NH$_2$- and COOH-Terminal Truncations of Murine Granulocyte Chemotactic Protein-2 Augment the In Vitro and In Vivo Neutrophil Chemotactic Potency

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NH₂- and COOH-Terminal Truncations of Murine Granulocyte Chemotactic Protein-2 Augment the In Vitro and In Vivo Neutrophil Chemotactic Potency¹

Anja Wuyts,² Anne D’Haese, † Valerie Cremers, * Patricia Menten, * Jean-Pierre Lenaerts, * Arnold De Loof, † Hubertine Heremans, † Paul Proost, * and Jo Van Damme*

Chemokines are important mediators of leukocyte migration during the inflammatory response. Post-translational modifications affect the biological potency of chemokines. In addition to previously identified NH₂-terminally truncated forms, COOH-terminally truncated forms of the CXC chemokine murine granulocyte chemotactic protein-2 (GCP-2) were purified from conditioned medium of stimulated fibroblasts. The truncations generated 28 natural murine GCP-2 isoforms containing 69–92 residues, including most intermediate forms. Both NH₂- and COOH-terminal truncations of GCP-2 resulted in enhanced chemotactic potency for human and murine neutrophils in vitro. The truncated isoform GCP-2(9–78) was 30-fold more potent than intact GCP-2(1–92)/LPS-induced CXC chemokine (LIX) at inducing an intracellular calcium increase in human neutrophils. After intradermal injection in mice, GCP-2(9–78) was also more effective than GCP-2(1–92)/LIX at inducing neutrophil infiltration. Similar to human IL-8 and GCP-2, murine GCP-2(9–78) and macrophage inflammatory protein-2 (MIP-2) induced calcium increases in both CXCR1 and CXCR2 transfectants. Murine GCP-2(9–78) could desensitize the calcium response induced by MIP-2 in human neutrophils and vice versa. Furthermore, MIP-2 and truncated GCP-2(9–78), but not intact GCP-2(1–92)/LIX, partially desensitized the calcium response to human IL-8 in human neutrophils. Taken together, these findings point to an important role of post-translationally modified GCP-2 to replace IL-8 in the mouse. The Journal of Immunology, 1999, 163: 6155–6163.

The migration of leukocytes from blood vessels into tissues is an essential part of the inflammatory response. Adhesion molecules and chemokines are key mediators for leukocyte migration. Chemokines are low m.w. proteins that specifically attract subsets of leukocytes. They are divided into four subfamilies depending on the position of the conserved cysteines (CXC, CC, C, and CX₃C chemokines) (1–3). The CXC chemokine subfamily can further be divided into ELR⁺ and ELR⁻ CXC chemokines depending on the presence or the absence of the Glu-Leu-Arg (ELR) motif, respectively. This motif, positioned in the NH₂-terminal region just in front of the first cysteine residue, is important for receptor binding (4). ELR⁺ CXC chemokines are the most selective chemotactic factors for neutrophil migration. In the human system, seven ELR⁺ CXC chemokines have been identified: IL-8; GROα, β, and γ; neutrophil-activating protein-2 (NAP-2); epithelial cell-derived neutrophil attractant-78; and granulocyte chemotactic protein-2 (GCP-2) (4). However, in the mouse, only three ELR⁺ CXC chemokines with neutrophil-activating properties are known: KC, the murine homologue of GROα (5–8); macrophage inflammatory protein-2 (MIP-2), the murine homologue of GROβ/γ (9); and GCP-2 (10). The murine counterpart of IL-8, the most potent human ELR⁺ CXC chemokine, has not been identified yet. It is likely that this chemokine does not exist in the mouse and that the other murine ELR⁺ CXC chemokines replace IL-8. The most potent murine neutrophil chemotactic protein is GCP-2. Different NH₂-terminally truncated forms of this chemokine were isolated from conditioned medium of thymic epithelial cells as well as of fibroblasts (10).

Human ELR⁺ CXC chemokines activate their target cells by binding to two receptors: CXC chemokine receptor 1 (CXCR1) and CXCR2 (11, 12). IL-8 and GCP-2 are highly efficacious ligands for both CXCR1 and CXCR2, whereas the other ELR⁺ CXC chemokines are efficient ligands for CXCR2 only (13, 14). In the mouse, only one homologue of CXCRs (IL-8R homologue (IL-8Rh); 68 and 71% similarity with CXCR1 and CXCR2, respectively) has been identified (15). IL-8Rh binds the murine chemokines KC and MIP-2 with high affinity (16, 17).

