Human and Bovine Granulocyte Chemotactic Protein-2: Complete Amino Acid Sequence and Functional Characterization as Chemokines[†,‡]

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ABSTRACT: Tumor cells are capable of simultaneously producing a number of related inflammatory peptides, now classified as chemokines. We have isolated a new human granulocyte chemotactic protein (GCP-2), coproduced with interleukin-8 (GCP-1/IL-8) by osteosarcoma cells. Furthermore, the bovine homologue of human GCP-2 was purified from kidney tumor cells using the same isolation procedure. Both chemokines occur in at least four NH_2-terminally truncated forms. These 5–6-kDa proteins do not differ in potency and efficacy as granulocyte chemotactic factors using a standard in vitro migration assay. The complete primary structures of human and bovine GCP-2 were disclosed by sequencing peptide fragments derived from the natural proteins. On the basis of the conservation of four cysteine residues, the two molecules are to be classified within the C–X–C chemokine family, including IL-8. Human and bovine GCP-2 are 67% similar at the amino acid level. Their sequences show only weak similarity with that of IL-8, and human GCP-2 does not cross-react in a radioimmunoassay for IL-8. Human and bovine GCP-2 are specific granulocyte chemotactic factors in that they do not attract human monocytes. Bovine GCP-2 is not species specific since it is at least as active as human GCP-2 on human granulocytes. Both chemokines can also activate postreceptor mechanisms leading to release of gelatinase B by granulocytes. This is indicative for a possible role in inflammation and tumor cell invasion.

Phagocytosis is an essential feature of the immune system and is preceded by chemotaxis of the phagocyte to the inflammatory focus. Bacterial and viral infections are often accompanied by local secretion of chemotactic substances, including cytokines. These selectively stimulate the migration of phagocytes in the chemotactic gradient to the site of infection. A recently discovered family of chemotactic cytokines, now designated chemokines, is composed of structurally related low molecular weight proteins [reviewed in Oppenheim et al. (1991), Van Damme (1991), Miller and Krangel (1992), and Baggiolini et al. (1992)]. The hallmark for this family of proinflammatory proteins is the conservation of four cysteine residues that are important for the tertiary structure. The chemokines can be divided into two subfamilies depending on whether the first two cysteines are adjacent (C–C chemokines) or not (C–X–C chemokines). A number of human genes for the C–X–C and C–C chemokines have been located on chromosomes 4 and 17, respectively. In contrast to classical chemotactic agents (such as platelet-activating factor, leukotriene B4, and complement factor 5a), the chemokines selectively attract monocytes or granulocytes. Monocyte chemotactic proteins (MCP)[1] predominantly belong to the C–C group, whereas the C–X–C subfamily contains a number of granulocyte chemotactic proteins (GCP). Human interleukin-8 (IL-8/GCP-1) is the best characterized neutrophil-activating protein of the C–X–C subfamily. The mature 7–8-kDa protein, secreted by cleavage of the signal peptide from its 99 amino acid precursor, acts as a dimer on neutrophils through at least two identified functional receptors (Holmes et al., 1991; Murphy & Tiffany, 1991). Activation of neutrophils by IL-8 results in secretion of proteases (Masure et al., 1991), adherence to endothelium, and emigration (Huber et al., 1991). This has been demonstrated by intradermal and intravenous injection of the chemokine, resulting in local and systemic accumulation of granulocytes, respectively (Van Damme et al., 1988).

Most chemokines, except for some platelet products, can be produced by different cell types after appropriate stimulation [reviewed in Oppenheim et al. (1991), Van Damme, (1991), Miller and Krangel (1992), and Baggiolini et al. (1992)]. IL-1 is a physiological inducer of both monocyte- and granulocyte-specific chemokines in fibroblasts, monocytes, and endothelial cells. In addition, a variety of tumor cell lines are reported to produce GCPs and/or MCPs. In this study we describe the biochemical and biological characterization of novel granulocyte chemotactic proteins (GCP-2) produced by human osteosarcoma cells and by bovine kidney cells. The complete amino acid sequence of both chemokines could be deduced from overlapping peptide fragments of the natural proteins. Human and bovine GCP-2 have a very similar primary structure and are both to be classified as C–X–C chemokines. Since the two cytokines are selectively chemotactic for granulocytes and also stimulate these cells to secrete proteases, they can be considered as authentic chemokines.

MATERIALS AND METHODS

Production and Purification of Human and Bovine Granulocyte Chemotactic Factors. Human MG-63 osteosarcoma cells and bovine MDBK (Madin Darby bovine kidney) cells

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[‡] Human and bovine GCP-2 sequences are available from EMBL/Gen Bank/DDB under accession numbers P80162 and P80221, respectively.

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[1] Abbreviations: GCP, granulocyte chemotactic protein; MCP, monocyte chemotactic protein; ENA, epithelial cell-derived neutrophil activator; NAP, neutrophil-activating peptide; IL-8, interleukin-8; FPLC, fast protein liquid chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography.

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were grown in Eagle's minimum essential medium (EMEM; Gibco, Paisley, Scotland) with Earle's salts containing 10% calf serum (Gibco). Confluent monolayers (175 cm^2; Nunc, Roskilde, Denmark) of MG-63 cells were stimulated in serum-free medium with silicic acid-purified human cytokine from mononuclear cells stimulated with LPS (*Escherichia coli* 0111.B4; Difco, Detroit, MI) and concanavalin A (Calbiochem, San Diego, CA) (Van Damme et al., 1988). MDBK cell cultures were washed and incubated with 25 mL of serum-free medium containing 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis, MO) for 48 h. Conditioned medium of three consecutive inductions was pooled, concentrated, and partially purified by adsorption to controlled pore glass (CPG-10-350; Serva, Heidelberg, Germany). Chemotactic activity was eluted with 0.3 M glycine–HCl, pH 2.0, and loaded on a heparin–Sepharose (CL-6B; Pharmacia, Uppsala, Sweden) column in 50 mM Tris–50 mM NaCl, pH 7.4. After washing with this equilibration buffer, the chemotactic activity was eluted in a linear NaCl gradient (0.05–2 M) in 50 mM Tris, pH 7.4. The heparin–Sepharose-derived chemotactic activity was further purified by Mono S cation-exchange fast protein liquid chromatography (FPLC; Pharmacia) in 50 mM formate, pH 4.0. Proteins were eluted in a linear NaCl gradient (0–1 M) in 50 mM formate, pH 4.0. The final purification step consisted in C8 RP-HPLC of the chemotactic FPLC fractions. FPLC fractions of 1 mL were injected on a C8 Aquapore RP-300 column (Applied Biosystems Inc., Foster City, CA) equilibrated with 0.1% trifluoroacetic acid (TFA) in H_2O (solvent A) and eluted with an acetonitrile gradient (solvent B, 80% CH_3CN–0.1% TFA in H_2O) at 0.4 mL/min.

