Circular Permutation of the Granulocyte Colony-Stimulating Factor Receptor Agonist Domain of Myelopoietin

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ABSTRACT: Myelopoietins (MPOs) are a family of engineered dual interleukin-3 (IL-3) and granulocyte colony-stimulating factor (G-CSF) receptor agonists that are superior in comparison to the single agonists in their ability to promote the growth and maturation of hematopoietic cells of the myeloid lineage. A series of MPO molecules were created which incorporated circularly permuted G-CSF (cpG-CSF) sequences with an IL-3 receptor (IL-3R) agonist moiety attached at locations that correspond to the loops that connect the helices of the G-CSF four-helix bundle structure. The cpG-CSF linkage sites (using the original sequence numbering) were residue 39, which is at the beginning of the first loop connecting helices 1 and 2; residue 97, which is in the turn connecting helices 2 and 3; and residues 126, 133, and 142, which are at the beginning, middle, and end, respectively, of the loop connecting helices 3 and 4. The N- and C-terminal helices of each cpG-CSF domain were constrained, either by direct linkage of the termini (L0) or by replacement of the amino-terminal 10-residue segment with a seven-residue linker composed of SGGSGGS (L1). All of the MPO molecules stimulated the proliferation of both IL-3-dependent (EC_{50} = 13−95 pM) and G-CSF-dependent (EC_{50} = 35−710 pM) cell lines. MPOs with the IL-3R agonist domain linked to cpG-CSFs in the first (residue 39) or second (residue 133) long overhand loops were found by CD spectroscopy to have helical contents similar to that expected for a protein comprised of two linked four-helix bundles. The MPOs retained the ability to bind to the IL-3R with affinities similar to that of the parental MPO. Using both a cell surface competitive binding assay and surface plasmon resonance detection of binding kinetics, the MPOs were found to bind to the G-CSF receptor with low nanomolar affinities, similar to that of G-CSF(S17). In a study of isolated cpG-CSF domains [Feng, Y., et al. (1999) *Biochemistry 38*, 4553−4563], domains with the L1 linker had lower G-CSF receptor-mediated proliferative activities and conformational stabilities than those which had the L0 linker. A similar trend was found for the MPOs in which the G-CSFR agonist activity is mostly a property of the cpG-CSF domain. Important exceptions were found in which the linkage to the IL-3R agonist domain either restored (e.g., attachment at residue 142) or further decreased (linkage at residue 39) the G-CSFR-mediated proliferative activity. MPO in which the IL-3R agonist domain is attached to the cpG-CSF(L1)[133/132] domain was shown to be more potent than the coaddition of the IL-3R agonist and G-CSF in stimulating the production of CFU-GM colonies in a human bone marrow-derived CD34+ colony-forming unit assay. Several MPOs also had decreased proinflammatory activity in a leukotriene C_4 release assay using *N*-formyl-Met-Leu-Phe-primed human monocytes. It was found that circular permutation of the G-CSF domain can alter the ratio of G-CSFR:IL-3R agonist activities, demonstrating that it is a useful tool in engineering chimeric proteins with therapeutic potential.

Hematopoietic growth factors have been intensively studied because of their potential to ameliorate the myelosuppresive states induced by cancer chemotherapies, as well as to mobilize peripheral blood progenitor (CD34+) cells for autologous transplantation following chemotherapy (see refs *1* and *2*). Clinically significant episodes of febrile neutropenia and thrombocytopenia are common following multiple cycles of dose-intensive chemotherapies. Growth factors used or under investigation for providing hematopoietic support include granulocyte colony-stimulating factor (G-CSF)[1] (*3*), granulocyte/macrophage colony-stimulating factor (GM-CSF) (*4*), erythropoietin (*5*), interleukin-3 (IL-3) (*6*−*8*), daniplestim (*9*), thrombopoietin (*10*, *11*), interleukin-11 (*12*), stem cell factor (*13*), flt3-ligand (*14*), and promegapoietin (*15*). Recombinant G-CSF is the current

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[1] Abbreviations: G-CSF, granulocyte colony-stimulating factor; G-CSFR, G-CSF receptor; cpG-CSF, circularly permuted G-CSF(S17); IL-3, interleukin-3; IL-3R, IL-3 receptor; MPO, myelopoietin, a chimeric dual IL-3 and G-CSF receptor agonist; MPO[new amino/new carboxy], myelopoietins in which the G-CSF domain has been circularly permuted with the new amino and carboxy groups of the cpG-CSF domain designated in the numbering of the G-CSF(S17) sequence; 3D, three-dimensional; CFU, colony-forming unit; LTC_4, leukotriene C_4.

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standard of care for addressing neutrophil deficit, while IL-3 has been shown to impact platelet production (*6*−*8*). Daniplestim is a multiply substituted IL-3 receptor (IL-3R) agonist engineered for increased potency and an improved side-effect profile (*16*, *17*) compared to those of native IL-3. In an irradiated rhesus model of myelosuppression, daniplestim was effective in preventing thrombocytopenia and eliminating the need for platelet transfusions. Daniplestim coadministered with G-CSF (filgrastim) is in advanced clinical testing for the mobilization of CD34+ progenitors for autologous transplantation following high-dose chemotherapy (*9*).