In our previous study on mouse GCP-2, mixtures of NH₂-terminally processed forms were evaluated in vitro (10). Here, we show that further analysis of natural GCP-2 additionally revealed COOH-terminal truncation. The biological significance of these post-translational modifications was studied both in vitro (micro-chamber assay, calcium release assay) and in vivo (intradural...
injection in mice) using pure recombinant GCP-2(1–92)/LPS-induced CXC chemokine (LIX) and synthetic GCP-2(9–78). Furthermore, the receptor usage of murine GCP-2 forms was determined using human neutrophils as well as CXCR1- and CXCR2-transfected cells.

Materials and Methods

Production, purification, and identification of natural murine GCP-2

The MO fibroblast cell line was grown in Eagle’s MEM with Earle’s salts (EMEM; Life Technologies, Paisley, Scotland) supplemented with 10% FCS (Life Technologies). To produce murine GCP-2, confluent monolayers (175 cm²; Nunc, Roskilde, Denmark) were induced for 72 h in EMEM containing 2% FCS and supplemented with the dsRNA pol(riboinosinic acid)pol(ribocytidylic acid) (poly r: rC; P-L Biochemicals, Milwaukee, WI) at 50 μg/ml plus LPS (Escherichia coli 0111:B4; Difco, Detroit, MI) at 10 μg/ml (10). Murine GCP-2 was purified from the conditioned medium by adsorption to controlled pore glass beads and by heparin-Sepharose affinity chromatography as previously described (18). As a third purification step, Mono-S cation exchange fast protein liquid chromatography (FPLC; Pharmacia, Uppsala, Sweden) at pH 4.0 was used. Proteins were eluted with a linear NaCl gradient (0–1 M) in 50 mM formate, pH 4.0 (1 ml/min, 1-ml fractions). Absorbance at 220 nm was measured as a parameter for the protein concentration (18). Alternatively, the heparin-Sepharose fractions containing murine GCP-2 were purified by Mono-S cation exchange chromatography at pH 6.4. Proteins were eluted with a linear NaCl gradient in 50 mM malonate, pH 6.4 (1 ml/min, 1-ml fractions). GCP-2 was further purified to homogeneity by reverse phase (RP-) HPLC on a C18 Aquapore RP-300 column (Perkin-Elmer, Norwalk, CT) and eluted with an acetonitrile gradient (0–80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid; 0.4 ml/min, 0.4-ml fractions). Absorbance at 220 nm was measured.

Purified proteins were analyzed for molecular mass and purity by SDS-PAGE under reducing conditions on Tris/tricine gels (19). The relative molecular mass markers used were phosphorylase b (M₉, 92,500), BSA (M₉, 66,200), OVA (M₉, 45,000), carbonic anhydrase (M₉, 31,000), soybean trypsin inhibitor (M₉, 21,500), and lysozyme (M₉, 14,400) (Bio-Rad, Richmond, CA) and the low molecular mass marker (Pierce, Rockford, IL), aprotinin (M₉, 6,500). Alternatively, the relative molecular mass markers OVA (M₉, 45,000), carbonic anhydrase (M₉, 31,000), β-lactoglobulin (M₉, 18,400), lysozyme (M₉, 14,400), bovine trypsin inhibitor (M₉, 26,000), and insulin (M₉, 3,400) (Life Technologies) were used.

To identify the NH₂-terminal amino acid sequence of GCP-2 forms that eluted at the same position during chromatographic purification, the proteins were separated by SDS-PAGE on Tris/tricine gels, electrobotted on polyvinylidene fluoride (PVDF) membranes (Problott, Perkin-Elmer), and stained with Coomassie brilliant blue R250. After destaining, membranes were rinsed five times with MilliQ water (Millipore, Bedford, MA). The protein bands were excised from PVDF blots and subjected to NH₂-terminal amino acid sequence analysis.

Murine GCP-2(2–78) was chemically synthesized by automated F-moc solid phase peptide synthesis on a 433A peptide synthesizer (Perkin-Elmer) as previously shown for human GCP-2 (20). After synthesis of the primary structure, the peptide was cleaved from the resin, and side-chain protecting groups were removed by stirring the resin-bound peptide under nitrogen in a cleavage mixture containing 0.75 g of phenol, 250 μl of ethanediol, 500 μl of thioanisole, 500 μl of MilliQ water, and 10 ml of trifluoroacetic acid for 90 min. Disulfide bridges were formed by incubation of RP-HPLC-purified unfolded peptide in 150 mM Tris-HCl, pH 8.7, containing 1 mM EDTA, 0.3 mM oxidized glutathione, 3 mM reduced glutathione, and 1 M guanidinium chloride for 90 min. The folded peptide was purified by C₅₃ RP-HPLC. Recombinant murine GCP-2(2–72)/LIX was purchased from PeproTech (Rocky Hill, NJ).