FPLC- and HPLC-derived fractions were checked for purity by SDS–PAGE on a linear gradient (10–25%) polyacrylamide gel and silver staining. The relative molecular mass markers (Bio-Rad Laboratories, Richmond, CA) phosphorylase *b* (M_r 92 500), BSA (M_r 66 200), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), and lysozyme (M_r 14 400) and the low relative molecular mass marker (Pierce Chemical Co., Rockford, IL) aprotinin (M_r 6500) were used.

Generation of GCP-2 Peptides and Amino Acid Sequencing. In order to identify the chemotactic proteins, the NH_2-terminal amino acid sequence was determined by Edman degradation on a pulsed liquid (477A/120A) amino acid sequencer (Applied Biosystems) with on-line PTH–amino acid analysis. Cysteine residues were determined by on-filter reduction and modification with tributylphosphine and 4-vinylpyridine (Aldrich Chemical Co. Inc., Wisconsin) (Andrews & Dixon, 1987).

To extend the sequence information, internal peptide fragments were prepared using different proteolytic enzymes. Chemotactic protein (4 μg) was incubated with 0.2 μg of enzyme in the suitable incubation buffer, and peptide fragments were separated on a C8 Aquapore RP-300 column as described for the final purification of the chemotactic factors. Endoproteinases (sequencing grade; Boehringer Mannheim, Mannheim, Germany) used were Lys-C (37 °C, 18 h in 25 mM Tris–HCl buffer, pH 8.5, 1 mM EDTA), Arg-C (37 °C, 18 h in 90 mM Tris–HCl buffer, pH 7.6, 8.5 mM $CaCl_2$, 5 mM DTT, 0.5 mM EDTA), Asp-N (37 °C, 18 h in 50 mM sodium phosphate buffer, pH 8.0), and Glu-C (25 °C, 18 h in 25 mM ammonium carbonate buffer, pH 7.8). All peptides were sequenced as described above.

Alternatively chemical digestion of the proteins was performed in 75% formic acid at 37 °C for 50 h. After the formic acid digestion, peptide fragments were dried on the cartridge filter of the protein sequencer. A solution of *o*-phthalaldehyde (Fluoropa, Pierce) and 2-mercaptoethanol in acetonitrile was added, and the fragments were incubated for 10 min in a continuous trimethylamine flow in order to block all peptide chains except for the one starting with an NH_2-terminal proline (Brauer et al., 1984). The remaining reagents were washed away with ethyl acetate and *n*-butyl chloride.

Assays for Chemotaxis and Enzyme Release. Human monocytes and granulocytes were isolated from heparinized peripheral blood from single donors. Sedimentation for 30 min in hydroxyethyl starch (Plasmasteril; Fresenius AG, Bad Homburg, Germany) was used to remove erythrocytes. Mononuclear cells and granulocytes were further separated by gradient centrifugation for 30 min at 400*g* on Ficoll–sodium metrizoate (Lymphoprep; Nyegaerd, Oslo, Norway). For monocyte chemotaxis experiments the total mononuclear cell fraction was used. The remaining erythrocytes in the granulocyte pellet were eliminated by lysis in bidistilled water for 30 s.

Migration of monocytes and granulocytes was measured with microchambers (Falk et al., 1980). The lower compartment of the microchamber (Neuro Probe Inc., Cabin John, MD) was filled with dilutions of test samples (27 μL). For monocyte chemotaxis the upper compartment was filled with 2×10^6 cells/mL (50 μL) in Hanks' balanced salt solution (HBSS) supplemented with 1 mg/mL human serum albumin. The lower and upper compartments were separated by a 5-μm-pore-size poly(vinylpyrrolidone)-treated polycarbonate membrane (Nuclepore, Pleasanton, CA). Granulocyte chemotaxis was performed for 45 min with 10^6 cells/mL (50 μL) in the same medium using a 5-μm-pore-size poly(vinylpyrrolidone)-free polycarbonate membrane. After fixation and staining of the filters with Diff-Quik (Harleco, Gibbstown, NJ), the assays were scored by counting the cells that had migrated through the membrane in 10 microscopic fields per well. Samples were run in duplicate in each assay. The chemotactic index was calculated by dividing the number of cells that migrated through the membrane to the sample by the number of migrated cells to the control medium.

Release of gelatinase B was used as a parameter to measure granulocyte activation. Purified granulocytes [(1–3) $\times 10^6$ cells/mL) were stimulated (in serum-free medium) with test reagents for 15–45 min. Supernatants were centrifuged to remove cells, and gelatinase activity was determined by SDS–PAGE zymography using gelatin as a substrate (Masure et al., 1991). Human IL-8 was used as a positive control for gelatinase B production. Gelatinase activity was detected as unstained bands on a Coomassie brilliant blue R-250 background. Quantitative determination of gelatinase activity was achieved by computerized scanning densitometry. Gelatinase activity was expressed in "scanning units", representing the scanning area under the curves, which is an integration ratio that takes into account both the brightness and the width of the substrate lysis zone.

