Characterization of a Receptor for Macrophage Inflammatory Protein 1α and Related Proteins on Human and Murine Cells

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Abstract
Macrophage inflammatory protein 1α (MIP-1α) is a potent stem cell inhibitor and a member of a large and expanding family of related cytokines. In an effort to understand the molecular basis of the activities of MIP-1α, we have sought to characterize the cellular receptors for this molecule. Our results demonstrate the presence of abundant MIP-1α receptors on both human and murine cells. The receptor on K562 cells can bind a range of members of the MIP-1α family and may thus be a general MIP-1α family receptor. Murine FDCPmix cells also bind a range of members of this peptide family, although the receptor(s) that they express appear somewhat more selective for peptides capable of displaying stem cell inhibitory properties. The human and murine receptors do not bind members of the related interleukin 8 family of peptides and are thus distinct from the recently cloned interleukin 8 receptor. We suggest that the receptor on the murine cell is a candidate for the receptor responsible for articulating stem cell inhibitory signals following MIP-1α binding.

Introduction
We have recently identified and characterized an inhibitor of hematopoietic stem cell proliferation [stem cells as defined by the in vivo CFU-S (1) and in vitro CFU-A (2) assays] and have shown it to be identical to a previously described cytokine, MIP-1α (3). MIP-1α is a member of a large and expanding family of related cytokines defined on the basis of limited sequence homology and the presence of four positionally conserved cysteine residues (Fig. 1; Refs. 4–6). The family is further subdivided on the basis of the cysteine motif, with the first family (containing MIP-1α) having the -CC-C-C- motif (the -CC- family) and the second subfamily having the -CC-C-C- motif (the -CXC- family).

A number of diverse functions have been attributed to the members of these two families such as monocyte activation [JE/MCAF (7)], T-cell activation [RANTES (8)], footpad swelling [TCA-3 (9)], and neutrophil activation [MIP-1α and MIP-1β (4)]. It has been suggested that, generally speaking, the -CXC- members affect neutrophils, whereas the -CC- members affect monocytes and T-cells (5). Only MIP-1α and the related human peptide LD78 have thus far been shown to be capable of exhibiting potent hematopoietic stem cell inhibitory activity (3, 10). In an effort to gain a more complete understanding of the mode of action of MIP-1α as a stem cell inhibitor and to attempt to define its role, if any, in the genesis of malignant disease, we have initiated studies aimed at characterizing the MIP-1α receptor.

Little is known about the receptors for members of this family. Initial characterization of the binding of one of the members of the family, ACT-2, has been carried out. This peptide is encoded by an immune activation gene cloned from an activated T-cell library (11) and binding to peripheral blood lymphocytes, and various cell lines indicate a single class of abundant receptor with dissociation constants in the nanomolar range (12). Zachariae et al. (13) reported the presence of specific MCAF (human JE) receptors on human peripheral blood mononuclear cells, although they have been unable to derive binding data from these studies. Recently, Oh and colleagues (14) have published preliminary reports on the characterization of receptors for MIP-1α on T-cells and macrophages, which appear to show similar dissociation constants to the receptors studied by Napolitano et al. (12).

To date, IL-8, a -CXC- family member, is the only one for which the receptor has been cloned (15–17). This receptor is a member of the G-protein coupled superfamilly of receptors, to which cells respond by transiently mobilizing calcium (18). It is probable, therefore, given the high degree of conservation of three-dimensional structure of the members of the -CC- and -CXC- families, based on modeling of MCAF and a knowledge of the structure of a number of -CXC- members (19, 20), that the receptors for the other members of this family also belong to this receptor subtype.

As a result of the lack of demonstrable stem cell inhibition by other members of the family, we have reasoned that the stem cell inhibitory receptor may be different from that responsible for the other functions of the family. The stem cell inhibitory activity of MIP-1α, and presumably that of the close human homologue LD78, has been shown to be specific for the stem cell compartment, with these cytokines showing no inhibitory activity on more mature hematopoietic cells (3, 10).

Therefore, to maximize the chances of detecting the stem...
cell inhibitory receptor, we have used cell lines which, as initially isolated, displayed a primitive phenotype. The two cell lines that we have selected for study are the human K562 cell line (21) and the murine FDCPmix cell line (22, 23).