Chemotaxis assay

The neutrophil chemotactic activity was tested in the 48-well microchamber (Neuro Probe, Cabin John, MD) chemotaxis assay. Human or murine neutrophils were purified from fresh heparinized peripheral blood from one donor or from pooled mouse blood obtained by cardiac punctures, respectively, as described previously (18). The lower compartments of the microchamber, filled with test samples or controls, were separated from the upper compartments, containing 10 × 10⁶ neutrophils/ml, by a 5-μm pore size polycarbonate filter (Nuclepore, Pleasanton, CA). After incubation at 37°C for 45 min, migrated cells were fixed, stained, and counted in 10 microscopic fields/well. The chemotactic activity is expressed as a chemo- tactic index, i.e., the number of cells migrated to the test sample divided by the number of cells migrated to the negative control (18). Statistical analysis was performed using the Mann-Whitney U test.

Measurement of intracellular calcium concentration (Ca²⁺)

Changes in [Ca²⁺], were measured using the fluorescent indicator fura-2 as described by Grynkiewicz et al. (21). Purified neutrophils (10⁷ cells/ml) were incubated with 2.5 μM fura-2/AM (AM) (Molecular Probes Europe, Leiden, The Netherlands) and 0.01% pluronic F-127 (Sigma, St. Louis, MO) for 30 min at 37°C in EMEM containing 2% FCS. After incubation, cells were washed twice and resuspended (10⁶ cells/ml) in HBSS (1 mM Ca²⁺; Life Technologies) supplemented with 0.1% FCS and buffered at pH 7.4 with 10 mM HEPES/NaOH. Fura-2 fluorescence was measured in an LS50B luminescence spectrophotometer fitted with a temperature-regulated stirred cuvette holder (Perkin-Elmer) after equilibration of the cells at 37°C for 10 min (20). The excitation wavelengths used were 340 and 380 nm, with an emission wavelength of 500 nm. The fluorescence ratio R340/380 was calculated using the Grynkiewicz equation (21). The Kₘ used for calibration was 224 nM. In desensitization experiments, buffer or chemokine was added to the cells as a first stimulus, followed by the addition of an active chemokine concentration after 2 min. The percent inhibition of the increase in [Ca²⁺], in response to the second stimulus by prestimulation of the cells was calculated.

In addition to calcium measurements in neutrophils, changes in [Ca²⁺], were determined in human embryonic kidney (HEK) cells transfected with either CXCR1 or CXCR2, supplied by Dr. J. M. Wang (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD) (22). HEK cells were cultured in DMEM (Life Technologies) with 10% FCS and 100 μg/ml geneticin (Life Technologies) to maintain the transfected characteristics. Cells were treated with pyrviniumPDE (Life Technologies), washed, and loaded with fura-2/AM in DMEM with 10% FCS as described for neutrophils.

In vivo inflammatory properties

To evaluate the effects of GCP-2(9–78) and GCP-2(1–92)/LIX in vivo, C57BL/6 mice were shaved on the abdomen, and chemokines or lysozyme (negative control peptide) (diluted in 0.9% NaCl) or 0.9% NaCl were injected intradermally. After 2 h, 50 sites were counted in 10 microscopic fields for each injection site. Results are expressed as the mean number of granulocytes per field minus the mean number of granulocytes per field at the saline injection site. Statistical analysis was performed using Student’s t test.

Results

Purification and identification of different isoforms of murine GCP-2

The MO fibroblast cell line was stimulated with LPS and poly rI:rC to produce murine GCP-2 (10). The chemokine was purified by adsorption to controlled pore glass beads, heparin-Sepharose affinity chromatography, cation exchange chromatography at pH 4.0, and C₅₃ RP-HPLC based upon neutrophil chemotactic activity.

Since in a previous study truncated GCP-2 forms remained as inactive forms as much as possible. SDS-PAGE analysis and mass determination by MALDI-MS revealed that 6- and 7-kDa proteins corresponded to different isoforms of murine GCP-2 (Table I). The 7-kDa protein contained NH₂-terminally intact GCP-2 (78 aa) and GCP-2 missing two NH₂-
N-glycosylation sites, and additionally, the cDNA-deduced amino
acid sequence of a protein called LIX is identical with that of
natural GCP-2(L), except for a COOH-terminal extension of 14 aa
(23). Thus, COOH-terminal extension is the most probable
explanation for the higher molecular mass proteins.