Radioimmunoassay for IL-8. Pure natural IL-8 (Van Damme et al., 1988) derived from human peripheral blood leukocytes was used to prepare an antibody in goat. [^{125}I]-IL-8 (2000 Ci/mmol) was purchased from Amersham (Buckinghamshire, United Kingdom). The radioimmunoassay for IL-8 was performed as described by Rampart et al. (1992). Briefly, column fractions, IL-8 (natural) standard, [^{125}I]IL-8 (1/1000), and IL-8 antibody (1/3000) were diluted in Tris-buffered saline, pH 7.4, containing 1% bovine serum

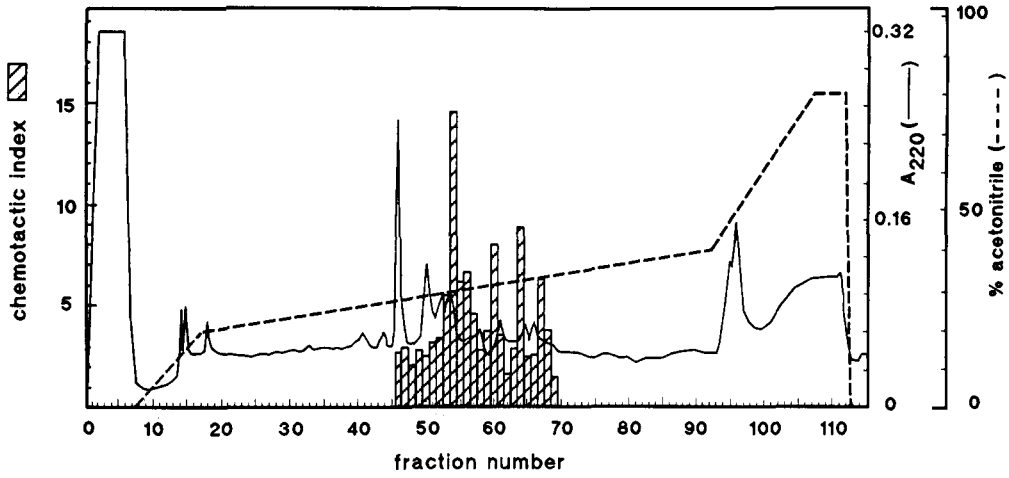

FIGURE 1: Separation of different forms of human GCP-2 by RP-HPLC. Partially purified granulocyte chemotactic activity from MG-63 cells was further fractionated by cation-exchange FPLC. The GCP-2 peak fraction devoid of IL-8 and GRO was finally applied on a C8 Aquapore RP-300 column and eluted with an acetonitrile gradient (---). Absorbance (—) was measured at 220 nm, and granulocyte chemotactic activity (histograms) was determined using the microchamber migration assay.

albumin and 0.2% gelatin. Column fractions or IL-8 standard (100 μL) was mixed with antibody (50 μL) and [^{125}I]IL-8 (50 μL) and incubated for 18 h at room temperature. Antibody-bound reactivity was precipitated by addition of protein-A bacterial absorbent and counted in duplicate. The detection limit of the assay was 0.1 ng/mL.

RESULTS

(1) Separation and Characterization of Multiple Forms of Natural Human GCP-2. Human GCP-2 from MG-63 osteosarcoma cells was concentrated and purified from large volumes (5 L per batch) of conditioned medium by adsorption to controlled pore glass, heparin–Sepharose chromatography, and cation-exchange FPLC (Proost et al., 1993). Although not completely pure at this stage, GCP-2 was separated from related proteins such as IL-8 and GRO. Figure 1 illustrates that, upon purification to homogeneity by HPLC, the GCP-2 activity dissociated in multiple peaks. Four 6-kDa proteins eluting in the corresponding active fractions were visualized by SDS–PAGE and could be identified as GCP-2 by NH_2-terminal sequence analysis (about 30 cycles for each form). Except for NH_2-terminal truncation, no differences were observed between the sequences of these proteins. Thus, elution on RP-HPLC probably separates the truncated NH_2-terminal forms of the mature protein due to cleavage of hydrophobic residues. This results in GCP-2 forms that are missing two (fraction 60), five (fraction 56), and eight (fraction 54) amino acids (Figures 1 and 2).

The four GCP-2 forms were further compared for their potency as granulocyte chemotactic factors. Table I confirms that all forms of GCP-2 are biologically active in the microchamber assay. These GCP-2 forms stimulate neutrophil migration in a dose-dependent fashion. Their potency (specific activity) and efficacy (maximal chemotactic response) were found to be similar. However, although GCP-2 and IL-8 also have an equal efficacy, GCP-2 was found to be at least 10-fold less potent when compared to IL-8.

Checkerboard analysis was used to determine whether GCP-2 can induce chemokinesis rather than chemotaxis. When both compartments of the microchamber were filled with GCP-2 at the same concentration, stimulation of cell migration was reduced to control levels. This indicates that GCP-2 is an authentic chemotactic factor (data not shown).

(2) Complete Sequence of Human GCP-2. In order to obtain internal sequences, pure natural protein was cleaved with three different endoproteinases. Proteolytic fragments were purified on a RP-HPLC column. After endoproteinase Lys-C digestion, four internal fragments could be sequenced (Figure 2). Endoproteinase Asp-N fragmentation confirmed sequence information obtained by NH_2-terminal sequencing and by endoproteinase Lys-C digestion. Cleavage of the protein with endoproteinase Glu-C yielded a fragment overlapping the gap between the Lys-C sequence stretches. The carboxy-terminal residues were determined by formic acid cleavage of the Asp–Pro bound. In this case, all peptides obtained, except those starting with a proline, were blocked at the NH_2-terminus with *o*-phthalaldehyde, allowing the COOH-terminal sequence of human GCP-2 to be determined without purification of the formic acid digest.

