Characterization of Two High Affinity Human Interleukin-8 Receptors*

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Interleukin 8 (IL-8) and melanocyte growth-stimulatory activity/gro (MGSA) are structurally related proinflammatory cytokines that are chemotaxtants and activators of neutrophils. Recently, cDNA clones encoding a high affinity IL-8 receptor (IL-8R-A) and a "low affinity" IL-8 receptor (IL-8R-B) have been isolated from human CDNA libraries. These two receptors have 77% amino acid identity and are members of the G protein-coupled superfamily of receptors with seven transmembrane domains. We have expressed these two receptors in mammalian cells and find that in this system both receptors bind IL-8 with high affinity (Kd ~ 2 nM). The receptor affinities differ for MGSA, however. IL-8R-A binds MGSA with low affinity (Kd ~ 450 nM); IL-8R-B binds MGSA with high affinity (Kd ~ 2 nM). The transfected cells respond to ligand binding with a transient increase in the intracellular Ca2+ concentration. A Ca2+ response is found for IL-8R-A following the binding of IL-8; no response is found for MGSA. A Ca2+ response for IL-8R-B follows the binding of both ligands. Blot hybridization with oligonucleotide probes specific for the two receptors shows that mRNA for both receptors is present in human neutrophils. Analysis of IL-8 and MGSA binding data on neutrophils as well as Ca2+ response and desensitization data shows that the presence of these two IL-8 receptors on the cell surface can account for the profile of these two ligands on neutrophils. Characterized (6, 7). We isolated clones for one receptor from a human neutrophil cDNA library by expression cloning and binding to I125-IIL-8 (6). Characterization of mammalian cells transfected with these clones shows that they encode a receptor that binds IL-8 with high affinity (6). A second, "low-affinity" IL-8 receptor was isolated by Murphy and Tiffany (7) from a neutrophil-like cell line, HL-60 (stimulated with dibutyryl cAMP). This receptor was reported to have a low affinity for IL-8 and for the ligands MGSA and NAP-2 (7). No high affinity ligand for this receptor has yet been identified. Since the two receptors sequences are quite similar (77% identical), it was suggested that the second receptor might be a high affinity receptor for one of the several cytokines that are related to IL-8 (6, 7). Both receptors are members of the superfamily of seven transmembrane domain-containing proteins that bind to guanine nucleotide binding proteins (G proteins).

Several groups have shown that there is a single class of high affinity IL-8 binding sites on human neutrophils (Kd ~ 1 nM (5, 8-10). Binding competition studies with I125-IIL-8 and unlabeled MGSA, however, have shown that the IL-8 binding sites on neutrophils can be resolved into two classes, one that binds MGSA with high affinity and another that binds MGSA with low affinity (5). Blot hybridization with cDNA probes for the two cloned IL-8 receptors have shown that mRNA for both receptors is present in neutrophils (6, 7), although cross-hybridization of the two probes could not be ruled out. The published binding profile of these two IL-8 receptors would not be expected to account for the IL-8 and MGSA binding profile of neutrophils, suggesting that additional IL-8 receptors would be present on these cells.

To fully characterize the binding and intracellular Ca2+ response of the two IL-8 receptors, we have transfected cDNA clones encoding both receptors into mammalian cells and have performed binding and Ca2+ response experiments. These results show that both receptors bind IL-8 with high affinity. One receptor binds MGSA with low affinity, the other binds MGSA with high affinity. This is just the profile expected from the binding competition studies on neutrophils suggesting that these are the two high affinity IL-8 receptors present on these cells. Since these data show that IL-8 is a high affinity ligand for both these receptors, we use the designation IL-8R-A for the receptor we previously identified (6) and the designation IL-8R-B for the receptor identified by Murphy and Tiffany (7).