Myelopoietins (MPOs) are a family of engineered chimeric hematopoietic growth factors that bind and activate the receptors for both IL-3 and G-CSF (*18*−*21*). The impetus for engineering the MPOs grew out of the observation that daniplestim potentiated suboptimal concentrations of G-CSF in stimulating myelopoiesis (see ref *19*). It was subsequently found that the combination of the early acting, multilineage IL-3R agonist with the lineage specific G-CSF receptor (G-CSFR) agonist was superior in its ability to promote the proliferation and differentiation of the neutrophilic−granulocytic lineage. As a result, two lead members of the MPO family were studied clinically and found to be well-tolerated and effective in their ability to mobilize neutrophil and multilineage precursors in patients undergoing chemotherapy (*22*−*24*).

The biochemical and pharmacological action of the prototypical MPO has been characterized in detail (*19*−*21*). Studies with cells positive either for each receptor alone or for both receptors together established that the MPO prototype was able to bind, signal, and stimulate proliferation using both receptors. A colony-forming unit assay in which human bone marrow-derived CD34+ cells were employed was used to evaluate the precursor type and frequency generated in response to treatment with the prototypical MPO or to coaddition of equivalent amounts of the single agonists. The prototypical MPO was found to have a pronounced improvement in activity compared to that of the coaddition, especially at low concentrations, in its ability to stimulate the proliferation and differentiation of cells in the myeloid lineage (CFU-GM). The MPO prototype was also evaluated in comparison to the coadministration of daniplestim and G-CSF in an irradiated rhesus model of myelosuppression (*20*). MPO was found to profoundly reduce both the neutrophil and platelet nadirs observed in control animals, an effect which was not matched by daniplestim or G-CSF, either alone or in combination.

As a first step toward clarifying the molecular requirements for chimera that bind and activate both the IL-3 and G-CSF receptors, we have engineered MPOs with different interdomain connectivities by incorporating circularly permuted G-CSF(S17) sequences. Conceptually, circular permutation (*25*−*33*) of a protein entails linking the termini of the original sequence (the "linker") and breaking the chain at a new location (the "breakpoint"), a process which both introduces and removes conformational constraints and ionizable groups. This results in swapping long-range and short-range interactions in the three-dimensional structure and provides a new aspect to structure−function relationships that cannot be made available through classical means of manipulating sequence.

Pastan and co-workers (*33*) first pointed out that using circularly permuted domains in chimeric molecules overcomes the limited spatial ensemble that is available when only the natural termini are used to connect sequences. Using a circularly permuted IL-4 linked to a *Pseudomonas* exotoxin, they demonstrated that the connectivity between domains, and therefore their spatial relationship, was important for optimal activity. Thus, by circularly permuting the G-CSFR agonist domain of the MPO molecule, we sought to clarify the spatial relationships that are important for the activities of MPO.

We report in this paper that the activity of the G-CSF receptor agonist domain of MPO can be altered by using circularly permuted G-CSF sequences. When cpG-CSFs are incorporated into MPOs, the G-CSF-dependent proliferation activities range from being fully active to being <10% of that of native G-CSF. A human bone marrow-derived CD34+ colony-forming assay was used to demonstrate that the hematopoietic activity of an MPO with a cpG-CSF domain is greater than equimolar concentrations of the IL-3R agonist and G-CSF. A detailed study of cpG-CSF sequences in the preceding paper (*34*) provided important information about the interpretation of the effects of their incorporation into the chimeric MPO molecules. Several fully active MPO molecules were found to have decreased proinflammatory activity in a human monocyte sulfidoleukotriene (LTC_4) priming assay.

EXPERIMENTAL PROCEDURES

Construction of MPO Genes Incorporating Circularly Permuted G-CSF Sequences. The MPOs described in this paper are composed of an N-terminal IL-3R agonist domain (SC-63032) connected by a chimeric linker to a C-terminal G-CSF receptor agonist domain. SC-63032, which is closely related to daniplestim (SC-55494; see ref *16*), consists of a 112-amino acid sequence derived from the human IL-3 sequence by truncating the 13 N-terminal residues and eight C-terminal residues and making the following substitutions: V14A, N18I, T25H, Q29R, L32A, F37P, G42D, Q45V, D46S, E50D, N51R, R55T, A60S, N62V, S67N, Q69E, A73G, S76A, K79R, L82Q, L87S, T93S, H98I, D101A, N105Q, R109E, K116V, N120Q, and A123E. The chimeric linker, which mimics the hinge region of mouse IgG2b (*35*), consists of the sequence YVEGGGGSPGEPSGPISTINP-SPPSKESHKSPNM. The G-CSF receptor agonist consists of either the G-CSF(S17) sequence or circularly permuted G-CSF(S17) sequences (see ref *34*).

MPOs which incorporate a cpG-CSF sequence are designated by descriptors in a simple extension of the nomenclature described in the preceding paper (*34*). For example, MPO and MPO(L1)[39/38] refer to MPOs with G-CSF(S17) and cpG-CSF(L1)[39/38] domains, respectively. cpG-CSF-(L1)[39/38] specifies a circularly permuted G-CSF(S17) sequence in which the amino- and carboxy-terminal residues correspond to residues 39 and 38, respectively, of the original sequence and with the L1 linker (defined below) connecting the original terminal segments. Whenever no linker designation is given, this corresponds to a peptide bond linker (L0) in which residue 174 is directly linked to residue 1. The L1 linker corresponds to deleting the amino-terminal 10-residue segment of G-CSF, and replacing it with seven-residue linker SGGSGGS.