Alignment of the sequences from the multiple GCP-2 fragments resulted in the complete elucidation of the primary structure of this novel granulocyte chemotactic protein. Most residues have been confirmed several times by repeated sequencing of overlapping fragments. The sequence of human GCP-2 allows the molecule to be classified as a member of the chemokine family, on the basis of the conservation of four cysteine residues. The protein is most related to human ENA-78 (Walz et al., 1991) (77% similarity), whereas the relationship with human IL-8 (31% similarity) is rather weak (Table II).

(3) Isolation and Sequencing of the Bovine Equivalent of Human GCP-2. For the isolation of bovine GCP-2, serum-free conditioned medium of bovine MDBK cells was processed as described to isolate human GCP-2. Granulocyte chemotactic activity purified by heparin–Sepharose chromatography was further fractionated by cation-exchange FPLC. The activity eluting in the NaCl gradient at the position corresponding to that of human GCP-2 (0.7 M) was purified to homogeneity by HPLC. Figure 3A illustrates that, similarly to human GCP-2, the bovine granulocyte chemotactic activity from FPLC dissociates after HPLC into several 5-kDa protein bands. In particular, four protein peaks eluted in the gradient at 31.5% (fraction 62), 32% (fraction 64), 32.5% (fraction 66), and 34% (fraction 72) acetonitrile. Figure 3 shows that in those GCP-2 fractions no other contaminating proteins were present (high molecular weight bands are artifacts). Running a gel with lower amounts of protein demonstrated that in each GCP-2 fraction only a single 5-kDa band could

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NH2-terminal  GPVSAVLTELRCTCLRVTLRVNPKTIGKLQVFPAG(3)
                VSAVLTELRCTCLRVTLRVNPKTIGKLQVFPAG(3)
                  VLTELRCTCLRVTLRVNPKTIGKLQVFPA(2)
                    ELRCTCLRVTLRVNPKTIGKLQVFPAG(1)

Glu-C digest    VSAVLTELRXTXLRVTLRVN(1)                    VVASLKNGKQVCLDPE(3)

Lys-C digest                                LQVFPAGPQCSK(1)        QVCLDPEAPFLK(1)
                                            LQVFPAGPQCSKVEVVA(1)
                                                      VEVVASLK(1)

Asp-N digest          ELRXTXLRVTLRVNPKTIGKLQVFPAGPQXSKVEVV(2)

HCOOH digest                                                          PEAPFLKKVIQKILDSGNK(2)
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Hu-GCP-2      GPVSAVLTELRCTCLRVTLRVNPKTIGKLQVFPAGPQCSKVEVVASLKNGKQVCLDPEAPFLKKVIQKILDSGNK
              *** **  **** **  •    ***   ***  ********** • ****  ********  **  *******
Bo-GCP-2      GPVAAVVRELRCVCLTTTPGIHPKTVSDLQVIAAGPQCSKVEVIATLKNGREVCLDPEAPLIKKIVQKILDSGKN
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NH2-terminal          ELRXVXLTTTPGIHPKTVS(1)
                    RELRCVCLTTTPGIHPKTVSDLQVIAAGPQCSKVEVIATLKNGRXV(1)
                  VRELRCVCLTTTPGIHPKTVSDLQVIAAGPQ(2)
              GPVAAVVRELRXVXLTTTPGIHPKTVSDLQVIAAGPQ(1)

Arg-C digest                                                  EVCLDPEAPLIK(1)
                                                                    IVQKILDSGKN(1)
                                                              EVXLDPEAPLIK(1)
                                                                   KIVQKILDSGKN(1)

HCOOH digest                                                  PEAPLIKKIVQKILDSGKN(1)
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FIGURE 2: Amino-terminal and internal sequencing of human and bovine GCP-2. Homogeneous (HPLC-grade) GCP-2 from MG-63 cells (Figure 1) and from MDBK cells (Figure 3) was directly subjected to NH_2-terminal sequence analysis. Alternatively, GCP-2 was first submitted to proteolytic cleavage, followed by separation of peptide fragments on RP-HPLC before sequencing. Values between brackets indicate the number of sequence runs performed for each peptide. Ambiguous or undetectable residues (e.g., underivatized cysteines) are marked with an X. Human (upper part) and bovine (lower part) GCP-2 peptide sequences are aligned (see box). Asterisks demonstrate identical amino acids in human and bovine GCP-2. Human and bovine GCP-2 sequences are available from EMBL/Gen Bank/DDB under accession numbers P80162 and P80221, respectively.

Table I: Comparison of Human GCP-2 Forms in Stimulating Neutrophil Chemotaxis[a]

chemokine form	HPLC fraction	NH_2-terminal sequence	concn (nM)	chemotactic index, mean ± SEM (*n*)[b]
GCP-2-I (75 AA)	67	GPVSAVLTELRCTC	30	18.8 ● 4.9 (4)
			10	5.4 ± 1.3 (8)
			3	3.0 ± 1.1 (6)
			1	1.1 ± 0.0 (2)
GCP-2-II (73 AA)	60	VSAVLTELRCTC	30	49.5 (1)
			10	10.2 ± 2.5 (7)
			3	2.5 ± 0.4 (6)
			1	1.8 ● 0.3 (5)
GCP-2-III (70 AA)	57	VLTELRCTC	30	18.0 (1)
			10	4.7 ± 1.3 (6)
			3	2.4 ● 0.4 (6)
			1	2.2 ± 0.4 (5)
GCP-2-IV (67 AA)	54	ELRCTC	100	49.6 (1)
			30	11.8 ± 1.8 (7)
			10	3.4 ± 0.5 (6)
			3	2.0 ± 0.4 (5)
IL-8 (72 AA + 70 AA)		KELRCQC	1	28.4 ± 7.8 (6)
		SAKELRCQC	0.1	7.2 ● 1.2 (10)
			0.01	3.0 ± 0.6 (4)

[a] HPLC-purified and NH_2-terminally sequenced forms of natural human GCP-2 (forms I–IV) were tested in the granulocyte microchamber assay. [b] Chemokine response expressed as average chemotactic index ± standard error of the mean; *n* represents the number of determinations.

