Cloning and Sequencing of a New *gro* Transcript from Activated Human Monocytes: Expression in Leukocytes and Wound Tissue

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Using cDNA cloning, we have identified a new member of the *gro*/KC inflammatory protein superfamily (*gro*- β) produced by activated human monocytes and neutrophils and expressed at sites of inflammation in vivo. The open reading frame encodes a protein with 91% sequence homology to the human *gro* protein. Interestingly, some of the amino acid substitutions are located at regions where proteolytic cleavage occurs in the *gro* protein generating biologically active peptides (neutrophil activating peptide III). These changes would likely alter the processing of the *gro*- β peptide and suggest that peptides with biological activities different from those produced by *gro*- α are encoded by this transcript.

Mouse KC and human *gro*/MGSA are members of a growing inflammatory protein superfamily which is defined by the presence of four conserved cysteine residues with a CXC motif (1, 2, 7, 19, 21). Other members include PF4 (20), PBP (11), CTAP-III (5), IP-10 (16), 9E3/CEF4 (3, 26), 3-10C (23), and IL-8 (18). The members of this superfamily are relatively short secretory proteins, some of which are found upon stimulation of leukocytes by lymphotoxin (23). Recent reports indicate that proteolytic cleavage products of *gro*, IL-8, and CTAP-III have biological activities as neutrophil chemoattractants (24, 28, 29), which suggests that these peptides may play an important regulatory role in wound healing and inflammation.

Expression of *gro* mRNA in human macrophages and neutrophils. We initially examined the expression of the *gro* gene in human leukocytes. Human peripheral blood monocytes and neutrophils were isolated from whole blood by centrifugation through a Hypaque-Ficoll gradient. Cells were incubated for 2 h at 37°C in an atmosphere of 90% air-10% CO₂ to allow for attachment, and the medium was changed to eliminate any nonadherent cells such as erythrocytes, lymphocytes, and platelets.

Total RNA was isolated from nonactivated and activated (lipopolysaccharide [LPS]-treated) macrophages and neutrophils by the method of Chirgwin et al. (6) and examined for *gro* gene expression by Northern (RNA) blot hybridization probing with a random primer ³²P-labeled mouse KC cDNA. The results demonstrated a substantial induction of *gro* mRNA in LPS-activated human macrophages (100 to 1,000-fold) (Fig. 1). Induction was also observed in activated neutrophils although at much lower levels than that seen in the macrophages (10-fold) (Fig. 1). The mRNA in the monocytes (1.2 kilobases) is slightly larger than that in the neutrophils (1.1 kilobases). The *gro* transcript is induced rapidly after LPS treatment, with an increase evident within 1 h and high levels by 3 h after addition of LPS (data not shown).

Nucleotide and amino acid sequences of human *gro*- β . Because the reported nucleotide sequences of both the *gro* and KC transcripts had been determined from RNA isolated from cells other than leukocytes, we wanted to determine whether the *gro* transcript in the leukocyte was identical. We

therefore isolated 1 mg of total RNA from LPS-activated macrophages (6) and prepared poly(A)⁺ RNA using oligo(dT)-cellulose chromatography, and a lambda *gt*11 cDNA library was constructed by using *Eco*RI linkers. Low-melting-point agarose gel-separated cDNAs larger than 0.8 kilobases were ligated into lambda *gt*11 arms, packaged by using the Stratagene Gigapack Plus kit, and plated on a lawn of Y1088 *Escherichia coli* (9). Tests with X-Gal (5-mono-4-

FIG. 1. Northern blot analysis of *gro* transcripts in human peripheral blood neutrophils (PMN) and monocytes (M Φ). Cells were isolated from fresh blood as described in the text. Northern blots were performed as described in the text. Each lane contained 20 μ g of total RNA. Cells were incubated for 24 h in the presence (+) or absence (-) of 1 μ g of LPS per ml. An approximate 10-fold induction of transcript was detected in the neutrophils after activation while a 100-1000 fold induction of transcript was seen in the activated monocytes. The two activated monocyte samples were from two different individuals. The film was exposed for 72 h at -70°C with an intensifying screen.

