation of the signaling function to the wild-type level (data not shown). In summary, the data suggest that (1) signaling of c-mpl is critically dependent on the presence of the membrane-proximal segment containing the box 1 motif; (2) maximal action requires the region of the box 2 motif and the carboxy terminal 22 amino acid segment; and (3) HRRE regulation is not strictly correlated with the ability of c-mpl to activate SIF.

JAK2 participates in c-mpl signal communication. It has been shown for several hematopoietin receptors, such as GHR, EPOR, and IL-3Rβ, that the box 1 motif was critical for activation of JAK2.7-5 The results of Fig 7 suggest that JAK2 may also play a role in signaling by c-mpl via interaction with the box 1 motif. Furthermore, we hypothesized that the cell-line–specific difference in c-mpl action described in Fig 5A may be caused by different levels of JAK2 expression or by differences in the availability of JAK2 for action of the transfected receptors. One indication for potential JAK2 deficiency in HepG2 cells was the inability of HepG2 cells to stimulate HRRE-CAT gene expression with transfected receptor constructs containing just box 1 motif, such as G-CSFR-mplΔ2 (Fig 8B, below).

We determined the level of immunoreactive JAK1 and JAK2 in HepG2 and H-35 cells by Western blot analysis and observed, under the condition used, an equal signal for JAK1. However, a several-fold higher signal for JAK2 was observed in H-35 cells than in HepG2 cells (Fig 8A). Because this analysis did not provide conclusive evidence about biologically relevant levels of JAK2 in these cell lines, we assessed the potential role of JAK2 in c-mpl signaling by two alternative approaches, either by enhancing c-mpl response through overexpression of active JAK2 (Fig 8A) or inhibition of c-mpl action by overexpression of the dominant-negative JAK2ΔVIII.35 HepG2 cells appeared to be the optimal system for applying these approaches because the c-mpl response in this cell line was expected to allow for the enhancement as well as inhibition.

Transfection of increasing amounts of expression vector for JAK2 showed that, at concentrations less than 1 μg/mL, expression vector had little enhancing effect on basal activity of cotransfected p(8XHRRE)CAT construct. However, at concentrations greater than 1 μg/mL, a significant increase in basal expression occurred (data not shown). Transfection of the combination of either c-mpl or G-CSFR–mpl with JAK2 resulted in a several-fold elevation of basal expression of the CAT construct, even with a JAK2 plasmid concentration as low as 0.05 μg/mL. We established empirically the concentrations of JAK2 and receptor expression vectors (as applied in Fig 8B) that yielded an optimal manifestation of the JAK2 action on c-mpl signaling. JAK2 enhanced the regulation of the HRRE-CAT construct by c-mpl and G-CSFR–mpl to a level equal or exceeding that achieved by...
G-CSFR-MPL Constructs

Fig 7. Mutational analysis of cytoplasmic domain of c-mpl. The chimeric G-CSFR-mpl constructs indicated at the top were transfected into COS-1 cells (for determining receptor protein expression and activation of SIF activity) and together with p(BsHRRE)-CAT into H-35 cells (for determining gene regulatory activity). The receptor transfected COS-1 cell cultures were treated for 15 minutes with G-CSF. The cell lysate of one quarter of the culture was subjected to Western blot analysis of the receptor protein. The autoradiogram was exposed for 3 days, and the region of the receptor bands is reproduced. Equal aliquots of the whole cell extract from the remaining 3/4 cell cultures were used for EMSA. The autoradiogram of the SIF complexes after 24 hours of exposure is shown. The CAT activities in the G-CSF-treated H-35 cells were quantitated relative to the untreated control. The mean and SD for all IL-6 treatments in this experimental series were calculated and are indicated. The relative signaling activities of the various G-CSFR-mpl constructs were confirmed in two additional, independent experimental series.