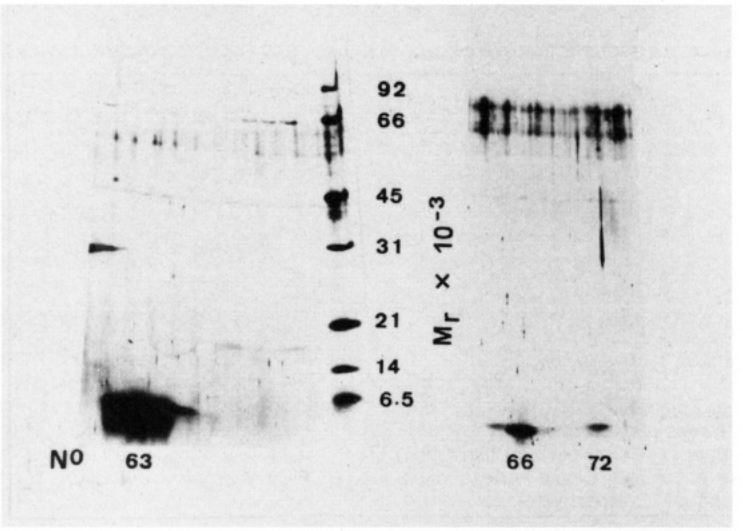
be visualized (data not shown). NH_2-terminal amino acid sequence analysis revealed that these 5-kDa proteins differed in truncation at the NH_2-terminus (Figure 3B). The complete sequence of bovine GCP-2 was deduced from overlapping COOH-terminal fragments obtained by endoproteinase Arg-C digestion and confirmed by formic acid digestion (Figure 2). It can be deduced that the four cysteines typical for chemokines are also conserved in bovine GCP-2. Moreover, the overall similarity with human GCP-2 was 67% at the protein level (Table II). In view of the similar amino acid sequence and elution pattern upon FPLC and HPLC, bovine GCP-2 is in all probability the equivalent of human GCP-2. However, at the protein level the molecule also shows an important similarity with the human platelet product NAP-2 (Begg et al., 1978) (60%) and with human ENA-78 (72%). In contrast, bovine GCP-2 has a weak similarity (39%) with human IL-8 (Table II). The shortest (67 residues) forms of human and bovine GCP-2 correspond in length to the shortest forms of human IL-8 and NAP-2. This indicates that these probably represent the ultimately NH_2-terminally processed natural chemokine forms. Indeed, from this position onward a significant increase in sequence similarity occurs which includes the two closely located cysteines.

(*4*) *Biological Activities of Human and Bovine GCP-2.* The four different forms of bovine GCP-2 were compared for their biological activity using the microchamber migration

Table II: Comparison (% similarity) of Amino Acid Sequence of Human and Bovine C–X–C Chemokines

	hu-GCP-2	hu-IL-8	hu-ENA-78	hu-NAP-2	hu-GRO-α	hu-GRO-β	hu-GRO-γ	hu-PF4	hu-IP-10	bo-PF-4
hu-GCP-2	100									
hu-IL-8	31	100								
hu-ENA-78	77	34	100							
hu-NAP-2	46	48	51	100						
hu-GRO-α	44	42	49	59	100					
hu-GRO-β	42	41	48	55	88	100				
hu-GRO-γ	44	40	51	52	85	84	100			
hu-PF4	41	34	43	51	46	42	41	100		
hu-IP-10	31	23	24	31	25	27	23	30	100	
bo-PF-4	39	37	40	50	47	47	46	76	24	100
bo-GCP-2	67	39	72	60	54	50	51	49	31	47

A



B

Bo-GCP-2 form	Fraction (HPLC)	% Aceto-nitrile	Sequences detected	conc. (μM)
III	62	31.5	RELRCVCLTTTPGIHPKTVSDLQV	2.0
	63	31.5	RELRCVCLTTTPGIHPKTVS	0.8
			VRELRCVCLTTTPGIHPKTV	0.7
II	64	32	VRELRCVCLTTTPGIHPKTV	1.0
IV	66	32.5	ELRCVCLTTTPGIHPKTVS	0.4
I	72	34	GPVAAVVRELRCVCLTTTPGIHPKTVSDLQV	0.25

FIGURE 3: Identification of NH_2-terminal forms of bovine GCP-2. MDBK cell-derived GCP-2 was purified to homogeneity by subsequent adsorption to controlled pore glass, heparin–Sepharose chromatography, FPLC, and RP-HPLC. GCP-2 forms present in the different RP-HPLC fractions (numbers indicated at the bottom of the gel) were visualized (panel A) by SDS–PAGE (20 μL/lane, reducing conditions, silver staining) and identified (panel B) by NH_2-terminal sequence analysis.

assay. Figure 4 shows that all GCP-2 forms have a comparable potency and efficacy on neutrophils. Although human cells were used as the test substrate, the bovine molecule was found to be as efficient as human IL-8 and human GCP-2 in that maximal chemotactic responses were similar (Figure 4). Because its minimum effective dose was as low as 1 nM (Table I and Figure 4), bovine GCP-2 was at least as potent as human GCP-2 in this test using human cells. For human GCP-2 about 3 nM was necessary to observe a chemotactic response, whereas IL-8 was still effective at a dose as low as 0.1 nM. When tested (concentration range of 1–100 nM) on human monocytes, human and bovine GCP-2 were found to be inactive (data not shown).

For further comparison of human and bovine GCP-2 with IL-8 the chemokines were tested as neutrophil-activating proteins in an enzyme release assay. Neutrophils have been reported to release gelatinase B, but not gelatinase A after stimulation with IL-8 (Masure et al., 1991). For this purpose secretion of gelatinase B activity was measured by zymography. Figure 5 illustrates that the three molecules can dose-dependently stimulate gelatinase B release from human neutrophils within 15 min. The lower specific activity of human GCP-2 when compared to bovine GCP-2 was also confirmed in this assay. The minimum effective concentration for GCP-2 is about 10 nM, suggesting that for gelatinase B release more chemokine is needed than for neutrophil migration.