As summarized in Table 1, a series of MPOs with cpG-CSF domains utilizing five breakpoint locations and two

Table 1: Summary of MPO Properties[a]

	competitive cell surface receptor binding		soluble G-CSF receptor binding[b]			cell proliferation	
growth factor	IC_{50}(G-CSFR) (nM)	IC_{50}(IL3Rα) (nM)	k_a ($\times 10^5$ s^{-1} M^{-1})	k_d ($\times 10^{-4}$ s^{-1})	K_D (nM)	EC_{50}(Baf3-G) (pM)	EC_{50}(TF-1) (pM)
SC-63032	nd	0.24 ± 0.17 (18)	nd	nd	nd	nd	5.9 ± 0.6 (2)
G-CSF[c]	0.95 ± 0.17 (4)	nd	10.0 ± 3.0	2.0 ± 0.4	0.20 ± 0.07 (7)	57 ± 13 (2)	nd
G-CSF(S17)[c]	nd	nd	8.8 ± 0.8	4.4 ± 0.6	0.5 ± 0.11 (9)	51 ± 8 (2)	nd
MPO	4.1 ± 1.5 (8)	5.9 ± 3.5 (7)	6.3 ± 0.5	1.7 ± 0.7	0.28 ± 0.13 (2)	50 ± 41 (2)	50 ± 1 (2)
MPO[39/38]	22.5 ± 2.3 (2)	nd	3.5 ± 0.5	10 ± 0.3	2.9 ± 0.4 (3)	280 ± 70 (2)	18 ± 4 (2)
MPO(L1)[39/38]	7.6 ± 3.0 (4)	4.1 ± 1.5 (4)	3.9 ± 0.6	7.3 ± 1.0	1.9 ± 0.6 (3)	710 (1)	45 ± 1 (2)
MPO[97/96]	8.3 ± 5.0 (3)	nd	nd	nd	nd	39 ± 6 (2)	37 ± 0 (2)
MPO(L1)[97/96]	5.2 ± 1.9 (2)	5.8 ± 0.7 (2)	nd	nd	nd	600 ± 30 (2)	74 ± 15 (2)
MPO[126/125]	2.9 ± 0.6 (2)	nd	nd	nd	nd	25 ± 2 (2)	13 ± 1 (2)
MPO(L1)[126/125]	nd	nd	nd	nd	nd	210 (1)	95 (1)
MPO[133/132]	4.7 ± 2.1 (3)	6.2 (1)	4.5 ± 1.2	4.6 ± 1.2	1.0 ± 0.3 (3)	53 ± 6 (2)	61 ± 3 (2)
MPO(L1)[133/132]	4.4 ± 3.0 (12)	5.3 ± 3.4 (4)	9.0 ± 1.7	1.0 ± 0.3	0.11 ± 0.02 (3)	35 ± 16 (4)	29 ± 5 (4)
MPO[142/141]	nd	nd	nd	nd	nd	nd	nd
MPO(L1)[142/141]	4.2 ± 1.2 (6)	5.4 ± 2.9 (2)	nd	nd	nd	103 ± 4 (2)	26 ± 11 (2)

[a] Note that the competitive binding experiments were performed at 4 °C while the plasmon resonance experiments were performed at 25 °C. nd means not determined. Numbers in parentheses indicate the number of independent experiments performed. [b] Binding to affinity-captured sG-CSFR-F_C detected by surface plasmon resonance. [c] These data were reported in the preceding paper (*34*).

different circularizing linkers (the L0 and L1 linkers) were examined in this study. To make MPO molecules which contain cpG-CSF sequences, DNA fragments encoding cpG-CSF sequences were first created as described in the preceding paper (*34*) and then cloned into an expression plasmid behind the IL-3R agonist and hinge sequences. The MPO expression vectors were used to transform *Escherichia coli* strain DH5α cells (Life Technologies, Gaithersburg, MD). The plasmid DNA was purified and the sequence confirmed. For protein expression, *E. coli* strain JM101 was transformed with the confirmed plasmids.

Folding and Purification of MPOs. Expression of recombinant proteins as inclusion bodies in *E. coli* has been previously described (*36*). A typical procedure for folding and purification consists of the following. Inclusion bodies were isolated by sonication of the *E. coli* cell pellets in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, followed by centrifugation (*37*). The inclusion bodies from a 1 L fermentation mixture were solubilized in a 30 mL solution containing 8 M urea, 50 mM Tris-HCl (pH 9.5), and 5 mM dithiothreitol and stirred at room temperature for 30 min. To this stirred mixture was added 210 mL of a cold solution containing 2.3 M urea and 50 mM Tris-HCl (pH 9.5). After the mixture was stirred at 4 °C for 24 h, the refolding reaction was terminated by adjusting the pH to 5.0 with 15% acetic acid and stirring the mixture for an additional 2 h at 4 °C. The solution was clarified by centrifugation at 8000*g* and filtration of the supernatant. The filtrate was chromatographed on a 20 mL cation-exchange column (CM Sepharose, #CCF-100, Sigma, St. Louis, MO) equilibrated with 50 mM sodium acetate (pH 5.0). The desired product was eluted from the column by a linear gradient of 0 to 300 mM NaCl containing 50 mM sodium acetate (pH 5.0). HPLC and SDS−polyacrylamide gel electrophoresis (PAGE) analysis were used to select fractions containing the desired product. The combined fractions from the CM column were diluted 10-fold with 50 mM Tris-HCl (pH 9.0) and loaded onto a 15 mL anion-exchange column (DEAE Sepharose, #DFF-100, Sigma) equilibrated with 50 mM Tris-HCl (pH 9.0). The product was eluted from the column at pH 9.0 with a 0 to 200 mM NaCl gradient. After HPLC and SDS−PAGE analysis, fractions were combined, dialyzed against 10 mM ammonium acetate (pH 4.5), and sterile filtered. The final products were characterized by HPLC, SDS−PAGE, electrospray mass spectrometry, and amino acid composition. The purity of each protein was >90%. Protein concentrations were determined by amino acid composition or absorption at 280 nm.