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GRO-alpha	M	A	R	A	A	L	S	A	A	P	S	N	P	R	L	L	R	V	A	L	L	L	L	L	V	A	A	G	R	R	A	A	G	A	E	V	A	T	E	40							
GRO-beta	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	R	-	A	-	-	-	-	-	-	V	-	A	S	-	-	-	-	-	-	-	-	-	-	-	-	40						
PBP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	T	K	G	Q	T	K	R	N	-	A	K	G	K	E	E	S	L	D	S	D	L	Y	A	-	26						
9E3/pCEF4	-	-	-	-	-	-	-	-	-	M	-	G	K	-	G	A	-	L	A	-	-	-	-	V	S	-	-	L	S	Q	G	R	T	L	V	K	M	G	N	-	30						
IP-10	-	-	-	-	-	-	-	-	-	M	N	Q	-	A	I	-	I	C	C	-	I	F	L	T	L	S	G	I	Q	G	V	P	L	S	R	T	27										
3-10C/IL8	-	-	-	-	-	-	-	-	-	M	-	S	K	L	A	V	A	L	L	A	A	F	-	I	S	-	-	L	C	-	G	-	V	L	P	R	S	-	K	-	31						
PF4	-	-	-	-	-	-	-	-	-	M	S	S	A	A	G	F	C	A	S	R	P	G	-	L	F	L	G	-	-	-	P	L	V	V	A	F	A	-	-	E	-	E	E	D	G	D	38
GRO-alpha	L	R	C	Q	C	L	Q	T	L	Q	G	I	H	P	K	N	I	Q	S	V	N	V	K	S	P	G	P	H	C	A	Q	T	E	V	I	A	T	L	K	80							
GRO-beta	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	80		
PBP	-	-	-	M	I	K	-	T	S	-	-	-	-	-	-	-	-	-	-	-	-	L	E	-	I	G	K	-	T	-	N	-	V	-	-	-	-	-	-	-	-	-	-	66			
9E3/pCEF4	-	-	-	-	-	S	-	H	-	K	F	-	-	-	-	-	-	-	-	-	-	D	-	K	L	T	P	S	-	-	K	N	-	-	I	-	-	-	-	-	-	-	-	-	70		
IP-10	V	-	-	T	-	-	S	I	S	N	Q	P	V	N	-	R	S	L	E	K	-	-	I	-	P	A	S	Q	F	P	R	-	-	I	-	-	-	-	-	-	-	-	-	M	-	67	
3-10C/IL8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	71		
PF4	-	Q	-	L	-	V	-	-	-	Q	V	R	-	R	H	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	P	T	A	Q	L	-	-	-	-	-	-	-	-	-	-	78	
GRO-alpha	N	G	R	K	A	C	L	N	P	A	S	P	I	V	K	K	I	I	E	K	M	L	N	S	D	K	S	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	109	
GRO-beta	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	109	
PBP	D	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	96	
9E3/pCEF4	-	-	-	E	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	104	
IP-10	K	K	-	E	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98	
3-10C/IL8	D	-	-	E	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	
PF4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	103	

FIG. 2. Comparison of amino acid sequences of *gro* (CXC) family members. The first two lines compare the encoded amino acid sequences of the *gro*- α and *gro*- β proteins. -, Identical amino acids and codons. Codon substitutions are indicated in boldface only where the codon change is conservative and does not alter the amino acid at that position in the sequence. Changes in amino acids are indicated by shading of the position. The underlined sequence at positions 35 to 40 in *gro*- α indicates the N-terminal sequence of NAP-III (24). Remaining lines compare other CXC family members with the *gro*- α sequence. Substitutions are indicated by the appropriate single-letter amino acid codes. *, Conserved cysteine residues (also indicated by shading). Other residues conserved in all members of the family are indicated by shading only.

chloro-3-indolyl- β -D-galactoside) plates indicated that the original packaging mixture contained 10^7 PFU/ μ g of lambda arms and 96% of the recombinant phage contained inserts. An initial screening of 10,000 plaques with a chicken actin probe yielded 0.2% positive plaques, which is near the expected level of abundance of this mRNA in these cells (10).

The LPS-activated human macrophage cDNA library was screened with a mouse KC cDNA (700-base-pair *Pst*I fragment). Eight positive clones in 140,000 plaques screened were isolated and exhibited the same restriction fragment pattern by *Eco*RI digestion. The largest insert was subcloned into M13mp18 and KS(+) vectors, and the nucleotide sequence was determined by dideoxynucleotide sequencing. This clone contained a 1,065 base-pair insert which encoded a single open reading frame of 107 amino acids and a long 3' untranslated region (GenBank accession no. M36964). Comparison of the sequence within the open reading frames of this clone and the *gro* cDNA previously characterized showed 93% identity. In both the 5'- and 3'-flanking regions there was less nucleotide sequence conservation between the two cDNAs (82% identity) than within the open reading frames. However, AUUUA pentamers, which have been suggested to be responsible for mRNA destabilization (4), appear in both mRNA transcripts. The open reading frame of the *gro*- β transcript contained 20 base substitutions compared to that of *gro*- α . Nine of these do not change the encoded amino acid, but eleven result in amino acid substitutions (Fig. 2).