(*5*) *Lack of Cross-Reactivity between Human IL-8 and GCP-2.* IL-8 has been detected in body fluids with a specific radioimmunoassay using labeled natural chemokine (Rampart et al., 1992). In order to exclude cross-reactivity with GCP-2, a batch of a MG-63 cell-derived chemokine mixture was fractionated by cation-exchange FPLC. In the microchamber assay granulocyte chemotactic activity is detectable corresponding to GRO, GCP-2, and IL-8, eluting at 0.6, 0.7, and 0.85 M NaCl in the FPLC gradient, respectively (Proost et al., 1993). However, with the radioimmunoassay for IL-8 no immunoreactivity could be measured in the fraction containing GRO, whereas the assay was quite sensitive for IL-8 (detection limit of 0.1 ng/mL). Since GCP-2 fractions (5 μg/mL)

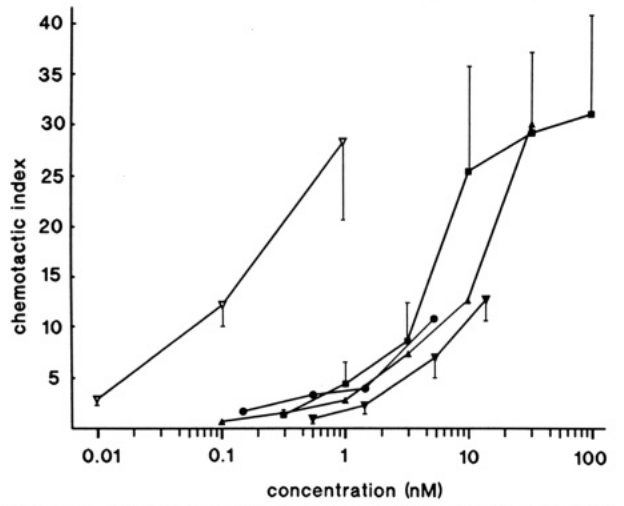


FIGURE 4: Granulocyte chemotactic activity of different NH_2-terminal forms of bovine GCP-2. Pure forms of MDBK cell-derived GCP-2 [form I, 75 residues (●); form II, 69 residues (▲); form III, 68 residues (■); form IV, 67 residues (▼) (see Figure 3)] were compared with pure natural human IL-8 (▽) in the microchamber assay for granulocyte chemotaxis. Average chemotactic indexes with standard errors of the mean were derived from four independent experiments.

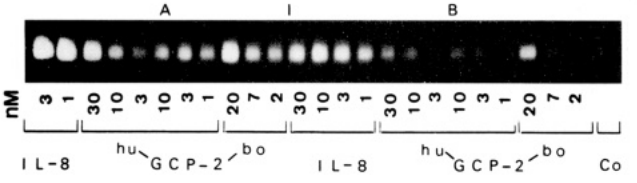


FIGURE 5: Stimulation of gelatinase B release from granulocytes by GCP-2. Purified granulocytes were activated with different doses of human (hu) GCP-2, bovine (bo) GCP-2, or human IL-8 or were left untreated (Co). Samples were harvested after 15 min (3×10^6 cells/mL, panel A) or 30 min (1×10^6 cells/mL, panel B) and analyzed by SDS–PAGE zymography. Gelatinase B activity is visible as unstained bands on a dark background.

contained only 20 ng/mL IL-8 immunoreactivity (probably due to contamination with authentic IL-8), it can be calculated that the IL-8 radioimmunoassay is at least 100-fold less specific for GCP-2 than for IL-8 (data not shown).

DISCUSSION

This study focuses on the identification of a human granulocyte chemotactic protein (GCP) and its equivalent in the bovine species. The complete primary structures (75 amino acids) of both chemotactic factors were disclosed by sequencing internal peptides derived from the natural proteins. The human and bovine factors could be classified as new members of the chemokine family on the basis of the conservation of four cysteine residues. In view of this structural relationship with human GCP-1/IL-8, the novel human chemokine and its bovine equivalent were designated GCP-2.

Human and bovine GCP-2 were purified from stimulated MG-63 and MDBK tumor cells, respectively. Both molecules have affinity for heparin and elute at an identical position upon cation-exchange chromatography. On SDS-PAGE (reducing conditions) pure human and bovine GCP-2 occur as 6- and 5-kDa proteins, respectively. NH_2-terminal processing of human and bovine GCP-2 results in multiple forms that differ in the number of deleted residues (from two to eight amino acids). This truncation of mainly hydrophobic amino acids allowed these forms to be separated by RP-HPLC. For the structurally related platelet product NAP-2 it has been demonstrated that NH_2-terminal processing by proteases is essential to generate a biologically active neutrophil-activating peptide from the inactive precursor molecule (Walz et al., 1989; Van Damme et al., 1989). We and others have previously observed a similar NH_2-terminal processing of natural IL-8 (Van Damme et al., 1989; Gimbrone et al., 1989). In addition, it has been demonstrated that the occurrence of these truncated forms of natural IL-8 is dependent on the producer cell type (Van Damme et al., 1991) and that such recombinant forms of IL-8 have a different specific activity in chemotaxis assays (Nourshargh et al., 1992). In contrast, for both the human and bovine species, truncated forms of GCP-2 were found not to differ individually in their capacity to stimulate granulocyte chemotaxis in vitro.