We report here the characterization of the human and murine receptors for MIP-1α on primitive hemopoietic cell lines. The receptors present on the human K562 cells, rather than being specific for MIP-1α, appear capable of binding the majority of members of the -CC- family. These cells may therefore express a general -CC- family receptor. The receptors on murine FDCPmix cells, on the other hand, display a more restricted repertoire of binding and may more specifically mediate stem cell inhibition. Binding competition studies indicate that these receptors are distinct from the previously cloned IL-8 receptor.

These results expand current knowledge concerning the receptors for this family and, as discussed below, are potentially more accurate than those previously reported, in terms of receptor numbers and binding constants, since the aggregation potential of the MIP-1α ligand has been taken into account. The multiple binding capabilities of the receptors (particularly those found on human cells) are intriguing, and this is discussed below in terms of mechanistic implications for the function of MIP-1α and related peptides.

**Results**

**Response of FDCPmix and K562 Cell Lines to MIP-1α.** Using a tritiated thymidine incorporation assay for proliferation, we sought to measure the response of our primitive cell lines to MIP-1α. As the results outlined in Fig. 2 demonstrate, both FDCPmix and K562 cells respond significantly to the inhibitory effects of MIP-1α. It must be pointed out that this response is weak and typically incomplete. One hundred ng/ml represents approximately 1 nM and is thus a considerably higher concentration than is required for complete inhibition of normal hemopoietic stem cells (3, 10). One further feature of this inhibition is that it is very variable, and one can observe, occasionally, potent inhibition by MIP-1α and also, occasionally, poor inhibition. The basis for this variability is not as yet known (see "Discussion"); however, it is for this reason that we have been unable to

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**Fig. 1.** Alignment of the primary sequences of members of the -CC- family of cytokines.

**Fig. 2.** Inhibition of FDCPmix and K562 cell lines by MIP-1α. Cells, either FDCPmix (a) or K562 (b), were incubated overnight (2.5 x 10⁴ in 100 µl) at 37°C with or without 100 ng/ml MIP-1α. Sixteen h later, 10 µl of 0.1 mCi/ml [³H]thymidine were added to the cells, and incorporation was allowed to continue for 4 h. Insoluble counts were trichloroacetic acid precipitated on glass fiber filters, and scintillation was counted. Values are expressed as means ± SEM and are representative of 5 replicate experiments. *, P < 0.05 (Student’s t test).
confidently define an ED\textsubscript{50} for the effects of MIP-1\textalpha on these cell lines.

**Binding of MIP-1\textalpha to Cell Surface Receptors.** We have used iodinated MIP-1\textalpha to obtain saturable and competitive binding to both FDCPmix and K562 cells following incubation with increasing concentrations of iodinated MIP-1\textalpha for 90 min at 37°C. Backgrounds of nonspecific binding in these studies have generally been 5% or lower. Representative results from a series of experiments are shown in Fig. 3, a and b.

Calculations of receptor numbers and binding affinities are based on a native molecular weight of 100,000 for the MIP-1\textalpha molecule. Binding of MIP-1\textalpha to FDCPmix cells (Fig. 3a) revealed the presence of a single class of receptor at 18,000 receptors/cell with a \(K_d\) of 330 pm. Similarly, K562 cells (Fig. 3b) were seen to possess a single class of receptor at 15,000 receptors/cell with a \(K_d\) of 600 pm.

**MIP-1\textalpha/MIP-1\textbeta Competition Analysis.** As discussed above, we hoped to identify a stem cell inhibitor specific receptor. MIP-1\textbeta is a cytokine which shares 65% identity at the amino acid level with MIP-1\textalpha and appears to share its inflammatory functions. It does not, however, appear to function as a stem cell inhibitor at equivalent concentrations to MIP-1\textalpha (3) (see below). Thus, if the cellular receptors detected on the K562 or FDCPmix cells were inhibitor specific, it may be assumed that they should not bind MIP-1\textbeta with the same efficiency as they do MIP-1\textalpha. To test this assumption, we have assessed the relative abilities of MIP-1\textalpha and MIP-1\textbeta to compete out the binding of iodinated MIP-1\textalpha to the cells.