**EXPERIMENTAL PROCEDURES**

Seventy-two amino acid IL-8 was expressed in Escherichia coli, purified, and labeled with I125 (typical initial specific activity, 930 Ci/mmol) as described (11). Full-length MGSA was expressed in E. coli,
purified, and labeled with \(^{125}\text{I}\)-labeled Bolton-Hunter reagent (12). The labeled MGSA was purified by a combination of reversed-phase HPLC and ion-exchange chromatography. The initial specific activity was 370–740 Ci/mmol.

The expression vector, pRK5.8rr.27-1.1, used to express IL-8-R-A, has been previously described (6). A DNA clone encoding IL-8-R-B was isolated from a \(1\times10^6\) cDNA library synthesized from oligo(dT)-primed human peripheral blood lymphocyte mRNA (13, 14). The library was screened at low stringency with a probe from IL-8-R-A, positive clones were subcloned into a plasmid vector, and the DNA sequence of the coding region matches that reported (7). The expression vector, pRK5.8rr.27-1.1, containing the IL-8-R-B coding region was constructed by ligating the 165-bp \(Hn1111/\text{Hi}1111\) fragment containing the coding region (after filling in the ends with the large fragment of DNA polymerase) into the SmaI site of the vector pRK5 (16, 17). DNA was transfected into human 293 cells by electroporation (18) and assayed for binding or Ca\(^{2+}\) response after 2 days.

Binding assays were performed as described (6); the incubations were in 0.2 ml at 0–4 °C for 2 h. Individual assay determinations are plotted. The binding data were curve fit with the computer program LIGAND (19) to determine the affinity \((K_d)\), number of sites, and nonspecific binding. The estimated standard error of the fit parameters for each curve is given in the figure legends. The number of sites per cell is not corrected for the transfection efficiency. The curves shown are the binding isotherms determined by LIGAND. The intracellular Ca\(^{2+}\) response was determined by flow cytometry as described (6).

Total RNA was isolated from human neutrophils as described (20). Poly(A) RNA was purified by oligo(dT) cellulose chromatography (17). RNA blot hybridization was performed by electrophoresing samples on a 1% agarose gel containing 2.2 M formamide followed by transfer to nitrocellulose (17). Duplicate blots were hybridized to chemically synthesized oligonucleotide probes that were end-labeled by transfer to nitrocellulose (17). Hybridizations used the IL-8R-A-specific probes ohumi18rl.l and 1.2, \(5'\text{CCAGAAATCTTCAAAGCTGTCACTCTCCATGTTAAAATC-10}GCACTTAGGCAGGAGGTCTTAGAGAG.\)

**RESULTS**

Clones encoding the IL-8-R-B (a “low-affinity” IL-8 receptor described by Murphy and Tiffany (7)) were isolated from a human peripheral blood lymphocyte cDNA library by low stringency hybridization. An alignment of the encoded amino acid sequence of this receptor and that of IL-8-R-A (a high affinity IL-8 receptor isolated from a human neutrophil cDNA library (6)) is shown in Fig. 1. Although the two receptors share a sequence identity of 77% overall, the sequence similarity is low in some regions (28% for the amino terminus) and high in others with two blocks of sequence identity of 105 and 64 amino acids. The DNA sequence encoding these blocks is highly conserved as well with only 8 of the 105 codons and 5 of the 64 codons differing between the two sequences (data not shown).

In order to compare their binding characteristics, cDNA clones encoding the A and B receptors were expressed in mammalian cells, and binding assays were performed with \(^{125}\text{I}\)-IL-8 and \(^{125}\text{I}\)-MGSA. Unlike the findings of Murphy and Tiffany (7) using \(Xenopus\) oocytes, IL-8-R-B binds IL-8 and MGSA with high affinity (Fig. 2). When the binding is performed with increasing concentrations of \(^{125}\text{I}\)-IL-8 or \(^{125}\text{I}\)-MGSA, a single class of sites is observed \((K_d = 1.2 \text{ or } 0.80 \text{ nM, respectively})\) (Fig. 2, A and B). Competition binding experiments show that unlabeled IL-8 and MGSA fully compete for the binding of \(^{125}\text{I}\)-IL-8 or \(^{125}\text{I}\)-MGSA to IL-8-R-B (Fig. 2, C and D).