Because the most extended NH_2-terminal forms of human and bovine GCP-2 (75 residues) correspond in length to those of IL-8 and related chemokines, we can conclude that in all probability they represent the largest biologically active forms of GCP-2. Alternatively, the 67-residue forms of human and bovine GCP-2 are likely to be the ultimately processed biologically active molecules. This is endorsed by published data showing that the subsequent three amino acids (ELR motif) before the first cysteine are essential in the NH_2-terminal sequence of IL-8 in order to retain chemotactic activity and receptor binding (Clark-Lewis et al., 1991). Since the GCP-2 forms do not significantly differ in molecular weight on SDS–PAGE, no significant processing at the COOH-terminus is to be expected. In addition, no COOH-terminal truncation has been reported for related chemokines. More evidence for this resides in the fact that the carboxy-terminal peptide of formic acid digests of GCP-2 always yielded the same terminal amino acid on the sequencer.

Comparison of GCP-2 with IL-8 allowed the conclusion that both chemokines were equally efficient in eliciting a maximal chemotactic response. However, IL-8 turned out to be more potent in that its minimum effective dose was lower. Human GCP-2 was found to be equally active as GRO/NAP-3 (Schröder et al., 1990), another granulocyte-activating factor of the chemokine family, and to be even more effective than NAP-2 (Proost et al., 1993). Since bovine GCP-2 was more potent on human cells than its human counterpart, it is to be expected that it represents a major granulocyte-activating factor in the bovine species. The biological significance of GCP-2 was further illustrated by the demonstration that, like IL-8, it is an efficient stimulator of protease secretion measurable by a prompt and significant gelatinase B release from activated granulocytes.

GCP-2 is to be structurally classified in the C–X–C chemokine subfamily because the two first cysteine residues are not adjacent (Figure 6). Most members of the C–X–C group, such as IL-8, are reported to be neutrophil-activating proteins, whereas the C–C chemokines seem to stimulate rather the chemotactic response of monocytes. In this context we have been able to identify two novel monocyte chemotactic proteins (MCP-2 and MCP-3) that do not attract granulocytes (Van Damme et al., 1992). Conversely it was found that human and bovine GCP-2 (like IL-8) are not effective on human monocytes. This is in agreement with the finding that these two proteins have a leucine and a valine residue at positions 29 and 31, respectively (Figure 6). Indeed, these amino acids are well conserved in the C–X–C chemokine family and are reported to be important for a specific neutrophil chemotactic effect (Beall et al., 1992). Both human and bovine GCP-2 are structurally most related (77% and 72% similarity) to human ENA-78, another neutrophil-activating factor of

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hu-IL-8                EGAVLPRSAKELRCQCIKTYSKPFHPKFIKELRVIESGPHCANTEIIVKLSD GRELCLDPKENWVQRVVEKFLKRAENS
hu-GRO-α                    ASVATELRCQCLQTLQG IHPKNIQSVNVKSPGPHCAQTEVIATLKN GRKACLNPASPIVKKIIEKMLNSDKSN
hu-GRO-β                    APLATELRCQCLQTLQG IHLKNIQSVKVKSPGPHCAQTEVIATLKN GQKACLNPASPMVKKIIEKMLKNGKSN
hu-GRO-γ                    ASVVTELRCQCLQTLQG IHLKNIQSVNVRSPGPHCAQTEVIATLKN GKKACLNPASPMVQKIIEKILNKGSTN
hu-IP-10                    VPLSRTVRCTCISISNQPVNPRSLEKLEIIPASQFCPRVEIIATMKKKGEKRCLNPESKAIKNLLKAVSKEMSKRSP
hu-PF-4                    EAEEDGDLQCLCVKTTSQ VRPRHITSLEVIKAGPHCPTAQLIATLKN GRKICLDLQAPLYKKIIKKLLES
hu-NAP-2                       AELRCMCIKTTSG IHPKNIQSLEVIGKGTHCNQVEVIATLKD GRKICLDPDAPRIKKIVQKKLAGDESAD
hu-ENA-78               AGPAAAVLRELRCVCLQTTQG VHPKMISNLQVFAIGPQCSKVEVVASLKN GKEICLDPEAPFLKKVIQKILDGGNKEN
hu-GCP-2                  GPVSAVLTELRCTCLRVTLR VNPKTIGKLQVFPAGPQCSKVEVVASLKN GKQVCLDPEAPFLKKVIQKILDSGNK
bo-GCP-2                  GPVAAVVRELRCVCLTTTPG IHPKTVSDLQVIAAGPQCSKVEVIATLKN GREVCLDPEAPLIKKIVQKILDSGKN
bo-PF-4    ESSFPATFVPLPADSEGGEDEDLQCVCLKTTSG INPRHISSLEVIGAGTHCPSPQLLATKKT GRKICLDQQRPLYKKILKKLLDGDES
po-AMCF-I                 DVLARVSAELRCQCINTHSTPFHPKFIKELRVIESGPHCENSEIIVKLVN GKEVCLDPKEKWVQKVVQIFLKRTEKQQQQQ
po-AMCF-II          SPIEAAEAAVVRELRCMCLTTTPG IHPKMISDLQVIPAGPQCSKAEVIATLKN GKEVCLDPKAPLIKKIVQKMLDSGKKKN
rab-IL-8                  AVLTRIGTELRCQCIKTHSTPFHPKFIKELRVIESGPHCANSEIIVKLVD GRELCLDPKEKWVQKVVQIFLKRAEQQES
rab-RPF2/GRO                   ALTELRCQCLQTVQG IHLKSIQSLKVLSPGPHCAQT...
ha-GRO                   RLATGAPVANELRCQCLQTMTG VHLKNIESLKVTPPGPHCTQTEVIATLKN GQEACLNPEAPMVQKIVQKMLKSGIRK
rat-CINC/GRO                 APVANELRCQCLQTVAG IHFKNIQSLKVMPPGPHCTQTEVIATLKN GREACLDPEAPMVQKIVQKMLKGVPK
rat-PF-4                VTRASPEESDGDLSCVCVKTSSSRIHLKRITSLEVIKAGPHCAVPQLIATLKN GSKICLDRQVPLYKKIIKKLLES
mu-KC/GRO                RLATGAPIANELRCQCLQTMAG IHLKNIQSLKVLPSGPHCTQTEVIATLKN GREACLDPEAPLVQKIVQKMLKGVPK
mu-MIP-2                      AVVASELRCQCLKTLPR VDFKNIQSLSVTPPGPHCAQTEVIATLKG GQKVCLDPEAPLVQKIIQKILNKGKAN
mu-MIG/M119                   TLVIRNARCSCISTSRGTIHYKSLKDLKQFAPSPNCNKTEIIATLKN GDQTCLDPDSANVKKLMKEWEKKINQKKKQ...
mu-CRG-2                      IPLARTVRCNCIHIDDGPVRMRAIGKLEIIPASLSCPRVEIIATMKKNDEQRCLNPESKTIKNLMKAFSQKRSKRAP
ch-9E3                   RTLVKMGNELRCQCISTHSKFIHPKSIQDVKLTPSGPHCKNVEIIATLKD GREVCLDPTAPWVQLIVKALMAKAQLNSDAPL
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FIGURE 6: Amino acid sequence alignment of human and bovine GCP-2 with other members of the C–X–C chemokine family. Different species are indicated as hu, human; bo, bovine; po, porcine; rab, rabbit; ha, chinese hamster; mu, murine; and ch, chicken. References: hu-IL-8 (Matsushima et al., 1988), hu-GRO-α and ha-GRO (Anisowicz et al., 1987), hu-GRO-β and hu-GRO-γ (Haskill et al., 1990; Tekamp-Olsen et al., 1990), hu-IP-10 (Luster et al., 1985), hu-PF-4 (Deuel et al., 1977), hu-NAP-2 (Begg et al., 1978), hu-ENA-78 (Walz et al., 1991), bo-PF-4 (Ciaglowski et al., 1986), po-AMCF-I and -II (Goodman et al., 1992), rab-IL-8 (Beaubien et al., 1990; Yoshimura & Yuhki, 1991), rab-RPF2/GRO (Jose et al., 1991), rat-CINC/GRO (Watanabe et al., 1989), rat-PF-4 (Doi et al., 1987), mu-KC/GRO (Oquendo et al., 1989), mu-MIP-2 (Tekamp-Olsen et al., 1990), mu-MIG/M119 (Farber, 1990), mu-CRG-2 (Vanguri & Farber, 1990), ch-9E3 (Sugano et al., 1987).