The results from such an analysis are shown in Fig. 4. As can be seen, MIP-1\textalpha and MIP-1\textbeta compete with the iodinated MIP-1\textalpha for binding to the K562 cell surface receptors with identical affinity, and thus we conclude that the receptor under study recognizes both cytokines and is not MIP-1\textalpha specific. Identical results, again show-

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**Fig. 3.** Binding of radiolabeled MIP-1\textalpha to FDCPmix and K562 cells. FDCPmix (a) and K562 (b) cells (5 x 10\textsuperscript{5} (point) were incubated at 37°C for 90 min in the presence of increasing amounts of radiolabeled MIP-1\textalpha with or without a 100-200-fold excess of cold competing MIP-1\textalpha. The cells were subsequently washed in PBS and gamma counted. Scatchard analysis (lower panels) was carried out using the LIGAND program (35). Results are representative of at least 5 replicate experiments.
binding identical affinity binding of MIP-1α and MIP-1β, have been obtained using FDCPmix cells (data not shown).

-CC/C-CXC Family Displacement Studies. To further analyze the specificity of the MIP-1α/MIP-1β receptor, we obtained samples of a number of members of the two subfamilies and used them to attempt to block binding of MIP-1α to its receptor. The results obtained using K562 cells (Fig. 5a) demonstrate that, far from being MIP-1α/MIP-1β specific, the receptor appears to be able to bind all of the members of the -CC- family with the exception of TCA-3 and thus appears to be a general -CC- family receptor. It is possible that the structure of TCA-3 may differ from those of other -CC- family members, as it contains six cysteine residues and thus may not be expected to bind to the same receptor. It is noticeable that MCAF and RANTES show a reduced capacity to compete with MIP-1α for binding when compared with the other binding cytokines. The receptor present on K562 cells does not bind members of the -CXC- family such as IL-8 or MIP-2 and is thus distinct from the previously cloned IL-8 receptor.

Results obtained using the FDCPmix cell line were similar (Fig. 5b), although, in this case, RANTES appears to be unable to displace MIP-1α from its receptor, and again, MCAF was relatively weak at displacing MIP-1α. This receptor present on the FDCPmix cell line does not bind TCA-3, nor does it bind members of the -CXC- family, IL-8 and MIP-2. It therefore seems that the receptor on the murine FDCPmix cells has a slightly more restricted pattern of recognition of the -CC- family proteins than that observed on the human K562 cells.

The Inhibitory Properties of the -CC- and -CXC- Family Members. Given the results from the binding studies, we set about studying the ability of the various members of the -CC- and -CXC- peptide families to function as stem cell inhibitory molecules. Results from CFU-A direct addition assay analysis of the inhibitory activity of these peptides are shown in Table 1 and indicate that MIP-1α, MIP-1β, LD78, and ACT-2 are all active as stem cell inhibitors. It is intriguing, however, that MIP-1β and its human homologue, ACT-2, appear to be 10–20 times less active (ED₅₀ values of 80 and 40 ng/ml, respectively) than MIP-1α and LD78 in the bioassays (ED₅₀ values of 5 and 2 ng/ml). The basis for this discrimination is not yet known. The remaining cytokines, RANTES, MCAF, TCA-3, IL-8, and MIP-2, are all inactive in the CFU-A assay system (data not shown).

We have also tested LD78, MIP-1β, and ACT-2 in the FDCPmix inhibition assay, and although we have observed inhibition by LD78 similar to that observed for MIP-1α, we have been unable to demonstrate significant inhibition by either MIP-1β or ACT-2 (data not shown).

Mapping of Putative Receptor Binding Sites. Alignment of the amino acid sequences of the cytokines has allowed us to construct a picture of the individual residues that are likely to be involved in receptor binding on either human or murine cells. This analysis has assumed that the receptor binding site is conserved among MIP-1α, MIP-1β, ACT-2, and LD78, as these cytokines completely compete out MIP-1α binding to both murine and human cells. Residues marked with an asterisk (Fig. 1) fulfill the above analysis restrictions and may therefore be involved in receptor binding.