Analysis shows that the competition data with \(^{125}\text{I}\)-IL-8 gives \(K_d (\text{IL-8}) = 1.5 \text{ nM}\) and \(K_d (\text{MGSA}) = 4.0 \text{ nM}\) (Fig. 2C). For the competitions with \(^{125}\text{I}\)-MGSA (Fig. 2D), the \(K_d (\text{IL-8}) = 0.87 \text{ nM}\) and \(K_d (\text{MGSA}) = 2.2 \text{ nM}\).

Previous work had shown that IL-8-R-A binds IL-8 with high affinity \((K_d = 3.6 \text{ nM})\) (6). Competition studies with unlabeled MGSA show that MGSA can compete for \(^{125}\text{I}\)-IL-8 binding to IL-8-R-A, but that MGSA binds with an affinity that is about 200-fold less than that for IL-8 (\(K_d (\text{IL-8}) = 1.7 \text{ nM}\), \(K_d (\text{MGSA}) = 450 \text{ nM}\) (Fig. 3). The affinity of IL-8 binding to IL-8-R-A determined by increasing concentrations of \(^{125}\text{I}\)-IL8 is similar to that determined by IL-8 competition
The binding of \(^{125}\text{I}-\text{IL-8}\) (0.12 nM) to IL-8R-A expressed in 293 cells. \(K_d\) (IL-8) = 1.7 (±0.24) nM; \(K_d\) (MGSA) = 450 (±84) nM; 360,000 (±46,000) sites/cell.

FIG. 4. Intracellular Ca\(^{2+}\) response of IL-8R-A and IL-8R-B expressed in 293 cells. The intracellular Ca\(^{2+}\) concentration was monitored in individual cells by flow cytometry. IL-8 or MGSA (100 nM) was added at the arrow.

\((K_d = 3.0\ \text{nM})\) (data not shown). Binding studies to IL-8R-A using \(^{125}\text{I}-\text{MGSA}\) were impractical because of the low affinity of MGSA for the receptor.

To show that both IL-8 receptors are capable of signal transduction upon ligand binding, the intracellular Ca\(^{2+}\) response was determined in transfected cells (Fig. 4). As would be expected based on the binding data, IL-8R-A responds to IL-8, but not to MGSA. IL-8R-B shows a Ca\(^{2+}\) response to both ligands.

Blot hybridization of human neutrophil RNA with cDNA probes encoding IL-8R-A and IL-8R-B has suggested that both receptors are present in these cells (6, 7). However, the experiments were done before the extent of the DNA similarity of these two genes was fully appreciated and cross-hybridization could not be ruled out. Fig. 5 shows that with specific oligonucleotide probes from divergent regions of the two receptors, specific bands are found in human neutrophil mRNA. With the IL-8R-A probe, bands of equal intensity are found (2.2 and 2.8 kb). With the IL-8R-B probe, a major band of 3.2 kb and a less intense band of 2.8 kb is observed. The hybridization is slightly more intense with the IL-8R-B probe.

