Molecular Cloning of Porcine Alveolar Macrophage-Derived Neutrophil Chemotactic Factors I and II: Identification of Porcine IL-8 and Another Intercrine-α Protein[†,‡]

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ABSTRACT: Alveolar macrophages (AM) mediate lung inflammation by producing lipid and peptide molecules that attract neutrophils (PMN) to the lung. Recently we described two porcine proteins called alveolar macrophage-derived chemotactic factors, AMCF-I and -II, that are potent, efficacious, and specific PMN chemoattractants both in vitro and in vivo. We report here the cloning of the full-length cDNAs which code for each protein. Porcine AM were stimulated for 4 h in vitro with *Escherichia coli* endotoxin (LPS), and a cDNA library was created from poly(A)$^+$-selected mRNA. Specific oligonucleotide probes for AMCF-I and AMCF-II were amplified from the porcine AM cDNA library by the polymerase chain reaction using degenerate oligonucleotide primer pairs derived from the N-terminal amino acid sequences of the proteins. These probes were used to isolate 2 full-length cDNAs of 1466 (AMCF-I) and 1515 (AMCF-II) base pairs. Both cDNAs code for proteins with four cysteine residues containing the C-X-C sequence characteristic of the intercrine-α family of neutrophil chemoattractants. AMCF-I shares 74% identity with human IL-8 and 84% identity with rabbit IL-8, and likely represents the porcine homologue of IL-8. By contrast, AMCF-II has no obvious human homologue. AMCF-II shares 53% identity with human neutrophil activating peptide 2. Its shared identity with the GRO-related proteins is as high as 61% (rat CINC/GRO), and its shared identity with the 78 amino acid epithelial cell-derived neutrophil activator (ENA-78) is 67%. AMCF-II may represent a new member of the intercine-α family of neutrophil chemoattractants. Steady-state mRNA levels for AMCF-I following LPS stimulation are detectable at 4 h, peak at 16–24 h, and persist for more than 72 h. Steady-state mRNA levels for AMCF-II are detectable immediately after adherence in vitro, peak at 8–16 h, and persist for greater than 72 h. Since both of these proteins are produced by porcine AM, strategies designed at limiting AM-mediated inflammation in the human lung should be aimed at the human homologues of AMCF-I and AMCF-II.

Alveolar macrophages (AM)[1] play a central role in host defense in the lung as a first line of defense from invading microorganisms. When stimulated, AM participate in the acute inflammatory response by producing chemotactically active substances that can recruit neutrophils (PMN) from the bloodstream. These chemotactically active substances include both lipids and proteins (Merrill et al., 1980; Hunninghake et al., 1980; Fels et al., 1982; Martin et al., 1984, 1987). The predominant lipid chemotaxin that is produced by AM is the arachidonic acid metabolite leukotriene B_4 (LTB_4), which can be released within minutes of stimulation (Martin et al., 1987; Rankin et al., 1990). However, with time and continued stimulation by substances such as *Escherichia coli* endotoxin (LPS), the contribution made by LTB_4 to the total chemotactic activity wanes and is replaced by protein chemotaxins (Rankin et al., 1990). As the protein chemotaxins produced by AM are not stored in the cell and require de novo protein synthesis (Merrill et al., 1980), they do not appear in culture supernatants until 3–5 h after stimulation. The identities of the human proteins responsible for this PMN chemotactic activity remain incomplete.

Neutrophil activating peptide 1/interleukin-8 (NAP-1/IL-8) is one of the most potent and effective neutrophil chemoattractants known (Leonard et al., 1991). It is a product of many cells, including alveolar macrophages (Sylvester et al., 1990), and its release is stimulated by a number of inflammatory conditions, including LPS [reviewed in Baggiolini et al. (1989), Leonard and Yoshimura (1990), and Goodman et al. (1992)]. However, antibody precipitation of NAP-1/IL-8 does not neutralize all of the PMN chemotactic activity released by AM (Sylvester et al., 1990). The identity of the protein(s) responsible for the balance of AM-derived PMN chemotactic activity remains unknown.

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[1] Abbreviations: AM, alveolar macrophage(s); PMN, polymorphonuclear leukocyte; LTB_4, leukotriene B_4; LPS, lipopolysaccharide; NAP, neutrophil activating peptide; AMCF, alveolar macrophage-derived chemotactic factor; PF-4, platelet factor 4; MGSA, melanoma growth stimulating activity; MIP, macrophage inflammatory peptide; ENA, epithelial cell-derived neutrophil activator; PCR, polymerase chain reaction; PBP, platelet basic protein; CINC, cytokine-induced neutrophil chemoattractant.