In an effort to separate the 8.5-kDa from the 6-kDa GCP-2(S)
and the 9.5-kDa from the 7-kDa GCP-2(L), fibroblast-conditioned
medium was purified using cation exchange chromatography at pH
6.4 as an alternative for Mono-S FPLC at pH 4.0. In these condi-
tions, GCP-2 forms were better separated and were further purified
by C8 RP-HPLC. Purification of the GCP-2 forms eluting during
FPLC at 0.4–0.45 M NaCl by HPLC yielded pure GCP-2 of 8.5
kDa as well as GCP-2 of 9.5 kDa, as determined by NH2-terminal
sequencing (Fig. 1B and Table I). However, by gel filtration a
15-kDa protein could not be separated from the 9.5-kDa GCP-
2(L). Possibly, the 15-kDa molecule corresponds to a dimer of this
protein. Indeed, blotting and sequencing of this 15-kDa protein in
the 9.5-kDa GCP-2(L) revealed the NH2-terminus of GCP-2(L) (data
not shown). To delineate the exact identity of the 8.5-kDa
GCP-2(S) and 9.5-kDa GCP-2(L), mass analysis was performed
by MALDI-MS. This revealed the presence of protein isoforms of
murine GCP-2 with an extended COOH-terminus for both 8.5-
and 9.5-kDa GCP-2. The 8.5- and 9.5-kDa GCP-2 contained several
COOH-terminally extended forms, including the one correspond-
ing to LIX. Therefore, the 8.5- and 9.5-kDa proteins were desig-
nated GCP-2(SL) and GCP-2(LL), respectively. In retrospective,
the 6- and 7-kDa GCP-2 forms were designated GCP-2(SS) and
GCP-2(LL), respectively. To obtain pure murine GCP-2(L)
(without the presence of the dimer), RP-HPLC fractions 51 and 52,
containing murine GCP-2(LS) and GCP-2(LL) (Fig. 1A), were fur-
ther submitted to cation exchange chromatography at pH 6.4,
yielding pure 7- and 9.5-kDa protein (Fig. 1C). Amino acid se-
quence analysis confirmed that the separated proteins contained
the same NH2-terminus corresponding to the longer form of
GCP-2.

Comparison of the neutrophil chemotactic activity of NH2- and/
or COOH-terminally truncated forms of natural murine GCP-2
After purification to homogeneity, the different natural isoforms of
murine GCP-2 were compared for their neutrophil chemotactic
activity in the microchamber chemotaxis assay using human neu-
rophils (Fig. 2A). The minimal effective concentrations for murine
GCP-2(SS), GCP-2(SL), GCP-2(LS), and GCP-2(LL) to induce
significant neutrophil migration (p < 0.01 compared with negative
control) were 15, 30, 60, and 100 ng/ml, respectively. These results
confirm the previous findings that GCP-2(SS) is 4-fold more active
than GCP-2(LS) (10) and in addition show that GCP-2(SL) is 3
times more potent than GCP-2(LL). Thus, NH2-terminal trunca-
tion induces an increase in chemotactic potency of murine GCP-2.
Furthermore, GCP-2(SS) and GCP-2(LS) are more potent than
GCP-2(SL) and GCP-2(LL), respectively, indicating that COOH-
terminal cleavage also increases the chemotactic potency of the
chemokine. Although the latter effect is less pronounced than that
of NH2-terminal truncation, the combined cleavage of murine
GCP-2 at both termini of the protein generates a chemokine with
a 10-fold higher potency than that of the intact protein (Fig. 2A).