Binding studies with human neutrophils (in particular the competition of \(^{125}\text{I}-\text{IL-8}\) binding with unlabeled MGSA) have suggested that more than one class of IL-8 receptor sites are present on these cells (5). In order to determine whether the A and B IL-8 receptors could account for the binding characteristics found on neutrophils, a full characterization of neutrophil binding with both \(^{125}\text{I}-\text{IL-8}\) and \(^{125}\text{I}-\text{MGSA}\) was performed (Fig. 6). The binding constants from these experiments were then compared with the values determined above for the transfected A and B receptors (IL-8R-A: \(K_d\) (IL-8) = 1.7 nM, \(K_d\) (MGSA) = 450 nM; IL-8R-B: \(K_d\) (IL-8) = 0.80 nM, \(K_d\) (MGSA) = 1.2 nM). With increasing labeled ligand (up to about 10 nM), both IL-8 and MGSA bind to neutrophils with a single affinity class of binding sites (\(K_d\) (IL-8) = 1.3 nM, \(K_d\) (MGSA) = 1.6 nM) (Fig. 6, A and B). This is as would be expected as IL-8 binds both receptors with about the same affinity, and MGSA would only be expected to bind to IL-8R-B. For the competition binding of \(^{125}\text{I}-\text{MGSA}\) with unlabeled IL-8 or MGSA, a single class of sites is found (\(K_d\) (IL-8) = 1.5 nM, \(K_d\) (MGSA) = 1.4 nM) (Fig. 6D). IL-8 fully competes with the \(^{125}\text{I}-\text{MGSA}\) binding excluding the possibility of an MGSA-specific receptor on these cells. For the competition binding of \(^{125}\text{I}-\text{IL-8}\) with unlabeled IL-8, again the expected single class of sites is found (\(K_d\) (IL-8) = 1.9 nM) (Fig. 6C). The competition of \(^{125}\text{I}-\text{IL-8}\) binding with MGSA resolves the IL-8 binding into two components (Fig. 6C) (see also Ref. 5), as would qualitatively be expected if both the A and B receptors are present. Fitting of this data to a two-site model gives sites of high and low affinity for MGSA (\(K_d\) (MGSA) = 4.7 and 1200 nM). Given the greater uncertainties inherent in fitting two-site data, these affinity values are in close agreement with those found for IL-8R-A and IL-8R-B expressed in mammalian cells (see above). The two-site fit of the \(^{125}\text{I}-\text{IL-8}\) data gives a ratio of A to B type sites of about 1 to 2 (see also Ref. 5). Replication of the \(^{125}\text{I}-\text{IL-8}\) competition data with additional preparations of neutrophils gave ratios of 1 to 1 and 4 to 1 of A to B type sites (data not shown) indicating some variation in the relative amounts of the two types of binding sites. Thus, the IL-8 and MGSA binding properties of neutrophils can be explained by having roughly comparable numbers of IL-8R-A and IL-8R-B on the cell surface.

**DISCUSSION**

By studying the binding properties and intracellular Ca\(^{2+}\) response of two human IL-8 receptors expressed in mammalian cells, we have demonstrated that there is a second high affinity receptor for IL-8. The affinity of IL-8 for the A and B IL-8 receptors is similar (\(K_d\) = 1.2–3.6 nM). These receptors differ considerably however in their affinities for the related ligand MGSA. IL-8R-B binds both IL-8 and MGSA with similar affinities (\(K_d\) ~ 2 nM), while IL-8R-A binds MGSA with a 200-fold reduced affinity (\(K_d\) = 450 nM). A lack of MGSA binding to the rabbit homologue of IL-8R-A had been demonstrated previously (21). The transfected receptors are
also able to signal in response to ligand binding. A transient increase in the intracellular Ca²⁺ concentration is found for IL-8R-B with both IL-8 and MGSA, while IL-8R-A responds only to its high affinity ligand, IL-8.

The initial characterization of IL-8R-B by expression in Xenopus oocytes led Murphy and Tiffany (7) to the conclusion that it is a low affinity receptor for IL-8 and MGSA. Binding studies with ¹²⁵I-IL-8 did not saturate at the highest concentrations used (100 nM), and a small Ca²⁺ response to MGSA was found only at the highest concentration tested (300 nM) (7). These results contrast with the high affinity binding that we find with both ligands when IL-8R-B is expressed in mammalian cells. Since the DNA sequence of the coding regions transfected is the same and since the assay conditions used are very similar, it appears that the differing results are due to the different expression systems that were employed. We find similar binding results for IL-8 and MGSA binding to IL-8R-B in buffers containing Ca²⁺. The differing results obtained from the two expression systems suggests that some factor in the cellular environment (possibly posttranslational modification or interaction with other signaling proteins) strongly influences the affinity of the receptor for its ligands.