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Using a biochemical approach, we found that two distinct proteins with specific chemotactic activity for PMN are produced by LPS-stimulated porcine alveolar macrophages. The N-terminal amino acid sequences of both of these alveolar macrophage-derived chemotactic factors (AMCF-I and -II) have partial identity with the intercrine-α (Oppenheim et al., 1991) or C-X-C (Sager, 1990) subfamily of the platelet factor 4 (PF-4) supergene family. This subfamily of human neutrophil chemoattractants consists of PF-4, NAP-1/IL-8, neutrophil activating peptide 2/connective tissue activating peptide III (NAP-2/CTAP-III), GRO/melanoma growth stimulating activity (GRO/MGSA), macrophage inflammatory peptide 2α/GRO-β (MIP-2α/GRO-β), MIP-2β/GRO-γ, and the 78 amino acid epithelial cell-derived neutrophil activator, ENA-78. We report here the molecular cloning of the full-length cDNAs of both of the porcine alveolar macrophage-derived chemotactic factor proteins, AMCF-I and AMCF-II, compare their primary structures with the other members of the intercrine-α subfamily, and examine the effect of endotoxin on the time course of steady-state levels of their mRNAs.

MATERIALS AND METHODS

cDNA Library Construction. Porcine alveolar macrophages were obtained by whole lung lavage (Goodman et al., 1991) and cultured for 4 h in the presence of 10 μg/mL *E. coli* endotoxin 026:B6 (LPS, Sigma). Total cellular RNA was isolated by CsCl density gradient centrifugation and purified by phenol–chloroform extraction as described (Mathison et al., 1990). Poly(A)$^+$ RNA (5 μg) was prepared from 200 μg of total RNA by adsorption and elution from oligo(dT)–cellulose. Double-stranded, complementary DNA was prepared by a modification of the method of Gubler and Hoffmann (1983) using T4 DNA polymerase to create blunt-ended cDNA. *Bst*XI nonpalindromic linkers were added to the blunt-ended cDNA, and the cDNA mixture was sized in agarose. Complementary DNAs with strand lengths of greater than 200 base pairs were recovered from the agarose by electroelution. The plasmid vector pcDNA-II (Invitrogen, San Diego, CA) was cut with the restriction enzyme *Bst*XI, and the cDNA mixture was ligated into the vector.

Isolation of Specific Probes For Library Screening. Degenerate oligonucleotide primers were designed from each end of the known N-terminal amino acid sequences of AMCF-I and -II (Figure 1) and were synthesized with *Eco*RI restriction sites at their 5′ ends to facilitate their eventual insertion into a cloning vector. Amplification of the specific AMCF-I and -II and N-terminal cDNA sequences was accomplished using the polymerase chain reaction (PCR) with Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) as described (Saiki et al., 1988) and using the LPS-stimulated porcine AM cDNA library as a template. The PCR products were precipitated, and digested with *Eco*RI to create sticky ends. The digested PCR products were purified by preparative electrophoresis in 3% agarose. The appropriately sized single bands (78 bp for AMCF-I, 108 bp for AMCF-II) were cut out, eluted from the agarose, and ligated into the *Eco*RI site of pUC19. Each ligated vector was expanded in *E. coli*, and the inserts were sequenced. The specific cDNA sequences between the primer ends were used to synthesize specific oligonucleotide probes for AMCF-I and -II for library screening (Figure 1). These specific antisense probes were 5′ end-labeled using T4 polynucleotide kinase (Bethesda Laboratories) and [γ-^{32}P]-ATP (ICN, Costa Mesa, CA) and used to screen the cDNA library.

Library Screening and cDNA Sequencing. Supercompetent *E. coli* (BRL, Gaithersberg, MD) were transformed by electroporation (Bio-Rad pulse control electroporator, Richmond, CA) with the pcDNA-II vector containing the LPS-stimulated porcine alveolar macrophage cDNA library. Two million primary clones were screened. Three clones (two for AMCF-I and one for AMCF-II) were sequenced.

Reduction, Alkylation, and Amino Acid Sequence Analysis of AMCF-I. Five micrograms of the same lot of purified AMCF-I protein previously reported (Goodman et al., 1991) was reduced with dithiothreitol and alkylated with 4-vinylpyridine according to published methods (Hermodson et al., 1973). The reaction mixture was desalted on Sephadex G-25 (Pharmacia). The N-terminal amino acid sequence of the pyridylethylated AMCF-I was determined by Edman degradation with a 475A pulse liquid protein sequencer (Applied Biosystems, Foster City, CA).

RNA Preparation and Northern Blot Analysis. Porcine alveolar macrophages from a single pig were plated at a density of 2 million macrophages/mL in 24 separate culture flasks. The cells were allowed to adhere for 1 h, and *E. coli* endotoxin 026:B6 (LPS, Sigma) was added to 12 of the flasks at a final concentration of 10 μg/mL. Paired flasks (with and without LPS) were incubated at 37 °C for 0, 0.5, 1, 2, 4, 8, 16, 24, 40, 48, 64, and 72 h. At the end of the incubation period, the supernatants were aspirated, and the cells were lysed with 4 M guanidine thiocyanate. The isolation of total cellular RNA, agarose gel electrophoresis (10 μg of total RNA/lane), and transfer of the RNA to nylon membranes were performed by methods described elsewhere (Mathison et al., 1990). Double-stranded DNA probes for AMCF-I and AMCF-II mRNA were prepared from synthetic oligonucleotides (Research Genetics, Huntsville, AL) based on the cloned cDNA sequences. Overlapping sense and antisense oligonucleotides were designed as follows (see Figure 2): the AMCF-I sense strand corresponding to base residues 163–187 had the sequence 5′-ATAAATACGCATTCCACACCTTTCC-3′; the AMCF-I antisense strand corresponding to base residues 176–201 had the sequence of 5′-GATAAATTTGGGGTG-GAAAGGTGTGG-3′; the AMCF-II sense strand corresponding to base residues 200–223 had the sequence 5′-ATGTGTTTAACCACCACACCCGGG-3′; the AMCF-II antisense strand corresponding to base residues 212–238 had the sequence 5′-CATCTTGGGATGAATCCCGGGTGTG-GT-3′. The probes were radiolabeled with [α-^{32}P]ATP using the Klenow fragment of DNA polymerase I and published methods (Mathison et al., 1990). The nylon membranes were hybridized with the [α-^{32}P]ATP-labeled probes overnight and then washed under stringent conditions (60 °C). Suitable autoradiograms were obtained in 4–6 h. The signals on the membranes were quantitated using a Molecular Dynamics 400A PhosphorImager System (Sunnyvale, CA) (Johnston et al., 1990).