IL-6 (Fig 8B, top two panels). The JAK2 effect was dependent on the box 1 motif in the cytoplasmic domain of c-mpl because the box 1-deficient mutant, G-CSFR--mplΔ1, remained inactive (data not shown), whereas the box 2-deficient mutant, G-CSFR--mplΔ2, showed a prominent JAK2 response (Fig 8B, bottom panel). The same experimental system also indicated that the regulation via the endogenous IL-6R was relatively unaffected by overexpressed JAK2. Similarly ineffective was JAK2 in modulating the maximal regulation by transfected IL-3R (data not shown).

To assess in HepG2 cells the contribution of resident JAK2 to the c-mpl signaling reaction, we transfected receptors together with relatively high amounts of dominant-negative JAK2ΔVIII into the cells (Fig 8C). The presence of inactive JAK2 caused a minor reduction in the basal activity of the reporter gene and attenuated the activation by c-mpl by threefold to sevenfold. In contrast, the action of the transfected IL-3R and the endogenous IL-6R was insignificantly affected.

The results in Fig 8 support a model that c-mpl requires JAK2 for producing a signal leading to gene regulation in hepatic cells. IL-3R is equally effective in controlling the same gene element as c-mpl, but uses an alternative mechanism that is not critically dependent on JAK2.

DISCUSSION

We have applied antisense oligonucleotides to c-mpl to assess the role of TPO in megakaryocytopenia. The results have supported the proposed function of c-mpl. However, the unexpected observation that AS-3 reduced the TPO and the IL-3 response of the cells in a similar fashion sparked a study in which we attempted to define signaling of c-mpl and its relationship to IL-3R action.

At present, we are unable to provide a molecular explanation for the differential effects of AS-3 and AS-6 (Fig 1). The antisense oligonucleotide experiments are not trivially explained by toxic effects of the AS-3 preparation because the experiments were reproducible with independently synthesized lots of the oligonucleotide (data not shown). The AS-3 sequence corresponds to the initiator methionine region of c-mpl, whereas the AS-6 sequence corresponds to the unusual hydrophilic insert domain of the first hematopoietin receptor repeat. A search of the GenBank by the BLAST algorithm did not yield any recorded sequence (besides human c-mpl) that contains a relevant match to the 18 nucleotides of AS-3 and AS-6. The hydrophilic insert is conserved between mouse and human c-mpl but is not found in any other member of the hematopoietin receptor family. c-Mpl is most like the IL-3R in overall structure, comprising two repeats of the hematopoietin receptor domain. Because the IL-3 subunits have no insert domain but is not found in any other member of the hematopoietin receptor family, c-Mpl is most like the IL-3R in overall structure, comprising two repeats of the hematopoietin receptor domain. Because the IL-3R subunits have no insert domain, a possible explanation for the inhibition of IL-3-induced CFU-MK-derived colony formation by AS-3 and not AS-6 is the existence of a variant or homolog of c-mpl that lacks the insert domain and signals in response to IL-3. We attempted to identify such a variant by RT-PCR of CD34+ bone marrow cells using oligonucleotides spanning the insert domain. We did not find evidence for any alternative splicing in this region leading to the formation of a membrane-bound variant of c-mpl that lacks the hydrophilic insert. Instead, a spliced variant was detected that corresponded to the deletion of the majority of the insert domain (nt 499-691) and that terminated at nt 701 (Ho, Gorvad, and Gearing, unpublished data). Because this variant truncated before the first Trp-Ser-Xaa-
Fig 8. The signaling by c-mpl is modulated by JAK2. (A) Expression of JAK1 and JAK2 in hepatoma cells. Duplicate aliquots of extracts from ~2 × 10⁷ H-35 and HepG2 cells were analyzed by Western blotting using anti-JAK1 and anti-JAK2. To show the expression of transfected JAK2, extract from HepG2 cells 36 hours after transfection with 5 µg/mL pEF-BOS-JAK2 was included in the Western blot analysis for JAK2. The section containing the immunoreactive bands representing JAK1 and JAK2 is reproduced. (B) Effect of overexpressed JAK2. HepG2 cell cultures were transfected with pEFXHRRE/CAT (6 µg/mL), expression vectors for the receptors listed at the right (1 µg/mL), and with or without pEF-BOS-JAK2 (0.2 µg/mL) serially diluted cytokines (TPO or G-CSF) or with IL-6. The CAT activity in each culture was calculated relative to the untreated control without transfected JAK2 in the respective series. (C) Effect of dominant-negative JAK2. HepG2 cells were transfected like the cells in (B) with cytokine receptors listed at the bottom and with or without pEF-BOS-JAK2ΔVIII (5 µg/mL). Subcultures were treated with the appropriate ligands indicated at the bottom. The relative stimulation of CAT expression relative to the untreated control without JAK2ΔVIII in each group was calculated. The mean and SD of three separate experiments are shown.
Trp-Ser motif, we assume that it does not represent a functional form of the soluble c-mpl, although this still remains to be tested.