The most active and most prominent (10) natural isoform of
murine GCP-2, i.e., GCP-2(9–78), was synthesized by solid phase
peptide synthesis, whereas the least active form, GCP-2(1–92),
corresponded to recombinant LIX. The neutrophil chemotactic
activities of pure synthetic GCP-2(9–78) and recombinant GCP-2(1–
92)/LIX were compared in the microchamber assay. As shown for
the natural GCP-2 isoforms, the truncated protein was much more
active than the intact protein (Fig. 2B). GCP-2(9–78) was active
terminal amino acids. These long isoforms were previously called
murine GCP-2(L). The smaller 6-kDa protein corresponded to ad-
tional truncated forms of murine GCP-2 that were missing four
to eight NH2-terminal residues and were previously designated
murine GCP-2(S) (10). Furthermore, HPLC fractions 47 and 48
(eluting at 27.5% acetonitrile) contained an 8.5-kDa protein, whereas fractions 51 and 52 (elut-
ing at 28.5% acetonitrile) contained a 9.5-kDa protein band in
addition to the 7-kDa GCP-2(L) (Fig. 1A). Blotting of the latter
fractions on PVDF membranes and sequencing of the proteins re-
vealed that the 6- and 8.5-kDa proteins, on the one hand, and the
7- and 9.5-kDa proteins, on the other hand, have the NH2-terminal
amino acid sequence, corresponding to the NH2-terminus of mur-
ine GCP-2(S) and GCP-2(L), respectively. The difference in rel-
ative molecular mass of the GCP-2(S) and GCP-2(L) doublets can
be explained by additional amino acids at the COOH-terminus or,
alternatively, by glycosylation. Murine GCP-2 does not contain
N-glycosylation sites, and additionally, the cDNA-deduced amino

![FIGURE 1. Separation of different isoforms of murine GCP-2 by FPLC and RP-HPLC. A, Murine GCP-2, isolated from MO cell-conditioned me-
dium by adsorption to CPG, heparin-Sepharose affinity chromatography, and cation exchange chromatography at pH 4.0, was further purified by
RP-HPLC. Proteins eluted from the column were evaluated for purity and relative molecular mass by SDS-PAGE on Tris/tricine gels (4 μl/lane;
reducing conditions; silver staining). B, Murine GCP-2, isolated from MO cell-conditioned medium by adsorption to CPG and heparin-Sepharose af-
finity chromatography, was further purified by cation exchange chroma-
tography at pH 6.4. Proteins eluting from the cation exchange column at
0.4–0.45 M NaCl were further purified by RP-HPLC. The eluted proteins
were analyzed by SDS-PAGE (20 μl/lane; reducing conditions; silver staining). C, The protein doublet containing GCP-2(LS) and GCP-2(LL),
purified from MO cell-conditioned medium by adsorption to controlled pore glass beads, heparin-Sepharose affinity chromatography, cation ex-
change chromatography at pH 4.0, and RP-HPLC (A), was submitted to cation exchange chromatography at pH 6.4. Eluted proteins were analyzed
by SDS-PAGE (20 μl/lane; reducing conditions; silver staining). Relative mo-
lecular mass markers (100 ng each) are as indicated in Materials and Methods.](http://www.jimmunol.org/ Downloaded from)
from 3 ng/ml onward \((p < 0.01)\), whereas 300 ng/ml of GCP-2(1–92)/LIX was necessary to obtain significant migration. Thus, a 100-fold difference in chemotactic potency between GCP-2(1–92)/LIX and GCP-2(9–78) was observed, whereas natural GCP-2(SSL) and GCP-2(LL) showed a 10-fold difference in potency. This can be explained by the presence of other isoforms in GCP-2(SS) and GCP-2(LL) in addition to GCP-2(1–92)/LIX and GCP-2(9–78). A 100-fold difference in chemotactic potency between GCP-2(1–92)/LIX and GCP-2(9–78) was observed, whereas natural GCP-2(SSL) and GCP-2(LL) showed a 10-fold difference in potency. This can be explained by the presence of other isoforms in GCP-2(SS) and GCP-2(LL) in addition to GCP-2(1–92)/LIX and GCP-2(9–78), respectively (see Table I).

The chemotactic activities of murine GCP-2(9–78) and GCP-2(1–92)/LIX were also compared in the microchamber assay using murine neutrophils (Fig. 2C). Again, it was found that GCP-2(9–78) is 100 times more potent than GCP-2(1–92)/LIX (minimal effective concentrations of 10 and 1000 ng/ml, respectively; \(p < 0.01\) compared with negative control).

### Table I. Identification of different natural isoforms of murine GCP-2

<table>
<thead>
<tr>
<th>Protein (Mᵦ)</th>
<th>NHᵢ-Terminal Amino Acid Sequence</th>
<th>Relative Amount (%)</th>
<th>Determined Molecular Mass</th>
<th>Corresponding COOH Terminus</th>
<th>Theoretical Molecular Mass</th>
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<td>9.5 kDa (LL)</td>
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<td>KKKAKRNAL</td>
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</table>

\(\text{**Note:** The accuracy of the experimentally determined masses is \pm 0.15%.}