Cell Surface Receptor Binding Assays. Specific binding assays were carried out with Baf3 and BHK cells transfected to express the G-CSFR (Baf3-G; *19*) and IL-3R α-subunit (BHK-IL3Rα; *16*), respectively, using procedures outlined by Feng et al. (*34*). For competitive binding assays, we employed [^{125}I]G-CSF(Y1, Y3, S17) (Baf3-G) or an ^{125}I-labeled hIL-3 variant (SC-65353; *16*) (BHK-IL-3Rα), both with a specific activity of 1000−2000 Ci/mmol. The extent of nonspecific binding was determined from the residual binding in the presence of 400 nM G-CSF (Baf3-G) or 800 nM daniplestim (BHK-IL3Rα).

Surface Plasmon Resonance Measurements. The kinetics of the binding of MPOs to the extracellular domain of G-CSFR (sG-CSFR) were determined using surface plasmon resonance. The capture phase consisted of an affinity-immobilized fusion of sG-CSFR and a mouse IgG F_C domain. Methods for collecting and analyzing binding data have been described previously (*34*).

Cell Proliferation Assays. G-CSFR-dependent proliferative activity was measured using the transfected Baf3-G cells (*19*) as described previously (*34*). The IL-3R-dependent proliferative activity was determined using TF-1 cells (C. Sanderson, Searle) seeded at 1.25×10^4 cells per well. All other culture conditions were identical to those of the Baf3-G assay. After 72 h, the TF-1 cells were incubated with [*methyl*-^{3}H]-thymidine for 4 h. Cell harvesting, counting, and analysis of the data were the same as described for the Baf3-G proliferative assay.

Far-UV CD Spectroscopy. The far-UV CD spectra of MPOs, G-CSF(S17) and SC-63032, were measured at 20 °C using a JASCO J-500C spectrometer at ambient temperature. The spectrometer was calibrated using *d*-(+)-10-camphorsulfonic acid. The protein concentrations of stock solutions were determined by absorbance at 280 nm or

quantitative amino acid analysis. Samples [concentration of 10−40 μM in 10 mM Tris-HCl (pH 7.5)] were placed in a quartz cell (path length of 2 mm). Spectra were collected by averaging four consecutive scans taken between 260 and 190 nm in 0.2 nm increments.

Colony-Forming Unit (CFU) Assays. Human bone marrow aspirates were obtained from healthy donors following informed consent. CD34+ cells were isolated from marrow using density gradient centrifugation and a Ceprate LC (CD34) kit (CellPro, Bothell, WA). Colony assays were carried out in methylcellulose (Stem Cell Technologies, Vancouver, BC). Cultures were incubated at 37 °C for 13−14 days in the presence of various concentrations of cytokines. Colonies (CFU-GM consisting of ≥50 cells) were counted using inverted phase microscopy on days 13 and 14.

LTC_4 Release Assays. An enriched population of basophils, eosinophils, and mononuclear cells was isolated from human peripheral blood by density gradient centrifugation. After incubation with cytokines at 37 °C for 15 min, cells were stimulated with 5 nM *N*-formyl-Met-Leu-Phe for 15 min and the release of LTC_4 was assessed using an ELISA (Clayman Chemicals, Ann Arbor, MI) as previously described (*16*).

RESULTS

MPOs used in this study consist of an IL-3R agonist domain connected to a G-CSFR agonist domain by a hinge sequence (*19*). In each case, the IL-3R agonist domain is SC-63032, a molecule that resulted from an extensive structure−function study of the human IL-3 sequence (*38*). SC-63032 is about 10-fold more potent than human IL-3 in IL-3R-mediated proliferation assays (unpublished data). When incorporated into chimeric MPOs, the highly engineered IL-3R agonists have an activity that is approximately equivalent to that of native IL-3 in TF-1 assays (unpublished data). The G-CSF domain of MPO was found to have Baf3-G activity that is similar to that of G-CSF (*19*). In colony-forming unit assays in which human bone marrow-derived CD34+ cells were used, a prototypical version of MPO is able to activate much larger numbers of responsive progenitor cells at far lower concentrations than does coaddition of equimolar amounts of the component IL-3R agonist and G-CSF (*19*). This prototype MPO has been shown to dramatically blunt both the neutrophil and platelet nadirs in the irradiated rhesus model of myelosuppression (*20*).

In this study, we have focused on the effect of the relative orientation of the IL-3R and G-CSFR agonist domains by using circularly permuted G-CSF sequences. G-CSF and cpG-CSF sequences have their termini in different spatial locations, thereby giving rise to different connectivities when incorporated into MPOs. MPOs with several different G-CSFR agonist domains have been studied here using a variety of biochemical and biophysical methods.