the C–X–C chemokine subfamily (Walz et al., 1991). Although only 67% similar, human and bovine GCP-2 probably represent the equivalent molecules in the two animal species, since the proteins showed similar elution patterns upon purification. As is the case for IL-8, the GCP-2 sequences do not contain N-glycosylation sites. This was experimentally evident since the calculated M_r (8070 and 7927 Da for human and bovine GCP-2, respectively) is higher than their apparent M_r deduced from SDS–PAGE.

The similarity of human GCP-2 with other C–X–C chemokines is lower (less than 50% similar residues) than with ENA-78. This indicates that these two chemokines might constitute a more related subgroup within this family. This is also the case with the neutrophil-activating proteins GRO-α, -β, and -γ (Anisowicz et al., 1987; Haskill et al., 1990; Tekamp-Olson et al., 1990) as well as with the monocyte chemotactic factors MCP-2 and MCP-3 that are structurally more related to MCP-1 than to other C–C chemokines (Opdenakker et al., 1993). Human GCP-2 is only 31% similar to IL-8. This weak similarity was also illustrated by the inability of GCP-2 to react in an IL-8 radioimmunoassay, indicating that antibodies against IL-8 are not able to recognize GCP-2. In addition to bovine GCP-2, another granulocyte chemotactic activity was recovered from bovine kidney cells. This factor elutes at the same position as human IL-8 on FPLC, but the responsible protein could not yet be identified (not shown). After bovine platelet factor-4 (Ciaglowski et al., 1986), the MDBK cell-derived GCP-2 is so far the second bovine C–X–C chemokine to be described.

With regard to regulation of production and cellular sources of GCP-2, our information is at present limited. Human GCP-2 is coproduced with IL-8 by osteosarcoma cells when stimulated with a semipurified leukocyte-derived cytokine preparation. So far, we have been unable to isolate GCP-2 from activated human mononuclear cells. This suggests that its production might be cell specific or strictly regulated. However, bovine GCP-2 was isolated from a kidney cell line stimulated with phorbol ester. The related human ENA-78 molecule was isolated from a human type II epithelial cell line (Walz et al., 1991). In contrast, IL-8 is known to be produced by multiple cell types including tumor cells, leukocytes, fibroblasts, and endothelial cells. Its production is regulated by cytokines (such as IL-1) and viral or bacterial products (Van Damme, 1991). Since the specific activity of IL-8 (units per milligram of protein) is higher than that of human GCP-2, the bioassays used (microchamber chemotaxis and enzyme release) do not allow the relative amounts of GCP-2 produced in cell cultures to be precisely estimated. From the purification data it can be deduced that IL-8 and GCP-2 are recovered in comparable amounts from MG-63 tumor cells. The production of GCP-2 could be more strictly regulated in that some IL-8 batches were totally devoid of GCP-2. Bovine GCP-2 seems to be important both in terms of specific biological activity and in amount of protein produced. It could therefore account for a major part of the granulocyte chemotactic activity measured in the bovine species. Specific immunoassays and probes for GCP-2 are essential in order to study gene regulation in various cell systems. Such tests will also allow GCP-2 to be measured in tissues and body fluids during acute or chronic inflammatory processes, such as rheumatoid arthritis, bacterial sepsis, etc. IL-8 has already been demonstrated in synovial fluids (Brennan et al., 1990; Peichl et al., 1991; Rampart et al., 1992), in psoriatic scales (Schröder & Christophers, 1986) and in the circulation (Redl et al., 1991; Hack et al., 1992) and has recently been reported to possess angiogenic activity (Koch et al., 1992). GCP-2 has

been found to cause granulocyte infiltration in the skin upon intradermal injection (Proost et al., 1993) and could therefore also play an important role in animal physiology and pathology.

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