To further investigate the regions of the molecule which are involved in receptor binding, and to complement previous studies on IL-8 and PF4 (see “Discussion”), we synthesized peptides representing the amino terminus (residues 1–11; P1) and the carboxy terminus (residues 51–69; P2). Using the CFU-A assay, we found that neither of these peptides displayed any inhibitory activity when used alone (data not shown), nor did they interfere with the activity and therefore the receptor binding capacity of MIP-1α (Table 2). Precise conclusions are difficult to draw from these studies. It may be that neither the amino nor the carboxy terminal regions of the molecule are involved in receptor binding. However, our data do not rule out the possibility that dimerization of the protein is essential for receptor binding and that monomeric peptide would be ineffective in either binding or blocking of binding, even should these peptides correspond to regions that are involved in binding (see “Discussion”). The remaining sections of the peptide form themselves into two distinct loop structures, one presumably extending from the first cysteine to the third and the second extending from the second cysteine to the fourth cysteine. Proteolytic digestion studies on MIP-1α and LD78 (using endoproteinase Lys-C and Staphylococcus aureus Protease V8) indicate that both loops must remain intact for the molecule to be active as a stem cell inhibitor (data not shown). Thus, the loops are critical for binding, but the specific contact points with the receptor remain to be elucidated. Residues 27–34 and 39–43 (underlined in Fig. 1) are hydrogen bonded as two antiparallel β strands in the three-dimensional structure of IL-8 and in the modeled structure of MCAF and are thus likely to be closely associated with each other in the native peptide. The conserved lysine and arginine residues (also underlined) may be exposed on the surface of the molecule and may therefore also contribute to the receptor binding.
**Discussion**

**Binding of MIP-1α to Cell Surface Receptors.** In an effort to gain a more complete understanding of the mode of action of MIP-1α as a stem cell inhibitor, we have sought to identify cell surface receptors for this molecule on murine and human cell lines which display a stem cell phenotype. Our demonstration of the inhibitory effects of MIP-1α on both the human K562 and the murine FDCPmix cell lines suggests that these cells must carry the necessary receptors for articulating inhibitory responses to MIP-1α. The inhibition observed is, however, weak, incomplete, and often irreproducible. Such weak and incomplete inhibition of FDCPmix cells has also been reported recently by Clements and colleagues (24). As discussed above, it is likely that the MIP-1α receptor is a member of the seven membrane spanning G-protein linked receptor family (18), and thus it may be that the variability in responsiveness to MIP-1α reflects alterations in the intracellular levels of the necessary signal transducing G-proteins. Alternatively, these G-proteins may be present but may not be associated with the MIP-1α receptor. We are currently investigating the role of specific G-proteins in the inhibitory response to MIP-1α and
Table 1  Inhibition by -CC- family members in the CFU-A assay

Cytokines were tested for inhibitory activity in the direct addition CFU-A assay. Materials to be tested were added directly to the feeder layers (0.6% agar in α-MEM with sources of macrophage-CSF and granulocyte-macrophage-CSF) of the CFU-A assays, and the target cells were normal bone marrow cells at 5 x 10^5/ml in the upper layer (0.5% agar in α-MEM). The assay plates were incubated for 11 days, following which CFU-A colonies were scored after fixing in 2% formaldehyde; the colonies are expressed as means and SD of at least 3 replicate experiments.

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>CFU-A colonies/10^5 bone marrow cells (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP-1α</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.9 ± 1.1</td>
</tr>
<tr>
<td>1 ng/ml</td>
<td>10.2 ± 1.5</td>
</tr>
<tr>
<td>5 ng/ml</td>
<td>7.3 ± 2.4</td>
</tr>
<tr>
<td>25 ng/ml</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>125 ng/ml</td>
<td>0.5 ± 0.8</td>
</tr>
<tr>
<td>LD 78</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.2 ± 0.4</td>
</tr>
<tr>
<td>1 ng/ml</td>
<td>7.8 ± 2.0</td>
</tr>
<tr>
<td>5 ng/ml</td>
<td>4.9 ± 2.7</td>
</tr>
<tr>
<td>25 ng/ml</td>
<td>1.6 ± 2.1</td>
</tr>
<tr>
<td>125 ng/ml</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>MIP-1β</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.3 ± 2.4</td>
</tr>
<tr>
<td>8 ng/ml</td>
<td>9.3 ± 0.7</td>
</tr>
<tr>
<td>40 ng/ml</td>
<td>7.4 ± 3.2</td>
</tr>
<tr>
<td>200 ng/ml</td>
<td>1.3 ± 0.7</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>ACT-2</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.5 ± 1.9</td>
</tr>
<tr>
<td>4 ng/ml</td>
<td>9.6 ± 0.8</td>
</tr>
<tr>
<td>20 ng/ml</td>
<td>4.9 ± 1.4</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>0.7 ± 1.2</td>
</tr>
<tr>
<td>500 ng/ml</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

presence of the cold competitor than in its absence. Such binding curves are similar to those described by Zachariae et al. (13) for MCAF.