Because we could not rule out the possibility that c-mpl and IL-3R subunits cooperate in CD34+ cells, we decided to characterize the signaling function of each of the participating receptor components in the heterologous systems of hepatoma cells and fibroblasts. The major advantages of choosing gene regulating signal as reporter for the receptor functions are that the receptor action can efficiently be quantitated in transiently transfected cells, categorized based on the specificity of the activated genetic targets, compared with the action of other related hematopoietin receptors, and the complexity of the suspected signaling molecules can be defined experimentally by complementation.

The reconstitution of c-mpl and IL-3R response in hepatoma cells (Figs 2 and 4) shows that the receptors are capable of recruiting the resident signal-transducing mechanisms. The magnitude and dose response of the activation of the HRRE-CAT constructs (Figs 5 and 8) illustrate that this recognition is equivalent to that mediated by endogenous IL-6-type cytokine receptors. This similarity of the cell response mediated by c-mpl and IL-3R suggests that the two receptors may use the same signaling mechanisms. However, comparison of gene element specificity (Figs 2 and 3) also indicated that neither c-mpl nor IL-3R execute the entire signaling program available in the hepatoma cells. The most distinguishing feature is the inability of c-mpl and IL-3R to activate the IL-6RE-containing reporter gene, a process that depends on a box 3 motif in the cytoplasmic domain of the signal transducing receptor subunit. Therefore, we conclude that neither c-mpl nor IL-3R cytoplasmic domain structures include a motif that is functionally equivalent to the box 3 motif of gp130.

The prominence and sensitivity of the gene regulation mediated by the transfected receptor subunits were used as evidence that c-mpl acts independently of IL-3Rα or β subunits. Moreover, because we could not ascribe a modulating activity to several other cloned hematopoietin receptor subunits, we propose that c-mpl functions as a homodimer analogous to G-CSFR, EPOR, PRLR, and GHR. The observation that G-CSFR–mpl elicits a c-mpl–like response (Fig 5) supports the model that homodimerized cytoplasmic domain of c-mpl has signaling activity. Although a c-mpl homodimer is strongly suggested, an alternative hetero-oligomeric complex that contains resident but undefined receptor subunits cannot formally be ruled out. Indeed, c-mpl seems to have the capability of signaling when present in an artificial heteromeric complex, as suggested by the report of Skoeda et al. In this system, the chimeric IL-4R–c-mpl receptor generates a proliferative response in transfected cells, probably via association with endogenous IL-2Rγ to be an alternative mechanism independent of IL-2Ry.

One caveat of testing hematopoietin receptors in a heterologous system is that the host cell may not contain cell-type–restricted signaling molecules that are needed for c-mpl action such as JAKs, STATs, src-related kinase, and their respective substrates. Moreover, the difference in the level of expression of limiting signal molecules that might be ubiquitous to all cells seems also to affect the regulatory phenotype of the receptor-transfected cell, as implied by JAK2 supplemental experiment in Fig 8. At present, we do not know of any signaling molecules that are used by c-mpl in homologous cell systems and that are not found in hepatic cells. If the heterologous systems should prove to be deficient in a component needed for c-mpl action in megakaryocytic cell types, then the cells studied here will be of value for testing the function of the component by complementation.