RESULTS

PCR Cloning of Specific Oligonucleotide Probes for AMCF-I and -II. Figure 1 shows the specific oligonucleotide sequences that were amplified by PCR from the porcine AM cDNA library using degenerate oligonucleotide primers designed from the amino acid sequences of AMCF-I and -II. The predicted amino acid sequence coded by the intervening cDNA for AMCF-II, between the degenerate primer ends, was identical to the N-terminal amino acid sequence of the purified protein determined previously (Goodman et al., 1991). Although the predicted amino acid sequence coded by the

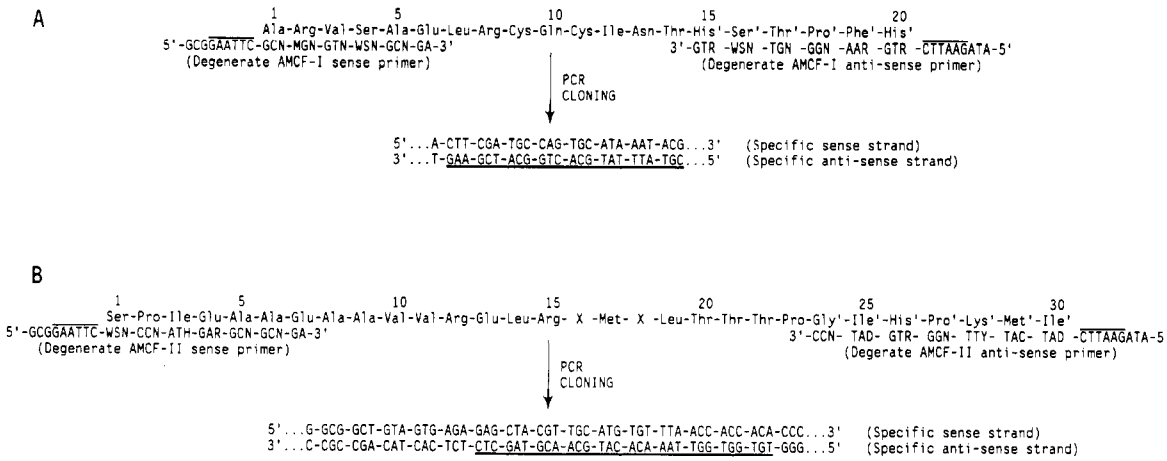

FIGURE 1: Strategy for cloning specific cDNA probes for AMCF-I and -II using PCR. The N-terminal amino acid sequences are shown for AMCF-I (A) and AMCF-II (B). The degenerate oligonucleotides containing *Eco*RI restriction sites (overlined) and used as primers for PCR are shown below each amino acid sequence. The intervening specific cDNA sequences that were amplified by PCR are shown below the arrows. The underlined sequences were synthesized and used as probes for screening the cDNA library.

intervening cDNA for AMCF-I, between the degenerate primer ends, was similar to the N-terminal amino acid sequence of the purified protein, it contained a sequence inversion. Whereas the unmodified, purified protein had the sequence "...(C)-R-Q-(C)...", the cDNA predicted the sequence "...R-C-Q-C...". To resolve this discrepancy, the purified AMCF-I protein was reduced, alkylated, and resequenced. The N-terminal amino acid sequence thus obtained is shown in Figure 1A and confirmed the cloned cDNA sequence of "...R-C-Q-C...". The sequences of the antisense strands for AMCF-I and AMCF-II shown in Figure 1 were used to synthesize specific oligonucleotide probes for library screening.

Characterization of the Full-Length cDNAs Coding for AMCF-I and -II. The cDNA coding for AMCF-I is composed of 1466 base pairs (Figure 2A). Two AMCF-I clones were isolated. Clone 4-2-6 was completely sequenced and contained bases 121–1466. Clone 4-4-4b was full-length, and its sequence allowed the determination of the first 120 bases of the AMCF-I cDNA. One full-length AMCF-II clone was isolated and sequenced. The cDNA coding for AMCF-II is composed of 1515 base pairs and is shown in Figure 2B.