\(\text{**b:** ND, not detected.}

To determine whether murine GCP-2 recognizes the same receptors as human IL-8 and murine MIP-2 on human neutrophils, calcium desensitization experiments were performed. Cross-desensitization was observed among murine GCP-2(9–78), MIP-2, and human IL-8 (Table II). IL-8 at 5 ng/ml completely inhibited the calcium increase in response to 50 ng/ml murine GCP-2(9–78). However, GCP-2(9–78) at 150 ng/ml and MIP-2 at 20 ng/ml could only partially desensitize for the IL-8 response (1.5 ng/ml). In contrast, murine GCP-2(9–78) at 50 ng/ml completely abolished the calcium increase induced by 7 ng/ml MIP-2, and, inversely, 20 ng/ml MIP-2 inhibited the GCP-2-induced calcium response (50 ng/ml). These data indicate that murine GCP-2 and MIP-2 use an identical receptor(s) to activate human neutrophils and that GCP-2 shares at least one receptor with human IL-8. In contrast to murine GCP-2(9–78), GCP-2(1–92)/LIX at concentrations up to 1000 ng/ml did not decrease the calcium response to IL-8, suggesting a lower affinity of the latter protein for the IL-8Rs.

**CXCR1 and CXCR2 receptor usage by murine GCP-2 and MIP-2**

The receptor usage by murine GCP-2(9–78) was further evaluated by performing calcium measurements in human CXCR1- or CXCR2-transfected HEK cells. As previously shown, human IL-8 and human GCP-2 efficiently induced an increase in \([\text{Ca}^{2+}]_i\), in both CXCR1- and CXCR2-transfected cells (Fig. 4). IL-8 was a more potent stimulus for CXCR1 than for CXCR2, whereas human GCP-2 showed similar minimal effective concentrations for calcium induction through both receptors. Both murine GCP-2 and MIP-2 induced an increase in \([\text{Ca}^{2+}]_i\), in CXCR1- as well as CXCR2-transfected cells, whereas the human homologue of murine neutrophils (Fig. 2) were also compared in the microchamber assay using GCP-2(9–78), respectively (see Table I).
MIP-2, GROβ/γ, failed to efficiently affect CXCR1 (13). In contrast to IL-8, both murine GCP-2 and MIP-2 were more potent at inducing an increase in \([\text{Ca}^{2+}]_i\), through CXCR2 than through CXCR1. Murine GCP-2 at 50 ng/ml increased the \([\text{Ca}^{2+}]_i\) in CXCR2-transfected cells, whereas 150 ng/ml was necessary to observe an effect in CXCR1-transfected cells. Murine MIP-2 was 10 times more potent at stimulating a calcium increase in CXCR2-transfected cells than in CXCR1-transfected cells; the minimal effective concentrations were 7 and 70 ng/ml, respectively. Because human IL-8 was more efficient at signaling through CXCR1 in this test, the results suggest a predominant CXCR2 usage by murine GCP-2 and MIP-2. This confirms that functionally the murine IL-8R is the equivalent of CXCR2.

**Comparison of the biological activities of murine GCP-2 (9–78) and GCP-2(1–92)/LIX in vivo**

To confirm the differences in potency between GCP-2 isoforms observed in vitro, mice were intradermally injected with different concentrations of murine GCP-2(1–92)/LIX or GCP-2(9–78) and with lysozyme and 0.9% NaCl as negative controls. Murine GCP-2(9–78) induced significant \((p < 0.01)\) granulocyte accumulation 2 h after injection at a dose of 10 ng, whereas GCP-2(1–92)/LIX was only active at a dose of 150 ng (Fig. 5). No cell types other than granulocytes were chemotactically induced at the injection site (Fig. 6). Lysozyme (1000 ng) did not induce infiltration of granulocytes (data not shown). These data indicate that GCP-2(9–78) is also more potent than GCP-2(1–92)/LIX in vivo. However, the difference in potency may be less pronounced in vivo than in vitro.

**Discussion**

In the human system, IL-8 is the most potent neutrophil-activating chemokine, whereas the other ELR \(^\text{CXC}\) chemokines GROα, -β, and -γ; NAP-2; epithelial cell-derived neutrophil attractant-78; and GCP-2 are weaker chemoattractants (4). In the mouse the equivalent of human IL-8 has not been identified. However, murine GCP-2 is equally potent to attract murine neutrophils as human IL-8 (10). This indicates that in the mouse other chemokines replace IL-8 during inflammation.