Selection and Preparation of the Circularly Permuted G-CSF Receptor Agonist Domain in MPO Sequences. The rationale for selecting the locations for the new termini (the breakpoints) and linkers used in this study is the same as that explained in the preceding paper (*34*). Transposed DNA sequences encoding cpG-CSF domains were used to prepare genes that encode the MPOs listed in Table 1. To avoid the potential for mispaired disulfides, the unpaired Cys-17 in wild-type G-CSF was replaced with Ser in all of the MPOs reported in this study. The *E. coli*-expressed MPOs were produced as inclusion bodies, solubilized, refolded, and purified using essentially the same procedure employed for other forms of MPO (*19*). The purified proteins were characterized using a number of analytical methods, including SDS−PAGE, electrospray mass spectrometry, quantitative amino acid analysis, and N-terminal sequencing (data not shown).

Receptor Binding Affinity of MPOs. Each MPO has been evaluated for its affinity for the IL-3R α-subunit and G-CSFR using competitive cell surface binding assays with either BHK or Baf3 cells transfected with cDNA encoding the respective receptor sequence (see Table 1). The affinities of the MPOs for the IL-3R α-subunit are the same as that of MPO within experimental error (4.1−6.2 nM), and are approximately 20-fold decreased compared to that of SC-63032.

The potencies for G-CSFR binding vary somewhat depending upon the particular G-CSF receptor agonist domain incorporated into the MPOs. All MPOs tested except for MPO[39/38] have potencies that are within a factor of 2 of one another (see Table 1), but are decreased by a factor of 4−8 compared with that of G-CSF. On the other hand, MPO-[39/38] had a significantly decreased ability for competitive binding with 5.5-, 24-, and 15-fold decreases in potency relative to those of MPO, G-CSF, and cpG-CSF[39/38] (*34*), respectively.

Surface Plasmon Resonance Studies of MPO Binding to the Soluble G-CSF Receptor. Surface plasmon resonance studies were carried out on a subset of MPOs using an affinity-immobilized chimeric receptor construct consisting of the extracellular portion of the G-CSF receptor linked to an F_C domain of mouse IgG, sG-CSFR-F_C. Association and dissociation kinetic rate constants were determined by studying the concentration dependence of MPO binding when passed over a surface coated with sG-CSFR-F_C captured by an anti-F_C antibody. Data were collected on molecules with breakpoints between residues 39 and 38 and 133 and 132 using both the L0 and L1 linkers (Table 1). G-CSF(S17) and MPO were included for comparison.

The association rate constants vary over a relatively narrow range of $3.5-9 \times 10^5$ M^{-1} s^{-1}. None of these rates differs by a factor of >2.5 from that of MPO or G-CSF(S17). The MPO dissociation rate constants exhibit a somewhat larger range, varying from 1 to 10×10^{-4} s^{-1}. The molecules with a breakpoint between residues 39 and 38 with either the L0 or L1 linker exhibit significantly larger first-order dissociation rate constants than the other MPOs examined. The effect on the k_d for the MPOs with a breakpoint between residues 39 and 38 is manifested in dynamic equilibrium dissociation constants ($K_D = k_d/k_a$) which are 7−10-fold greater than that of the unpermuted G-CSF domain (2.9 and 1.9 nM vs 0.28 nM, Table 1). This trend of decreased affinity determined by surface plasmon resonance is qualitatively similar to that observed for cell surface binding to the G-CSFR; MPOs with the breakpoint at residues 39 and 38 were 2−5.5-fold weaker than MPO in G-CSFR binding (Table 1). The differences between the results from these two binding experiments may be due to the difference in the temperatures that were used (25 °C for surface plasmon resonance vs 4 °C for G-CSFR binding). MPOs containing cpG-CSFs with the breakpoint between residues 133 and 132 exhibit affinities within a fac-

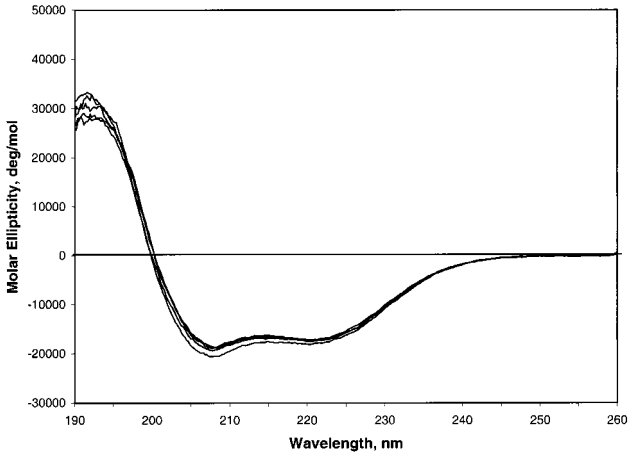

FIGURE 1: Far-UV circular dichroism spectra of MPO, MPO(39/38), MPO(L1)[39/38], MPO[133/132], and MPO(L1)[133/132]. Spectra are presented as the average of four consecutive scans.

tor of 4 of that of MPO by surface plasmon resonance, but are nearly equivalent in the cell surface competition assay.

Cell Proliferation Activity of MPOs. The MPOs are potent stimulators of proliferation of the IL-3-dependent TF-1 cell line, exhibiting EC_{50}s that are within 4-fold of that of the parental MPO (see Table 1). The IL-3R agonist activities of the MPO molecules are decreased to a level that is about equivalent to that of wild-type IL-3 (data not shown). This decrease in potency observed with TF-1 cells is consistent with observations made with other forms of MPO (*19*).