MIP-1α exists in physiological buffers, at the concentrations used in the present studies, in a stable noncovalently aggregated form with a native molecular weight of 100,000. The molecule is, however, capable of further extensive noncovalent self-aggregation, to the extent that it has been reported as having a native molecular weight in excess of 1,000,000 in certain buffers (4). We believe that the occasional unsaturable and uncompetitive binding curves reflect this strong self-aggregation potential of the MIP-1α molecule. Thus, when all available receptors have been occupied by MIP-1α molecules, the receptor bound molecules may be able to act as nucleating events for the further binding of MIP-1α by self-aggregation on the receptors. In this way, binding is seen to be neither saturable nor substantially competent. Why such results are only seen occasionally is at present unknown; however, it is generally observed following prolonged storage of the radiolabeled peptide, and not using freshly prepared material. No loss of activity is observed in the MIP-1α stored for prolonged periods, and thus it is unlikely that gross structural alterations are responsible for the unusual binding curves. It may be, therefore, that the extensive self-aggregation is a rare event in dilute solutions (such as the concentrations of the radiolabeled MIP-1α solutions) but that it is increasingly favored on prolonged storage or at higher concentrations. We are at present attempting to investigate the basis for this phenomenon.

The Binding Specificity of the MIP-1α Receptors. Our results demonstrate that the receptors present on both the human and murine cell lines bind MIP-1α and MIP-1β, a close homologue of MIP-1α, with identical affinity. Further analysis of the binding specificities of the receptors indicates that, far from being specific for MIP-1α and MIP-1β, these receptors, particularly those on the human cells, are capable of binding a range of -CC- family members. Of particular interest is the observation that only those members of the -CC- family which display stem cell inhibitory properties [i.e., MIP-1α, MIP-1β, LD78, and ACT-2 (Table 1)] strongly compete with MIP-1α for binding to the murine receptor. MCAF and RANTES compete less effectively and are inactive as stem cell inhibitors. We therefore propose that this receptor is a likely candidate for the stem cell inhibitor receptor and are in the process of attempting to expression clone.
this molecule, which may have a profound role to play in stem cell regulation.

To attempt to investigate the occurrence of this receptor in normal murine bone marrow, we have performed Scatchard analysis on binding isotherms obtained using murine bone marrow cells. The results indicate a complex binding pattern for whole bone marrow with two major classes of binding sites. The first is a low affinity site (Kd 16.5 nM), which appears to be abundant (approximately 40,000 receptors/cell). The second major receptor class detected occurred within the bone marrow at an apparently lower frequency (560 receptors/cell) than the low affinity receptor and had a dissociation constant that was similar to that detected on the FDCPmix cells (Kd 150 pm). It is difficult to say whether or not this is the in vivo equivalent of the receptor detected on FDCPmix cells, although the dissociation constant is similar. It is interesting to speculate that, if this receptor is present on normal bone marrow cells with a frequency similar to that seen on FDCPmix (18,000 receptors/cell), it may be present on only a small proportion (3%) of normal bone marrow cells. It may be, therefore, that in the bone marrow, this receptor has a relatively restricted distribution, being observed largely on the more primitive components of the hemopoietic system. Although this conclusion is at present speculative, it is clear from the analysis on normal bone marrow cells that the receptor detected on FDCPmix cells is only one of at least two different receptors for MIP-1α and that it is relatively rare. It is envisaged that confirmation of the relative distribution of receptors within normal bone marrow will come from binding studies on FACS sorted populations of murine bone marrow cells.