The AMCF-I cDNA has a single open reading frame beginning with the ATG start codon at base position 55 and ending with the TAA stop codon at base position 366, and codes for a 103 amino acid precursor protein. After the N-terminal methionine, there is a basic amino acid (lysine) at amino acid position 4, followed by 15 hydrophobic residues (amino acids 5–19) typical of a signal peptide (Perlman & Halvorson, 1983). The predicted signal peptidase cleavage site (von Heijne, 1984) occurs between residues 22 and 23 and follows the –3, –1 rule with Ala-22 at the –1 position and Cys-20 at the –3 position. This predicted cleavage site is three amino acids upstream from the N-terminal residue (Ala-26) found in the purified protein and suggests that the molecule undergoes further N-terminal processing (either intracelluar or extracellular) after cleavage of the signal peptide. The predicted amino acid sequence exactly matches the N-terminal amino acid sequence of the reduced and alkylated AMCF-I protein shown in Figure 1A. The base pair sequence from C-148 to G-171 (Figure 2B) exactly matches the internal cDNA sequence derived by PCR (Figure 1A). The predicted molecular mass of the mature protein (Ala-26 through Gln-103) is 9098 daltons. Using this molecular mass, the measured amino acid composition of purified AMCF-I protein and the cDNA-predicted amino acid composition of the peptide Ala-26 through Gln-103 are in excellent agreement (Table I).

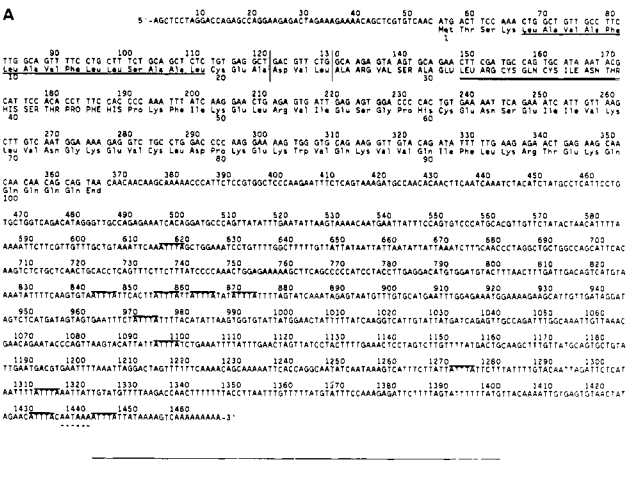

FIGURE 2: Full-length cDNA and deduced amino acid sequences of AMCF-I (panel A) and II (panel B). The N-terminal amino acid sequences of the purified proteins are shown in all-capital letters. The single-underlined segments denote the putative hydrophobic core region. The predicted signal peptidase cleavage sites are shown with double vertical lines. The single vertical line denotes the N-terminus of purified AMCF-I. The double underlines indicate the oligonucleotide probe sequence. The dotted underlines mark the polyadenylation signal sites. The overlined sequences denote ATTTA sequences in the 3′-untranslated regions.

Similarly, the AMCF-II cDNA has a single open reading frame beginning with the ATG start codon at base position 44 and ending with the TAA stop codon at base position 397, and codes for a 117 amino acid precursor protein. A basic residue (Arg-10) immediately precedes a 23 amino acid

Table I: Amino Acid Compositions

amino acid	AMCF-I cDNA predicted composition	AMCF-I composition by analysis[a]	AMCF-II cDNA predicted composition	AMCF-II composition by analysis[a]
Asx	4	4.5	5	5.5
Thr	3	3.4	4	4.3
Ser	4	3.7	4	4.0
Glx	16	15.3	8	8.1
Pro	4	3.9	7	5.9
Gly	2	3.1	4	5.3
Ala	2	2.7	8	7.6
Cys	4	(4)[b]	4	(4)
Val	8	7.7	6	5.3
Met	0	0.0	3	1.9
Ile	6	4.8	7	5.2
Leu	5	5.5	7	6.6
Tyr	0	0.0	0	0.0
Phe	3	3.3	0	0.0
His	3	2.8	1	1.8
Lys	9	8.6	11	10.2
Arg	4	4.4	2	4.6
Trp	1	(1)	0	(0)
	total 78	78.8	81	80.3

[a] Composition analysis (Goodman et al., 1991) expressed as residues per molecule using the cDNA-derived predicted molecular masses for AMCF-I (9098 daltons) and AMCF-II (8695 daltons). [b] Numbers in parentheses were not determined on composition analysis.

hydrophobic core sequence (amino acids 11–33) typical of a signal peptide (Perlman & Halvorson, 1983). The predicted signal peptidase cleavage site (von Heijne, 1984) occurs between residues 36 and 37 and follows the −3, −1 rule with Ala-36 at the −1 position and Ala-34 and the −3 position. This predicted cleavage site corresponds to the N-terminal residue (Ser-37) found in the purified protein. The predicted amino acid sequence exactly matches the N-terminal amino acid sequence of the purified protein reported previously (Goodman et al., 1991). The base pair sequence from G-172 to C-220 (Figure 3B) exactly matches the internal cDNA sequence derived by PCR (Figure 1B). The predicted molecular mass of the mature protein (Ser-37 through Asn-117) is 8695 daltons. Using this molecular mass, the measured amino acid composition of purified AMCF-II protein and the cDNA-predicted amino acid composition of the peptide Ser-37 through Asn-117 are in excellent agreement (Table I).