The MPOs are also effective activators of G-CSF-dependent proliferation of the transfected Baf3 cells, exhibiting similar maximal proliferative responses (data not shown). MPO, MPO[97/96], MPO[126/125], MPO[133/132], MPO(L1)[133/132], and MPO(L1)[142/141] exhibit EC_{50} activities similar to that of G-CSF(S17), whereas MPO(L1)[39/38], MPO[39/38], MPO(L1)[97/96], and MPO(L1)[126/125] remain potent but displayed 4−14-fold lower activities (see Table 1). The tendency of the L0 linker molecules to retain nearly full activity, while L1 linker molecules experience some loss in activity [except for MPO(L1)[133/132] and MPO(L1)[142/141]], is also observed for the individual cpG-CSF molecules (*34*).

Far-UV CD Spectra of MPOs. The far-UV CD spectrum of MPO is shown in Figure 1. The magnitude of the characteristic double-trough minima at 207 and 221 nm is consistent with the presence of two four-helix bundles in the MPOs. CD spectra were collected for several representative MPOs so the effects of the breakpoint and linker on structural integrity could be studied. The spectra of MPO(L1)[39/38], MPO(L1)[133/132], MPO[39/38], and MPO[133/132] are seen in Figure 1 to be nearly identical to the spectrum of MPO. These results establish that MPOs which incorporate cpG-CSF domains are able to fold into the helical bundle motif with little perturbation of secondary structure.

Colony-Forming Unit Activity of MPO(L1)[133/132]. The CFU-GM activity of MPO(L1)[133/132] was assessed by culturing human bone marrow-derived CD34+ cells and enumerating GM colonies greater than or equal to 50 cells. MPO(L1)[133/132] produced a higher precursor frequency at lower concentrations (0.1, 0.05, and 0.01 nM) than did coaddition of equimolar concentrations of SC-63032 and G-CSF (Figure 3).

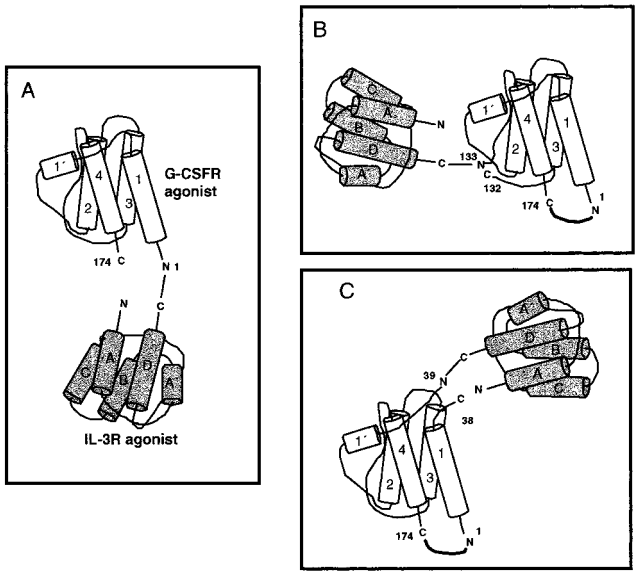

FIGURE 2: Schematic representation of the MPO structure indicating the location (arrows) of some of the points at which the hinge−IL-3R agonist (SC-63032) moiety is connected to the cpG-CSF domains used in this study: (A) parental MPO, (B) MPO[133/132], and (C) MPO[39/38].

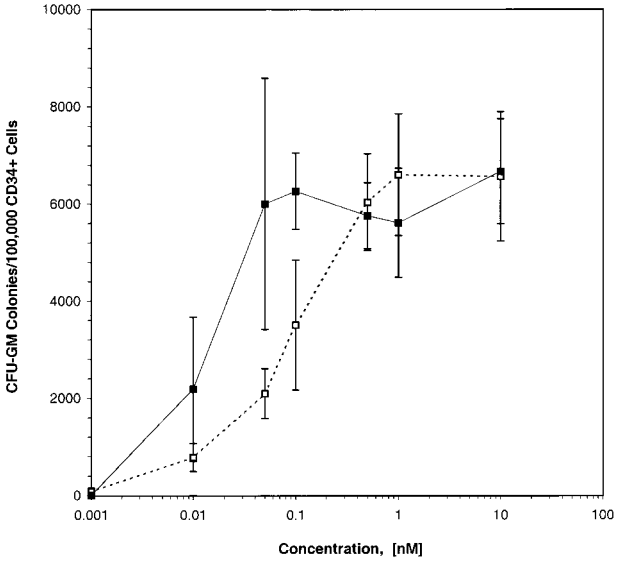

FIGURE 3: CFU activity of MPO(L1)[133/132] and coaddition of equimolar combinations of SC-63032 and G-CSF. Inverted-phase microscopy was used to determine the number of CFU-GM per 100 000 input cells by scoring colonies greater than or equal to 50 cells as positive. The error bars represent the standard deviation for counting of triplicate wells. A separate experiment produced a similar result (data not shown).

Measurement of LTC_4 Release by IL-3R Agonists. The production of LTC_4 has been linked to the dose limitation of native IL-3 in human studies (*39*). It is therefore of interest to assess the proinflammatory activity of the IL-3R agonist component of MPOs. Three of the MPO molecules [MPO, MPO[133/132], and MPO(L1)[133/132]] were tested for their ability to stimulate the synthesis and release of LTC_4 (Table 2). Despite the increase in their growth-promoting activities, the MPOs showed 3.8-fold (MPO), 8.9-fold (MPO[133/132]), and 6.3-fold [MPO(L1)[133/132]] decreased potencies in stimulating the LTC_4 release compared to that of their IL-3 receptor agonist component, SC-63032. G-CSF did not

Table 2: Effect of MPO on LTC_4 Release[a]

growth factor	EC_{50} (pM)	growth factor	EC_{50} (pM)
SC-63032	0.49 ± 0.08 (20)	MPO[133/132]	4.35 ± 1.02 (7)
MPO	1.87 ± 0.11 (3)	MPO(L1)[133/132]	3.11 ± 0.44 (22)

[a] Numbers in parentheses indicate the number of independent experiments performed.

stimulate leukotriene release at concentrations up to 10 nM (data not shown).