Murine GCP-2, isolated from conditioned medium of epithelial cells or fibroblasts, occurs in several NH2-terminally truncated forms (10). In this study we show that fibroblasts stimulated with LPS plus poly rI:rC produce not only different NH2-terminally, but also COOH-terminally truncated forms of murine GCP-2. These NH2- and/or COOH-terminal truncations give existence to murine GCP-2 isoforms containing 69 (GCP-2(10–78)) (10) to 92 residues (GCP-2(1–92)/LIX), including intermediate forms. Naturally truncated forms shortened (S) at the NH2-terminus or COOH-terminus compared with other human and murine ELR \(^\text{CXC}\) chemokines (Fig. 7). This is also the case for the murine ELR \(^\text{CXC}\) chemokine LIX.
and human ELR\(^+\) CXC chemokine MIG (26, 27). The COOH-terminal truncation of murine GCP-2 gives rise to a protein that is more similar in length to the other ELR\(^+\) CXC chemokines (Fig. 7).

Previously, only the murine isoforms GCP-2(SS) and GCP-2(LS) were identified and compared for their bioactivity. It was found that the NH\(_2\)-terminally truncated GCP-2(SS) forms are more potent than the longer GCP-2(LS) forms. In this study we also compared the neutrophil chemotactic and activating potencies of COOH-terminally processed forms of murine GCP-2. Both NH\(_2\)-terminal and COOH-terminal truncation result in an increase in neutrophil chemotactic potency, but COOH-terminal truncation has a somewhat less pronounced effect (2-fold) than cleavage at the NH\(_2\)-terminus. The higher potency of the truncated isoforms of GCP-2 was confirmed in intracellular signaling experiments, yielding a 30-fold difference in sp. act. NH\(_2\)-terminal cleavage has previously been reported for other CXC chemokines, including human IL-8 (24, 28), NAP-2 (24, 29), and GCP-2 (30, 31) and murine GCP-2 and KC (10). No natural chemokine truncation beyond the ELR motif, necessary for biological activity (32), has been observed. NH\(_2\)-terminal truncation of ELR\(^+\) CXC chemokines often induces an increase in biological potency. The various natural NH\(_2\)-terminally processed forms of IL-8 have never been completely separated chromatographically (33). However, using defined synthetic and recombinant material, the NH\(_2\)-terminally truncated IL-8 forms were shown to be more active than the longer forms (32, 34). Platelet basic protein and connective tissue-activating protein-III, precursors of NAP-2, need to be processed at the NH\(_2\)-terminus to become neutrophil attractants (29, 33). In contrast, the different NH\(_2\)-terminal isoforms of human GCP-2 did not show differences in potency in the chemotaxis assay (31).

<table>
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<tr>
<th>First Stimulus</th>
<th>Concentration (ng/ml)</th>
<th>Second Stimulus</th>
<th>Concentration (ng/ml)</th>
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FIGURE 4. Induction of an increase in \([\text{Ca}^{2+}]_i\) in CXCR1 and CXCR2 transfectants by murine GCP-2 and MIP-2. HEK cells, transfected with CXCR1 or CXCR2, were stimulated with different concentrations of human IL-8 and GCP-2 and murine GCP-2(9–78) and MIP-2. The \([\text{Ca}^{2+}]_i\) was calculated according to the Grynkiewicz equation (21). The detection limit for the increase in \([\text{Ca}^{2+}]_i\) (15 nM) is indicated by a dotted line. Data are derived from two experiments.
increased biological activity of ELR≤ CXC chemokines by NH₂-terminal processing is in contrast with data on other chemokines, such as the ELR≤ CXC chemokine stromal cell-derived factor-1α and the CC chemokines RANTES, macrophage-derived chemokine, and eotaxin, all showing impaired chemotactic activity and receptor binding after removal of their NH₂-terminal dipeptide by CD26 (35, 36). COOH-terminal truncation has only been described for a few CXC chemokines. Natural COOH-terminally truncated forms of NAP-2 missing four and seven residues were isolated from the conditioned medium of platelet-containing mononuclear cells (37–39). These isoforms were 3 and 5 times more potent than intact NAP-2 to degranulate neutrophils and to compete for NAP-2 receptor binding. Recombinant forms of NAP-2 missing five to seven residues showed a 5-fold increase in potency (38, 39). Using synthetic isoforms of IL-8, it has been shown that removal of the three COOH-terminal amino acids induces an increase in biological activity, whereas further removal of amino acids gradually decreases the potency (32). A 3.6-kDa form of IL-8, purified from conditioned medium of fibroblasts, with a 50-fold lower potency than intact IL-8 may represent a COOH-terminally truncated form (40). Finally, for the ELR≤ CXC chemokine MIG, COOH-terminally truncated forms (78–103 aa) were isolated from monocytes and THP-1 cells. This truncation resulted in a decrease in lymphocyte-activating potency (27).