The demonstration of stem cell inhibitory properties for MIP-1β and ACT-2 is the first report of such activity by these cytokines. We have previously been unable to detect inhibitory activity with batches of MIP-1β produced in mammalian cells (3); however, these studies used crude supernatants from transient COS cell transfections, and the actual levels of MIP-1β present were never accurately estimated. It may be, therefore, that we have never reached sufficiently high levels of MIP-1β in our assay systems to detect inhibitory activity. MIP-1β has a glycosylation site and is believed to be glycosylated in its native form. One further possibility, therefore, is that the absence of glycosylation in the peptide, which was produced in bacterial systems for the present study, unmask the receptor binding site on MIP-1β or, more likely, removes a steric hindrance to binding. ACT-2 has no such potential glycosylation site and may thus be expected to be active in both the mammalian or bacterial produced forms. We have tested both and found this to be the case. We have thus far been unable to identify the glycosylation residues (if any) on mammalian MIP-1β, and therefore, we have as yet no clear insight into the apparent contradiction between the present report of inhibitory activity of MIP-1β and previous reports (3, 25) suggesting no such effects of MIP-1β.

The competition studies reported here are essentially qualitative, although they do serve to demonstrate the ability of diverse family members to compete for binding to the MIP-1α receptor. The problems of accurately defining the extent of aggregation and therefore the stoichiometry of the various family members would make more detailed displacement studies uninterpretable and calculations of inhibition constants of little value.

One further possibility is that the apparent cross-competition observed in the present studies results from the cytokines interacting with one or more chains in a receptor complex. Such a phenomenon has been observed with the hemopoietin receptor family where competition can be observed for binding to shared subunits without necessarily involving the specific ligand binding receptor (26). Such complex competition is unlikely with the members of the -CC- family of peptides, as the receptors for these cytokines, as discussed above, are likely to be members of the seven membrane spanning receptor family and are not believed to require more than one extracellular binding event for signal transduction.

It is intriguing and puzzling that a range of members of the -CC- family with apparently differing activities (5) can bind to the same receptor on the human cell line. How is it possible to argue for specificity of action on the basis of this single receptor subtype? Clearly, multiple receptor subtypes may exist, although we have only been able to detect a single class of binding affinity on either the human or murine cells. It may be that the specificity of action of the individual peptides is dictated by intracellular events downstream of receptor binding and that the receptor acts as much as a gateway into the cell as a signal transducer. The tendency for some members of this family of peptides to form large self-aggregates may simultaneously increase the intracellular stability and act as an intracellular pool of active cytokine. There is some circumstantial evidence to support this notion from the work of Broxmeyer and colleagues (25), who have demonstrated that a cell pulsed with MIP-1α and subsequently washed to release unincorporated cytokines, is still capable of showing inhibition. This is seen in long term assays and thus is not simply a case of surface bound ligand transducing a prolonged inhibitory signal. There are examples in which full biological function following binding of cytokines to extracellular receptors also requires intracellular events such as nuclear localization (27). This additional mechanism may also be required for the biological activity of MIP-1α and related peptides.

An Assessment of the Residues on -CC- Family Members Involved in Receptor Binding. We have used the information gained from the competition experiments to compare the primary sequences of the -CC- family members binding to both the human and murine cells, in an effort to elucidate residues which may be involved in receptor binding. Based on the peptide competition studies (Table 2), we have tentatively concluded that isolated NH2-terminal and COOH-terminal peptides are not sufficient for binding, but we cannot exclude the possibility that these regions of the protein contribute to binding. The latter possibility would fit with the proposals of Gronenborn and Clore and colleagues (19, 28), who suggest, on the basis of three-dimensional structure, that the long carboxy terminal α-helix in both IL-8 and MCAF is intimately involved in receptor binding. Furthermore, studies on the chemotactic activity of platelet factor 4 (a -CX- family member), indicating that the carboxy terminal tridecapeptide of the molecule is functional as a monocyte chemotatic agent (29, 30), lend credence to these conclusions and support the use of peptide frag-
ments as probes to investigate receptor binding of this family of peptides.

The success of these and other peptide studies with members of the -CXC- family suggest that this approach is a valuable one for studying potential ligand receptor interactions within members of the -CC-/CXC- superfamily. These results also imply that the peptide studies reported in Table 2 may have functional significance and that, for MIP-1α and presumably other members of the -CC- family, the carboxy terminus is not in itself sufficient for receptor binding. In this respect, it is interesting to note that results from Hébert et al. (31) and Clark-Lewis et al. (32), again using a synthetic peptide based study, suggest that the carboxy terminal α-helix is not singularly responsible for IL-8 receptor binding, although both of these groups propose that the amino terminal region is required for receptor binding. Clearly, precise conclusions cannot be drawn from the peptide data in Table 2; however, these observations indicate that the binding of MIP-1α and the other inhibitory peptides may not simply involve either the amino or carboxy terminal regions alone. Structural analysis and mutagenesis studies are required to further elucidate the portions of MIP-1α which are required for receptor binding.