***gro* gene expression at sites of wound repair.** Because the *gro* gene was expressed by cells which participate in the inflammatory response, we wanted to examine an in vivo situation in which these cells should be activated. We chose a wound repair model developed by Schilling et al. (22) which consists of a stainless steel mesh chamber implanted subcutaneously in rats. The chamber induces a wound repair response which exhibits the classic phases of inflammation, connective tissue infiltration, neovascularization, and matrix deposition (12). Total RNA was isolated at various times

from tissue dissected from the wound chambers which had been implanted subcutaneously in 200-g female Fisher 344 rats. Total RNA (20 μ g) from wound tissue, normal rat skin, and rat peritoneal macrophages was analyzed by Northern blot hybridization with a mouse KC cDNA probe. These experiments demonstrated the presence of the rat homolog of the *gro* transcript in rat macrophages and in wound tissue on day 3 (Fig. 3) but not in normal uninjured rat skin. Histological analysis of day 3 tissue indicates inflammation with large numbers of leukocytes in the wound tissue (8). These studies demonstrate that high levels of *gro* transcripts are present at sites of acute inflammation in vivo and that the duration of this expression is short-lived over the time course of the repair response (normally 30 days).

We have identified a unique *gro* transcript, which we now call *gro*- β , expressed at high levels in LPS-activated human macrophages. This transcript encodes a protein which is highly homologous to the previously described cell cycle-

FIG. 3. Northern blot analysis of total RNA isolated from wound tissue. Wound tissue and RNA were prepared as described in the text. Total RNA was also prepared from uninjured skin (S) and from mouse rat peritoneal macrophages (M ϕ). Samples were analyzed as described in the legend to Fig. 1 by using the mouse KC PST I fragment as the cDNA probe. The films were developed for 1 week at -70°C with an intensifying screen. The region of the gel at 1.2 kilobases is shown. Only the peritoneal macrophages and day 3 wound tissue exhibited *gro* transcripts.

regulated mouse KC (7) and human *gro* (1) transcripts (*gro-α*). Both *gro-α* and *gro-β* transcripts encode proteins containing 107 amino acids. There are 20 base substitutions within the open reading frames of these transcripts that result in 11 amino acid substitutions between these two protein products. Most of the amino acid substitutions do not change the secondary structure significantly, and none of the four conserved cysteine residues are changed. However, a change from serine to proline at position 36 and from proline to leucine at position 54 alters the predicted secondary structure of these regions of the molecule. Importantly, protease cleavage of the *gro-α* protein at position 34 produces NAP-3, a potent neutrophil chemotactic factor (24). The substitution of proline at position 36 and leucine at position 37 in *gro-β* for serine and valine in *gro-α* would most certainly alter this cleavage site. Also, the proline at position 54 is conserved in all other members of this gene family which have been described to date (Fig. 2). Thus, these two peptides may possess different biological activities.

The discovery of the *gro-β* transcript suggests the existence of a multigene family consisting of two or possibly more genes. The expression of the *gro* genes is precisely regulated at the mRNA level in human macrophages and neutrophils. In both cell types examined, a low level of transcript was present constitutively. However, upon stimulation by endotoxin the inducibility of *gro* mRNAs differed between these cells. Upon activation of neutrophils by LPS, a low but significant induction (5- to 10-fold) of the *gro* transcript was observed. In contrast, macrophage activation by LPS resulted in a superinduction (>100-fold) of *gro* mRNA. It is not yet known what mechanism(s) would selectively induce the *gro* transcripts in LPS-activated macrophages as opposed to activated neutrophils. Moreover, the selective expression of *gro-α* and *gro-β* could not be determined in Northern blot analyses because of the high nucleotide sequence identity between two mRNA transcripts. Several attempts to amplify the transcripts by using polymerase chain reaction primers were not successful, possibly because of the high GC content of the 5' region and the extensive secondary structure of mRNA in the 5' region.

Analysis of the 3' untranslated region of the *gro* mRNA sequence reveals sequences which may act to regulate the level of *gro* transcript present in the expressing cell types. We find conservation of a nucleotide sequence in the 3' untranslated region containing repeated AUUUA pentamers which is found in other cytokine and lymphokine mRNAs (4). This consensus sequence is thought to serve a regulatory function by targeting these mRNAs for rapid degradation (4). Several possible mechanisms have been suggested for the regulation of mRNA decay (17, 25, 30). Other work has suggested that these sequences may regulate the translational efficiency of these messages (13, 14). As with other cytokines, there appears to be multiple mechanisms to fine tune the expression of the *gro-β* gene.

The *gro* transcripts are induced in human cells such as macrophages and neutrophils, which are involved in immune response and tissue regeneration. During the first 72 h of wound healing, the most abundant inflammatory cells are neutrophils, followed by macrophages (15). We find that the rat homolog of the *gro* mRNA is present only during the early stages of wound repair, corresponding to the presence of leukocytes at the wound site. These data suggest a potential role for *gro* in tissue repair. Macrophages are known to produce other cytokines that regulate endothelial cells and fibroblast growth and function (27). The *gro-α* gene product cleaved at AA34 has been reported to act as a

neutrophil chemoattractant secreted from LPS-activated monocytes (24) and may have other growth regulatory functions (2, 19). Future studies directed at the regulation of expression of the *gro* genes and the structure and function of their respective products will help us develop a clearer understanding of the role of these peptides during inflammation.

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