Differing results between Xenopus oocytes and mammalian cell expression of IL-8R-A have also been reported. The rabbit homologue of IL-8R-A was initially described as an fMLP receptor based on binding and Ca²⁺ response data in Xenopus oocytes (22). Subsequent mammalian cell expression data show that rabbit IL-8R-A does not bind or respond to fMLP and is in fact an IL-8 receptor (21, 23, 24). Thus, differing results have been found for both IL-8R-A and IL-8R-B. The study of gene expression in Xenopus oocytes is a widely used tool that has been used recently to expression clone and characterize mammalian receptors. However, in view of the simplicity of mammalian cell transfection and the differences found when using oocyte expression (7, 22), we favor the use of mammalian cells to provide a valid characterization of mammalian proteins and receptors.

Given that the two receptors show several regions of high as well as low amino acid sequence similarity (Fig. 1), it is tempting to speculate that the conserved regions are more likely to be directly involved in ligand binding than the other regions. We had suggested previously that the acidic amino terminus of IL-8R-A might participate in the binding of IL-8 which is quite basic (6). The lack of sequence similarity between the two receptors in this region makes this possibility seem unlikely. However, even when a large number of related sequences are known, the difficulties of inferring the locations of binding regions based on sequence similarity has been pointed out (25). The high degree of conservation of long blocks of DNA sequence for the two receptors interspersed with regions of lower conservation could also suggest that the two receptors might be derived by alternative splicing of a single gene product. Hybridization to genomic blots and to a genomic DNA library shows, however, that these two receptors are likely to be encoded by separate genes that have single exons for the coding regions.

We show here the expression in human neutrophils of mRNA for both IL-8 receptors by using oligonucleotide probes specific for each receptor. Characterization of the binding of IL-8 and MGSA to neutrophils by saturation and competition analysis shows that approximately equal amounts of IL-8R-A and IL-8R-B on the cell surface can account for the binding profile of these two ligands. Resolution of the binding to the two receptors is only observed for the competition of ¹²⁵I-IL-8 binding by unlabeled MGSA. Others have previously suggested based on similar data that there would be two IL-8 receptors on human neutrophils, one that would bind MGSA and NAP-2 with high affinity and one that would bind MGSA and NAP-2 with low affinity (5). Our data provides evidence that IL-8R-A and IL-8R-B are in fact these two receptors. Their relative amounts appear to differ among neutrophil preparations. We find ratios ranging from 1 to 2 (IL-8R-A to IL-8R-B) to 4 to 1. Others have suggested a ratio of 1 to 2 (5). Given the uncertainties in analyzing the binding data these ratios do not differ greatly.

Cross desensitization of the intracellular Ca²⁺ response in neutrophils by IL-8 and MGSA corroborates the binding properties of the two receptors. Initial stimulation by IL-8 blocks the subsequent Ca²⁺ response induced by MGSA, but initial stimulation by MGSA does not block a subsequent Ca²⁺ response to IL-8 (5). These are the results expected if both receptors are present on neutrophils. IL-8 would bind to

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3. K. Wong, J. Lee, and W. I. Wood, unpublished data.
4. G. Rice, unpublished data.
and desensitize both receptors, while MGSA could only desensitize IL-8R-B.

Of course, additional ligands may distinguish more receptor types on neutrophils. We want to stress that, while our data show that the binding properties of IL-8R-A and IL-8R-B can account for the binding observed on neutrophils, they do not unequivocally demonstrate that this is the case. Additional specific ligands or antibodies are needed to provide a conclusive demonstration. The complete competition of 125I-MGSA binding to neutrophils by unlabeled IL-8 excludes the possibility of an MGSA specific receptor on these cells (at least to the sensitivity of the assay). Since NAP-2 binds to and desensitizes neutrophils much like MGSA (5), the suggestion would be that this ligand will bind to the two IL-8 receptors as MGSA does with a low affinity for IL-8R-A and a high affinity for IL-8R-B.

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