Both AMCF-I and AMCF-II have 5′-noncoding regions whose lengths fall within the range of 20–100 nucleotides, commonly seen in vertebrate mRNAs and which conform to many of the consensus features shared by higher eukaryotes (Kozak, 1986, 1987). For example, in the vicinity of the ATG initiation codon, AMCF-I has cytosine at positions −1 (C-54) and −4 (C-51), adenine (A-52) at position −3, and guanine (G-49) at position −6. Although AMCF-I contains adenine (A-58) rather than guanine at position +4, adenine is the second most commonly observed base in this position. Similarly, AMCF-II has the most commonly occurring residues, adenine (A-41) at position −3, cytosine (C-40) at position −4, and guanine (G-38) at position −6, and the second most commonly occurring adenine (A-47) at position +4. The favorable environment surrounding this ATG at position 44 in AMCF-II, as well as the classic signal peptide sequence which follows it, identifies it as the likely start codon rather than ATG at position 113 which exhibits none of these expected properties.

Both the AMCF-I and AMCF-II cDNAs have long 3′-untranslated regions which contain the eukaryotic consensus polyadenylation signal sequence AATAAA (bases 1437–1441 in AMCF-I, bases 1469–1474 in AMCF-II), each followed 15 or 16 bases downstream by a poly(A)$^+$ tail (Birnstiel et al., 1985). Additionally, the 3′-untranslated regions of both the AMCF-I and AMCF-II cDNAs repeatedly contain the consensus sequence ATTTA (overline in Figure 2A,B) which is prevalent among mRNAs encoding proteins related to the inflammatory response and may mediate rapid message degradation (Shaw & Kamen, 1986). AMCF-I also repeatedly contains the complete octomeric sequence TTATTTAT, described by Caput (Caput et al., 1986), which is shared among inflammatory mediators (Figure 2A, base positions 852, 859, 1092, and 1269). This suggests that AMCF-I and -II can be regulated at the posttranscriptional level of mRNA stability.

Homology Comparisons of Porcine (AMCF-I and AMCF-II. Both AMCF-I and -II are members of the intercrine-α family of genes which code for proteins with four cysteine residues whose positions are highly conserved. Figure 3 shows this family of proteins which contains the sequence ...C-X-C... near the N-terminus of each of the secreted proteins. PMN chemotaxis is a functional feature of these molecules and has been demonstrated with NAP-1/IL-8 (Yoshimura et al., 1987; Schroder et al., 1987; Walz et al., 1987; van Damme et al., 1988), ENA-78 (Walz et al., 1991), the cleavage product of PBP, NAP-2 (Walz & Baggiolini, 1989; Walz et al., 1989), GRO/MGSA (Schroder et al., 1990), CINC/GRO (Watanabe et al., 1989a,b), MIP-2 (Wolpe et al., 1989), and PF-4 (Deuel et al., 1981). An alignment of their primary structures was performed by the method of Higgins and Sharp (1988, 1989) using the program CLUSTAL (Intelligenetics, Geneva) which first calculates pairwise similarity scores for all possible pairs of sequences (Wilbur & Lipman, 1983) and then performs the alignment beginning with the most similar sequences and proceeding to the least similar ones (Myers & Miller, 1988). The putative signal peptidase cleavage sites are shown with a vertical line in Figure 3.

Porcine AMCF-I has remarkable sequence similarity (Figure 2) with rabbit and human IL-8, as well as chicken 9E3, in terms of the total length of the proteins, their signal peptide lengths and sequences, and their nearly identical positions of the predicted signal peptidase cleavage sites. AMCF-I shares 84% identity with rabbit IL-8 and 74% identity with human IL-8 (Table II). If only the secretory portion of each protein is considered and chemically conservative substitutions (Feng et al., 1985) are allowed, AMCF-I shares 91% similarity with rabbit IL-8 and 81% similarity with human IL-8. These comparisons suggest that porcine AMCF-I, rabbit IL-8, and human IL-8 are homologues from different species.

By contrast, identification of a homologue of porcine AMCF-II is less obvious. AMCF-II shares its highest identity (67%) with a recently described protein from A549 cells, ENA-78 (Walz et al., 1991). However, AMCF-II also shares between 52 and 61% identity with rat CINC/GRO, murine KC/GRO, hamster GRO, murine MIP-2, and the human proteins GRO/MGSA, GRO-β/MIP-2α, GRO-γ/MIP-2β, and platelet basic protein/CTAP-III/NAP-2 (Table II). Although AMCF-II is clearly a member of the intecrine-α family, which specific protein represents its homologue or whether AMCF-II is a unique member of this family is not certain.