In the human system, two ELR≤ CXC chemokine receptors (CXCR1 and CXCR2) have been identified. Human IL-8 and GCP-2 can efficiently activate cells through binding to both receptors, whereas the other ELR≤ CXC chemokines are better ligands for CXCR2 (13, 14). In the mouse, only the functional homologue of CXCR2, IL-8Rh, but not that of CXCR1, is known (15). The IL-8Rh binds the murine chemokines KC and MIP-2 with high affinity (16, 17) and is important for neutrophil migration to inflammatory sites (41). Furthermore, mice lacking the IL-8Rh show lymphadenopathy and splenomegaly (41). KC efficiently binds to human CXCR2, but not to CXCR1 (8, 42). Substitution of the amino acid sequence of KC between cysteines 2 and 3 with the corresponding domain of IL-8 confers binding to CXCR1 (42).

**FIGURE 5.** In vivo inflammatory properties of GCP-2(9–78) and GCP-2(1–92)/LIX. Different amounts of GCP-2(9–78) and GCP-2(1–92)/LIX in 0.9% NaCl were injected intradermally in mice. Saline (0.9% NaCl) was injected as a negative control. After 2 h, mice were sacrificed. Granulocytes were counted in 20 microscopic fields/injection site. Data are derived from eight mice. Results are expressed as the mean number of granulocytes per field minus the mean number of granulocytes per field at the saline injection site ± SEM. Asterisks indicate a significant effect of GCP-2 compared with 0.9% NaCl: * p < 0.01; ** p < 0.001.

**FIGURE 6.** In vivo recruitment of granulocytes by GCP-2(9–78) and GCP-2(1–92)/LIX. Mice were injected intradermally with 900 ng of GCP-2(9–78) (A), 100 ng of GCP-2(9–78) (B), 500 ng of GCP-2(1–92)/LIX (C), or 0.9% NaCl (D). After 2 h, mice were sacrificed, and injection sites were excised. Skin biopsies were stained with hematoxylin-eosin. Magnification, ×350.
Furthermore, it has been shown that the presence of a basic residue, Arg20, in human GCP-2 and Lys21 in IL-8, is essential for signaling through CXCR1 (43). In the murine chemokines GCP-2 and MIP-2, a basic amino acid is present at this position, i.e., Lys21 in murine GCP-2 and Arg17 in murine MIP-2 (Fig. 7). This basic residue is not present in KC (Gly17). According to these predictions, we indeed found that murine GCP-2 and MIP-2 can bind both CXCR1 and CXCR2 to activate cells. However, both chemokines were more potent to signal through CXCR2 than through CXCR1, but the efficiencies for both receptors were comparable. In contrast, others have shown that recombinant MIP-2 has only low affinity for CXCR1, whereas it bound CXCR2 with high affinity (44). This can be explained by the observation that high agonist potency and high affinity binding are distinct functions (45).

Human GCP-2 induces neutrophil accumulation and plasma extravasation in rabbit skin (20, 30). Murine KC and MIP-2 were also shown to have chemotactic properties in vivo (8, 9). After intradermal injection of murine GCP-2(9–78) and GCP-2(1–92)/LIX in mice, both isoforms induced significant neutrophil accumulation after 2 h; GCP-2(9–78) was more potent than GCP-2(1–92)/LIX. It has been shown that IL-8(6–77) is a more potent chemoattractant and activator of neutrophils than IL-8(1–77) in vitro, but in vivo both isoforms are equipotent, possibly due to rapid proteolytic processing of the 77-aa form (34). In addition to different proteases being involved in the processing of IL-8 and GCP-2, the fact that the effects of IL-8 were evaluated after 4 h, whereas the chemotactic activity of murine GCP-2 was evaluated after 2 h, may also explain the difference between these two experiments. The period of 2 h may be too short to completely convert the intact GCP-2 into truncated forms. However, the difference in potency between the two GCP-2 isoforms may be lower in vivo than in vitro. Because multiple intermediately processed forms (26 in total) have been purified from natural cellular sources, this might be due to partial cleavage of intact GCP-2 into truncated GCP-2. In conclusion, murine GCP-2 occurs as 28 different NH2- and COOH-terminally truncated forms. Truncation yields more potent GCP-2, both in vitro and in vivo. This indicates that during accumulation and activation, the presence of both chemokines and proteases that can cleave chemokines will determine the efficiency of leukocyte accumulation and activation.

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References


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