A part of the signaling reaction of c-mpl involves the activation of SIF-A in hematopoietic cells (Fig 6D). The DNA binding activities of the STAT protein induced by c-mpl were identified with SIE as substrate, a sequence known to be recognized by several STAT proteins (STAT-1, -3, -4, and -5). We had also applied other DNA sequences representing the IFN-γ activation site (GAS) of the Fe-receptor-γ gene (bound by STAT-1 and -3), and the PRL-response element of the β-casein gene (bound by STAT5). However, none of these elements indicated any STAT complexes in addition to those already defined by SIE (data not presented). The SIF-A activation process seen in hepatoma cells could be faithfully reproduced in c-mpl–transfected L cells (Fig 6B). The SIF-A complex in both cell types includes the STAT3 protein (Fig 6E), a known target of the EGFR and IL-6R pathway. We failed to observe a c-mpl–mediated response via SIE or IL-6RE (Fig 3) despite the capability of c-mpl to activate STAT proteins, suggesting that either the SIF activity is not sufficient for regulation through these elements or the c-mpl effect on STAT proteins is below the threshold for being effective.

Although c-mpl transfected into COS-1 cells is abundantly expressed and capable of activating SIF (Figs 6C and 7), the contributing component to the SIF complex is STAT1, not STAT3. This switch in STAT isofrom is not caused by the lack of STAT3 in COS-1 cells as seen by the abundance of SIF-A after IL-6 treatment. A possible explanation might be seen in the massive overexpression of receptor proteins in the individually transfected cells, and that in those cells STAT3 is limiting and STAT1 acts as the default target. Ongoing complementation experiments using cotransfection of expression vectors for STAT1 or STAT3 and c-mpl show that c-mpl can similarly activate STAT1 and STAT3 (Lai, Morella, Ripperger, Fey, and Baumann, unpublished data).

Aside from cell-type–specific variations in STAT protein usage, the reconstitution experiments showed convincingly that the signaling functions of c-mpl to STAT proteins and to gene elements depend on specific cytoplasmic domain regions (Fig 7). It is not surprising that the box 1 motif plays such a critical role, because equally critical requirements have been ascribed to the box 1 motif of other hematopoietin receptors inducing IL-3Rα, gp130, G-CSFR, IL-3Rβ, EPOR, and GHR for controlling proliferation and activation of immediate growth-response genes. As described previously, the deletion of box 1 from v-mpl (Δ1) abolished pathogenicity that coincides with the loss of gene regulation in our system, suggesting overlap in control mechanisms that is related to proliferation and gene expression in different assay systems. This apparent concordance in control of separate cell responses by c-mpl is not perfect, as noted by...
the analysis of deletion mutants (Fig 7). Whereas Δ3 and Δ4 mutants of env-mpl supported similar pathogenicity, the same mutant pair showed substantially different abilities to activate SIP and HRRE in our assays (Fig 7). We conclude from these results that the carboxy terminal sequence of c-mpl is needed for gaining maximal gene regulation and SIP induction but is not essential for proliferation control.

A similar organization of distinct control elements in the cytoplasmic domain has been observed for other hematopoietin receptors such as IL-3R, gp130, JAK2, and EPOR. A C-MPL was from these results that the carboxy terminal sequence of c-MPL restricted to the control of gene transcription and the activation of ras-dependent pathways for control of proliferation or CytRE appear to be comparable. Delinination of the biochemical basis of these pathways and determination of the compatibility of the signaling mechanisms among hepatic cells, fibroblasts, and hematopoietic cells will be required before postreceptor effects stimulated by TPO can be fully understood.

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Signal transduction by the receptors for thrombopoietin (c-mpl) and interleukin-3 in hematopoietic and nonhematopoietic cells

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