AMCF-I and AMCF-II mRNA Expression. Figures 4 and 5 show the time-dependent steady-state mRNA levels of AMCF-I and AMCF-II in the presence and absence of LPS (10 μg/mL). AMCF-I mRNA levels are detectable at 4 h, peak at 16–24 h, and persist for 72 h (Figure 4). AMCF-II mRNA levels are detectable immediately (0 h), peak at 8–16 h, and persist for 72 h (Figure 5). LPS stimulation results

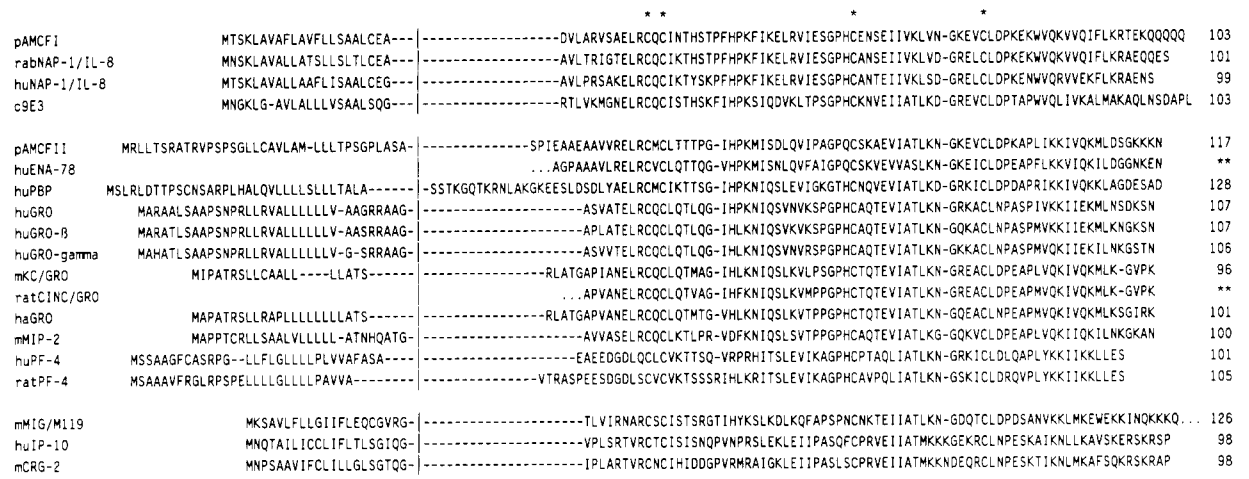

FIGURE 3: Sequence alignment of AMCF-I and -II with the intercrine-α family. Asterisks denote the four conserved cysteines characterizing this family. Sequences were obtained from the GenBank or SwissProt databases. The vertical line divides each sequence at its putative signal peptidase cleavage site. Lower case letters denote species: p, porcine, rab, rabbit; c, chicken; hu, human; m, murine; ha, hamster. References: rabNAP-1/IL-8 (Yoshimura & Yuhki, 1991), huNAP-1/IL-8 (Matsushima et al., 1988), c9E3 (Sugano et al., 1987), huENA-78 (Walz et al., 1991), huPBP (Wenger et al., 1989), huGRO (Anisowicz, et al., 1987), huGRO-β (Haskill et al., 1990; Tekamp-Olson et al., 1990), huGRO-γ (Haskill et al., 1990; Tekamp-Olson et al., 1990), mKC/GRO (Oquendo et al., 1989), ratCINC/GRO (Watanabe et al., 1989b), haGRO (Anisowicz et al., 1987), huPF-4 (Poncz et al., 1987), ratPF-4 (Doi et al., 1987), mMIG/M119 (Farber, 1990), huIP-10 (Luster et al., 1985), mCRG-2 (Vanguri & Farber, 1990).

Table II: Percent Identity Comparisons of AMCF-I and -II with the Intercrine-α Family Members[a]

	pAMCF-I	pAMCF-II
pAMCF-I	100	42
rabNAP-1/IL-8	84	38
huNAP-1/IL-8	74	42
c9E3	44	41
pAMCF-II	42	100
huENA-78	41	67
huPBP/CTAPIII	37	53
huGRO	36	55
huGRO-β	37	53
huGRO-γ	41	52
mKC/GRO	47	58
ratCINC/GRO	50	61
haGRO	45	58
mMIP-2	43	56
huPF-4	33	51
ratPF-4	36	45
mMIG/M119	34	43
huIP-10	27	29
mCRG-2	23	33

[a] Comparisons were performed on amino acid sequences obtained from translation of GenBank, EMBL files, or from the SwissProt database. The comparisons exclude the signal peptide of each sequence.

in higher steady-state levels of both AMCF-I and AMCF-II mRNA. However, non-LPS-stimulated cells also produce both AMCF-I and AMCF-II mRNA. Assay of LPS in the non-LPS-stimulated cell supernatants using a chromogenic limulus amebocyte lysate assay (Whittaker Bioproducts, Inc., Walkersville, MD) showed undetectable levels of endotoxin (<0.1 ng/mL). Because traces of endotoxin in the animal's airway may have activated the cells during the lavage and been diluted to undetectable levels in the cell culture, a second animal was studied. The experimental protocol was identical except that the bronchoalveolar lavage was done in the presence of polymyxin B (10 μg/mL) to adsorb any contaminating endotoxin. Similar results were obtained. The presence of a detectable signal in non-LPS-stimulated alveolar macrophages suggests that in vitro manipulation of these cells stimulates the production of mRNA for AMCF-I and AMCF-II. A similar phenomenon is seen with human IL-8 mRNA production from blood monocytes and has been attributed to stimulation by adherence (Kasahara et al., 1990).