Conclusions. In conclusion, we have identified cellular receptors for the -CC- family of cytokines on both human and murine cells. These abundant receptors are distinct from the IL-8 receptor and appear to recognize the majority of members of the -CC- family. The receptor detected on human cells appears to be capable of binding the majority of members of the -CC- family of peptides and may therefore be a general -CC- family receptor. The more restricted binding characteristics of the murine receptor on FDCPmix cells, i.e., the strong binding of MIP-1α, ACT-2, LD78, and MIP-1β [all shown to be stem cell inhibitors (Table 1)], the relatively weak binding of MCAF, and the absence of binding of RANTES and TCA-3 (inactive on stem cells), indicate that this receptor may be a candidate for the stem cell inhibitory receptor.

We also suggest possible receptor binding areas within the peptides on the basis of primary sequence analysis and peptide competition studies. These areas differ from those proposed for IL-8 and may therefore suggest alternate regions of the -CC- family peptides to be involved in receptor binding. Conclusions from our analysis are further substantiated by recent observations suggesting tyrosine 28 and arginine 30 to be critical for biological activity, and presumably for receptor binding, in MCAF (33). As shown in Fig. 1, these residues lie within the region of the molecule which we propose to be involved in receptor binding. The precise role of the receptors in mediating the varying biological effects of this family has yet to be elucidated. We are currently investigating possible mechanisms whereby specificity of action can be conferred on these proteins.

Finally, these observations establish the possibility that the stem cell inhibitor receptor can be isolated by expression cloning using the FDCPmix line as a source of mRNA.

Materials and Methods

Cytokines. Recombinant MIP-1α, MIP-1β, LD78, and IL-8 were obtained from R&D Systems (Minneapolis, MN). MIP-2 was a gift from Dr. Steve Wolpe (Genetics Institute, Cambridge, MA). ACT-2 was prepared as described previously (12). TCA-3 was kindly supplied by Dr. Martin Dorf (Harvard University), and RANTES was a gift from Dr. Tom Schall (Genentech, San Francisco, CA). Human JE (MCAF) was obtained from PeproTech (Rocky Hill, NJ). Peptides P1 (residues 1-11) and P2 (residues 51-69) were custom synthesized for us at the Microchemical Facility, AFRC Institute of Animal Physiology and Genetics Research, Cambridge, United Kingdom.

We have found that recombinant MIP-1α has a strong tendency to form large, noncovalent, self-aggregates of varying size (10). In our hands, the average size of the aggregates in physiological buffers (such as PBS) is 90-100 kilodaltons, and thus, for calculation of molecular weights, a mean aggregated molecular weight of 100,000 has been assumed for the native protein. MIP-1β also appears to exist in such high molecular weight aggregates, and thus, for molarity calculations involving MIP-1β, a similar native molecular weight of 100,000 is assumed. In our experience, neither cold MIP-1α nor cold MIP-1β will aggregate with the radiolabeled MIP-1α protein as measured using gel filtration chromatography (data not shown). Also, the ability of both MIP-1α and MIP-1β and a range of other -CC- peptides to displace MIP-1α from its receptor attests to the lack of aggregation between the radiolabeled MIP-1α and the cold molecules (see "Results").

Cells and Cell Culture. K562 cells were maintained in special liquid medium (GIBCO, Ltd., Paisley, United Kingdom) with 10% donor horse serum. FDCPmix cells (subclone 17/S) were a generous gift from Professor Mike Dexter and Dr. Elaine Spooner (CR Paterson Laboratories, Manchester, United Kingdom) and were maintained in special liquid medium with 10% donor horse serum and 10% WEHI conditioned medium.