DISCUSSION

All the MPO molecules employing circularly permuted G-CSF receptor agonists exhibited a potent ability to stimulate the proliferation of both IL-3R-dependent and G-CSFR-dependent cell lines. Consistent with this biological activity is the observation that a representative subset of the MPOs were shown to be folded with helical contents similar to that expected from the two correctly folded components and the hinge sequence. The MPOs also retain the ability to bind to the IL-3R α-subunit with affinities similar to that of MPO, and most of them are also able to bind to the G-CSFR with low nanomolar affinities, as does G-CSF(S17). These results demonstrate that connections to the hinge−IL-3R agonist domain can be made at different locations on the surface of G-CSF while still maintaining both IL-3R and G-CSFR agonist activities. Using the CD34+ colony-forming assay, MPO(L1)[133/132] was shown to have superior hematopoietic activity relative to equimolar concentrations of the IL-3R agonist (SC-63032) and G-CSF. The MPOs were also shown to have decreased EC_{50}s in stimulating LTC_4 release from *N*-formyl-Met-Leu-Phe-primed mononuclear cells. Improved hematoipoietic activity and decreased proinflammatory activity are key improvements in these engineered chimeric growth factors.

Despite the ability of MPOs to induce G-CSFR-mediated responses, there are nonetheless variations in potency at both the receptor binding and cell proliferation level. The range of activities is dependent on both the breakpoint and the linker. The effect of breakpoint location on the connectivity of the hinge and the IL-3R agonist (SC-63032) moiety is represented schematically in Figure 2. The discussion of the results is aided by considering the effects of the location of attachment, along with results for isolated cpG-CSF domains reported in the preceding paper (*34*).

In attempting to explain the decreases in the G-CSFR agonist activity of dual-agonist MPO molecules, we consider two plausible situations that represent extremes of the continuum of possibilities. First, the cpG-CSF domain may be conformationally deranged to an extent that it lacks the complementarity required to efficiently ligate the receptor. Alternatively, each domain may be folded correctly, but the proximity of the IL-3R agonist domain causes steric masking of the G-CSFR binding site. The results of this study, when interpreted with the results of the effects of circular permutation on isolated cpG-CSF domains, provide examples of both. As discussed below, an additional and unexpected result was that the IL-3R agonist domain stabilized and/or enhanced the biological activity of the cpG-CSF domain when linked at certain breakpoints. Although the exact origin of this "restoration" effect is unknown, the addition of the hinge−IL-3R agonist residues may stabilize the cpG-CSF domain, either by the addition of stabilizing interactions or by removal of destabilizing interactions. Unfortunately, a comparison between the biological activity and the thermodynamic stability is not feasible for these chimeric molecules because of complex urea denaturation curves. Some of the MPOs exhibited unfolding curves which deviated significantly from a two-state process, while others exhibited apparent two-state denaturation behavior. Interpretation of these results was not straightforward.

Both MPO[133/132] and MPO(L1)[133/132] function in a manner essentially identical to that of MPO, suggesting that in this case circular permutation had little effect on either the 3D fold or the receptor contact area of the G-CSFR agonist domain. Support for the lack of structural perturbation comes from the fact that the CD spectra for both of these MPOs are indistinguishable from that of parental MPO. Furthermore, Feng et al. (*34*) have shown using $^1H-^{15}N$ HSQC spectral fingerprinting that cpG-CSF[133/132] and cpG-CSF(L1)[133/132] are folded into structures which are very similar to G-CSF(S17). Residues 133 and 132 are in the middle of loop 34 (Figure 2B), a region which is known to be flexible (*40*−*43*). Residue 133 is a glycosylation site (*44*), indicating that this region is tolerant to attachment of large molecular entities, which is consistent with the results of this study. The proliferative activity for cpG-CSF(L1)[133/132] is 10-fold decreased relative to that of G-CSF (*34*). Taken together, the results for the cpG-CSF and MPO molecules with a breakpoint between residues 133 and 132 suggest that linkage through residue 133 is well tolerated in MPO, that the L1 linker introduces a destabilizing effect that diminishes the G-CSFR-mediated proliferative activity of cpG-CSF(L1)[133/132], and that this destabilizing effect is substantially reversed by connecting to the hinge−IL-3R agonist moiety.