DISCUSSION

We have cloned both of the full-length cDNAs which code for the porcine alveolar macrophage-derived neutrophil chemotactic factors, AMCF-I and AMCF-II. Previously, we used a biochemical approach to purify to homogeneity and characterize all of the recoverable PMN chemotactic activity produced by LPS-stimulated porcine alveolar macrophages (Goodman et al., 1991). We found that these cells produce two peptides with PMN chemotactic activity, designated AMCF-I and -II. Both proteins are potent PMN chemoattractants, with activity detectable at nanomolar concentrations, but neither has chemotactic activity for monocytes. Both proteins have molecular weights of approximately 10 000, but they are clearly distinct from each other on the basis both of amino acid composition analysis and of N-terminal amino acid sequence. Both of these porcine PMN chemoattractants have amino acid sequence similarity with the intercrine-α family of PMN chemoattractants. We hypothesized that AMCF-I and -II may be the porcine homologues of two of the human members of this family.

Porcine AMCF-I and human NAP-1/IL-8 appear to be homologous proteins on the basis of both functional and structural comparisons. In vitro, porcine AMCF-I and zymosan-activated porcine serum have similar chemotactic dose–response profiles for porcine PMN. Similarly, human NAP-1/IL-8 and zymosan-activated human serum have similar chemotactic dose–response profiles for human PMN. Since zymosan-activated serum contains the species-specific C5a as the predominant PMN chemoattractant, it can be used as a reference chemotaxin to make interspecies comparisons. Thus, porcine AMCF-I and human NAP-1/IL-8 have similar chemotactic potency and efficacy (Goodman, 1991). In vivo, porcine AMCF-I and human NAP-1/IL-8 exert similar effects. Instillation of AMCF-I into pig lungs causes an intense PMN infiltration on biopsy, and a 250-fold increase in total PMN recovered in bronchoalveolar lavage fluid. Additionally, a 25-fold increase in total lymphocytes is observed in the bronchoalveolar lavage fluid of the AMCF-I-instilled lung segments. Similarly, human NAP-1/IL-8 has

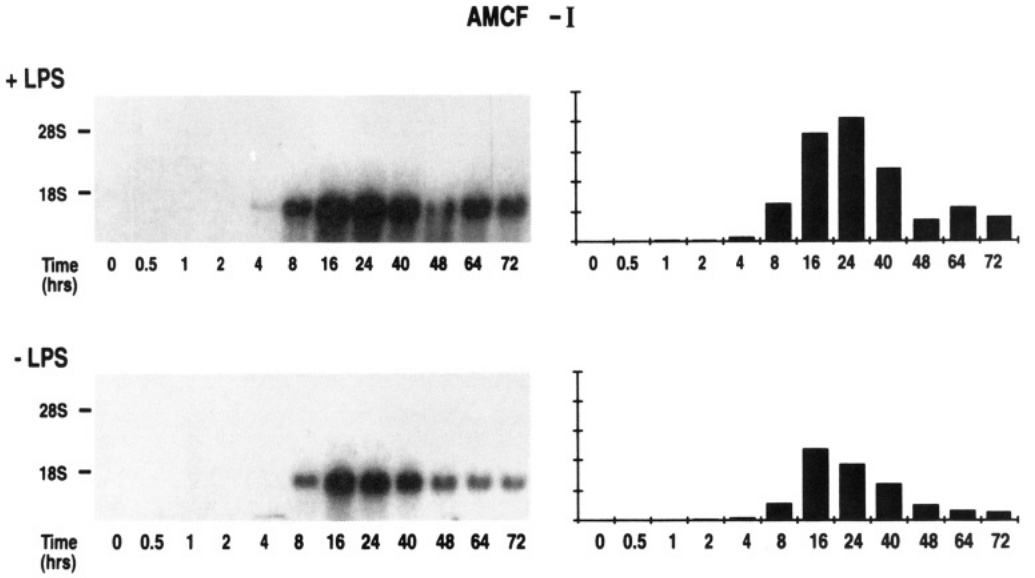

FIGURE 4: AMCF-I mRNA time course by Northern analysis. Porcine alveolar macrophages were incubated in the presence (top two panels) or absence (bottom two panels) of LPS for the times indicated, and total RNA was extracted as described under Materials and Methods. Each lane contains 10 μg of total RNA. Membranes were hybridized with a probe complementary to the mRNA sequence of AMCF-I. The upper and lower panels on the left show the autoradiograms, and the upper and lower panels on the right show a graphical representation of the signals obtained by phosporimage analysis of the radioactivity in each lane. The vertical axis is an arbitrary scale showing the relative strength of the signals in each lane. The vertical axes of both graphs are the same. The migration positions of the 28S and 18S rRNA bands are indicated.