Tritiated Thymidine Proliferation Assays. Tritiated thymidine assays were set up in 96-well plates (GIBCO). Briefly, either FDCPmix or K562 cells were washed thoroughly in PBS and resuspended at 2.5 x 10⁶ cells/ml in special liquid medium with 10% donor horse serum. To each well, 100 μl of this cell suspension were added (2.5 x 10⁵ cells), and the MIP-1α was then added in a final volume of 50 μl PBS. FDCPmix cell suspensions, which are dependent on interleukin 3 for survival, were brought to 10% with conditioned medium from the cell line WEHI. The plates were incubated overnight at 37°C in a humidified incubator (16 h).

The following morning, the cells in each well were resuspended by pipetting, and 100 μl of each cellular suspension were added to fresh wells containing 10 μl of 0.1 mCi/ml tritiated thymidine and incubated at 37°C for a further 4 h. Contents of these wells were then applied to 2.5 cm Whatman GF/C filters, and insoluble counts were precipitated with 15 ml of 5% trichloroacetic acid. Filters were then counted in a standard scintillation counter.

The Direct Addition CFU-A Assay. Inhibitory activity of MIP-1α and related cytokines was measured using the direct addition CFU-A assay exactly as described previously (3, 10). Briefly, feeder layers containing conditioned medium from the cell lines L929 and AF1 (sources of macrophage-CSF and granulocyte-macrophage-CSF, respectively) in 0.6% agar in α-MEM were added to 3-cm dishes (1 ml/layer). The putative inhibitory material to be tested was added to the dish and incorporated into the feeder layer. An upper layer of 0.3% agar in α-MEM...
containing normal bone marrow cells at 5 × 10^7/mL was then added. The assay plates were incubated for 11 days in a humidified incubator at 10% CO_2, 5% O_2, 85% N_2, following which CFU-A colony numbers were assessed by counting macroscopic colonies >2 mm in diameter (2, 34).

Radio-labeling of MIP-1α. MIP-1α was radio-labeled using iodogen (Pierce, Rockford, IL). Briefly, 10 μg of MIP-1α in PBS were incubated with 10 μg iodogen and 1 mCi Na^125I (New England Nuclear) in Eppendorf tubes for 15 min on ice. Following this procedure, the unincorporated iodine was separated from the labeled protein by applying the reaction mixture to a disposable desalt column (GF5 cellulose columns; Pierce) and eluting with PBS. Then, 500-μl fractions were collected and measured in a gamma counter to detect the peak of protein associated radioactivity, and the active fractions were pooled.

The labeled protein was checked for integrity on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and for activity on the previously described CFU-A inhibitory assay (2, 34). Labeled MIP-1α was stored at 4°C in polypropylene tubes and used within 1 month.

Using the method outlined above, MIP-1α was labeled to high specific activity (2.5 × 10^7 cpm/μg), and the 125I was observed to remain stably bound to the protein for periods of up to 1 month. MIP-1α exists in a native state as a noncovalently aggregated form with a molecular weight of 100,000 (10) (see "Discussion"). The iodination appeared to have no effect on the native molecular weight of the aggregated MIP-1α molecule, and the iodinated molecule displayed full biological activity in the CFU-A assay (data not shown).

Receptor Binding Assays. Cells in logarithmic growth phase (either FDCPmix or K562) were washed three times in PBS prior to use in the binding assays. They were then incubated (5 × 10^5 cells/point) in binding buffer [special liquid medium (GIBCO) with 10% donor horse serum (GIBCO) and 0.2% azide] with increasing concentrations of radiolabeled MIP-1α in the presence or absence of a 100-200-fold excess of unlabeled MIP-1α. The binding reactions were carried out at 37°C for 90 min, following which the unbound protein was separated from the receptor bound protein by three washes with PBS. Incorporation of radioactivity was assessed by gamma counting, and the results were analyzed using the LIGAND program (35).

Displacement Studies. The relative affinities of MIP-1α and MIP-1β for the receptor were tested by binding 0.5 nm radiolabeled MIP-1α to K562 or FDCPmix cells in the presence of increasing amounts of competing cold MIP-1α or MIP-1β. Binding reactions were carried out for 90 min at 37°C, and the cell pellets were gamma counted following extensive washing as described above.

The ability of various members of the -CC- and -CXC- families to displace MIP-1α from its receptor was analyzed by incubating cells with 0.5 nm radiolabeled MIP-1α in the presence or absence of a 100-fold molar excess of the competing cytokine. The cells were washed and counted as described above.

References