In contrast, both MPO[39/38] and MPO(L1)[39/38] displayed sharply decreased Baf3-G proliferative activity compared with that of MPO (18 and 7%, respectively), indicating that the new termini significantly perturbed either the structure or the binding site for the receptor. Examination of Figure 2C reveals that in this case the connectivity to the hinge−IL-3R agonist domain will be within loop 12, a site that is adjacent to one of the putative receptor binding sites (*45*). Both cpG-CSF[39/38] and cpG-CSF(L1)[39/38] were shown by CD and NMR spectroscopy to adopt a 3D fold similar to that of G-CSF(S17) (*34*). Their G-CSFR affinities are within a factor of 2-fold of that of G-CSF, although their Baf3-G proliferative activities are 36 and 4% of that of G-CSF. Thus, the cpG-CSF domains with a breakpoint at residues 39 and 38 in the chimeric MPOs are likely to be structurally intact, and their diminished activity is largely due to perturbation of the receptor binding site. The G-CSF proliferative and receptor binding activities of MPO[39/38] are further diminished in comparison to those of cpG-CSF-[39/38], suggesting a small effect due to the connection to the hinge−IL-3R agonist moiety. While such an effect may also be present in MPO(L1)[39/38], the relatively low remaining activity may have made this effect difficult to observe. In contrast to the G-CSFR activity, the IL-3R-mediated activities of the molecules with a breakpoint between residues 39 and 38 are close to that of MPO (Table 1). This suggests that the hinge−SC-63032 moiety may

interfere with the G-CSFR binding site, but that the cpG-CSF domains do not significantly interfere with the IL-3R binding site of MPO[39/38] molecules.

For MPOs with new termini at residues 97 and 96, the molecule with the L1 linker exhibited 8% of the G-CSFR proliferative activity compared to that of MPO, while the molecule with the L0 linker retained full activity. Again, the IL-3R activities of these molecules remain similar to that of MPO. With regard to Figure 2, the molecules with a breakpoint at residues 97 and 96 attaches the hinge−IL-3R agonist moiety at a turn between helices 2 and 3 at the end of the helical bundle that is distal to the original amino and carboxy termini. Since the L0 linker molecule (MPO[97/96]) was fully active compared to MPO, this suggests that the breakpoint was well tolerated but that the L1 linker is destabilizing. Unfortunately, the corresponding cpG-CSFs with either the L0 or L1 linker was not expressed in *E. coli* so that there is no detailed information about their 3D conformation to explicitly address this point. These cpG-CSF molecules were, however, transiently expressed in BHK cells and were found to have Baf3-G proliferation activity (data not shown). In this study, we are unable to distinguish between structural distortion and masking of the receptor contact site for MPO(L1)[97/96].

Similar to the case of the breakpoint at residues 97 and 96, MPO(L1)[126/125] has only 24% of the G-CSFR activity of MPO, while the L0 linker molecule was fully active. A similar but much less pronounced trend is seen for IL-3R activity. The connection site in this construct occurs in loop 34 just after the end of helix 3 (Figure 2). Biophysical studies on cpG-CSF[126/125] and cpG-CSF(L1)[126/125] reveal that they adopt the appropriate fold, but that cpG-CSF(L1)-[126/125] has lower stability toward urea denaturation. Both cpG-CSF[126/125] and cpG-CSF(L1)[126/125] exhibited decreased cell proliferative activity [40 and 3% of that of G-CSF(S17), respectively]. The MPO results for linkage through residue 126 are qualitatively similar to those for linkage through residue 133 (see above), but there is a smaller restoration of the G-CSFR-mediated proliferative activity as a result of connecting to the hinge−IL-3R agonist moiety.

Another example in which the hinge−IL-3R agonist moiety partially restores activity in MPO is MPO(L1)(142/141). In the G-CSFR-dependent Baf3 assay, MPO(L1)(142/141) retains 49% of the activity of MPO while cpG-CSF(L1)[142/141] retains only about 1% of the G-CSF activity. Residue 142 occurs at the end of the loop 34 and is adjacent to a hydrophobic core in G-CSF (see ref *34* for further discussion). Biophysical studies on cpG-CSF(L1)-[142/141] reported in the preceding paper (*34*) revealed that this molecule has a severely distorted 3D fold and a greatly diminished thermodynamic stability. The connection to the hinge−IL-3R agonist moiety appears to have substantially reversed the detrimental effect of the L1 linker in MPO-(L1)(142/141), which led to a dramatic and unexpected increase in the G-CSFR-mediated activity. Thus, MPO(L1)-[97/96], MPO(L1)[126/125], and MPO(L1)[142/141] provide examples where circular permutation impacted stability and proliferative activity, with a smaller effect on G-CSFR binding.

A situation involving steric masking in a chimera has been demonstrated by the elegant studies of Pastan and co-workers using IL-4−exotoxin chimeras (*33*, *46*). The point of attaching the toxin domain to the IL-4 domain altered the IL-4 receptor binding activity by as much as 1 order of magnitude (*33*), and this effect was translated into differences in the in vivo efficacy of IL-4−toxin and cpIL-4−toxin chimera (*46*). In the study described here, the MPO(39/38) molecule shows evidence of a small steric effect. The cpG-CSF studies (*34*) were key for distinguishing the effect of masking from structural perturbation.

In summary, the G-CSFR agonist activity of the MPOs largely parallels that of the corresponding cpG-CSFs. In comparison to the L0 linker, the L1 linker exerts pronounced effects in MPO as well as in cpG-CSF. Connectivity effects due to linking to the hinge−IL-3R agonist moiety have been observed that can either further diminish (e.g., MPO[39/38]) or enhance [e.g., MPO(L1)[126/125] and MPO(L1)[142/141]] the G-CSFR agonist activity in MPOs relative to those of the corresponding cpG-CSFs. This study established that the range of G-CSFR agonist activities in MPO is predominantly a property of the cpG-CSF domain, with a lesser effect arising from the connectivity between domains. It was also found that circular permutation can be used to modulate the relative activity of domains in MPO molecules, a capability which can be used for optimizing the therapeutic potential of chimeric proteins.

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