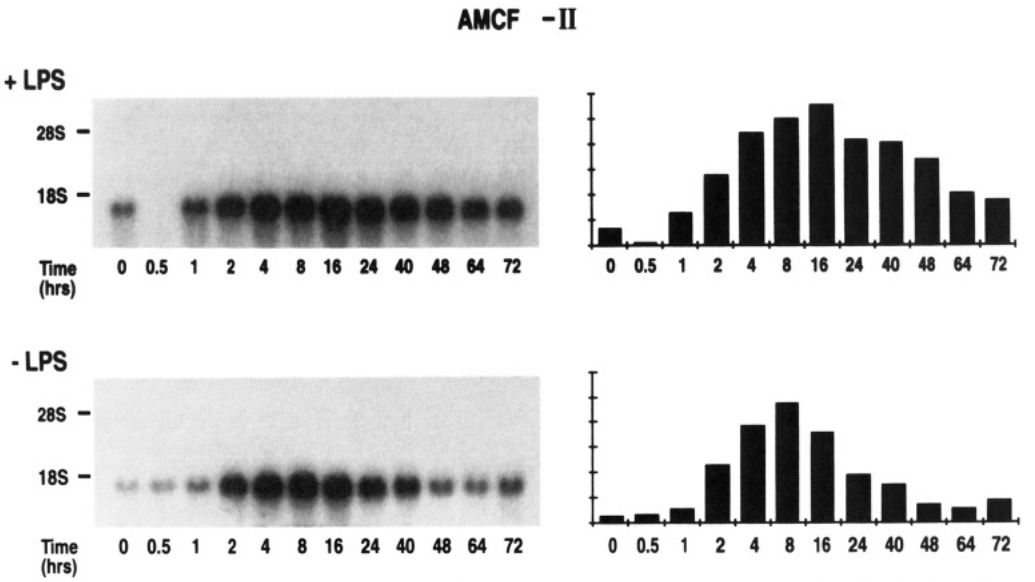

FIGURE 5: AMCF-II mRNA time course by Northern analysis. The data are shown in a presentation identical to Figure 4. Blots were hybridized with AMCF-II probe. The relative intensities of signals in this figure cannot be compared with those in Figure 4 because of differences in the specific activities of the probes.

been shown to have chemotactic activity for lymphocytes (Larsen et al., 1989) in addition to its well-described activity for PMN both in vivo and in vitro. Finally, comparison of the deduced amino acid sequences of porcine AMCF-I, human NAP-1/IL-8, and rabbit NAP-1/IL-8 shows remarkable similarities. All three proteins contain the four highly conserved cysteine residues that characterize this family of chemoattractants, with the first two cysteines conforming to the C-X-C configuration. Additionally, all three have predicted signal peptides of identical length and similar sequence. The predicted mature peptides have long stretches of internal sequence identity (Figure 3). Porcine AMCF-I shares 84% identity with rabbit NAP-1/IL-8 and 74% identity with human NAP-1/IL-8. Thus, it appears likely that AMCF-I is the porcine homologue of NAP-1/IL-8.

The identity of porcine AMCF-II is less clear. Structurally, it is most similar either to platelet basic protein, ENA-78 (Walz et al., 1991), or to the GRO subfamily of chemoattractants comprised of human GRO/MGSA, human GRO-β, human GRO-γ, mouse KC/GRO, rat CINC/GRO, hamster GRO, and mouse MIP-2. Platelet basic protein is a platelet-specific protein which is not produced by blood monocytes (Walz & Baggiolini, 1990). In contrast, GRO/MGSA has been observed as a product of blood monocytes (Schroder et al., 1990). Since blood monocytes are the precursors of alveolar macrophages, the fact that we purified and cloned AMCF-II from alveolar macrophages also suggests similarity with the GRO subfamily, rather than platelet basic protein. AMCF-II shares 61% identity with rat CINC/GRO. By contrast, AMCF-II shares only 53% identity with human platelet basic protein. However, since humans appear to have three highly similar genes which code for GRO/MGSA, GRO-β/MIP-2α, and GRO-γ/MIP-2β, which of these three might represent the human homologue of porcine AMCF-II remains

enigmatic. Alternatively, ENA-78 may represent the human homologue of porcine AMCF-II, as they share 67% amino acid sequence identity. This degree of identity is higher than that seen when either AMCF-II or ENA-78 is compared with any of the other members of the C-X-C family. If chemically conservative substitutions are allowed, AMCF-II and ENA-78 share 81% similarity. Thus, porcine AMCF-II may be the homologue either of ENA-78 or of one of the members of the GRO subfamily. Finally, it remains possible that porcine AMCF-II represents a yet undiscovered human protein.

We report here that steady-state mRNA levels of AMCF-I and AMCF-II are increased by LPS stimulation. We have previously observed that both AMCF-I and AMCF-II can produce an infiltration of neutrophils when instilled into porcine lungs in vivo (Goodman et al., 1991, and unpublished results). These observations provide a mechanism whereby the chemotactic signals from invading Gram-negative bacteria could be amplified in the lung of the host. AMCF-II mRNA levels are detectable earlier than AMCF-I mRNA levels. These differences suggest that these two signals for neutrophil migration into the lung are time-specific and that AMCF-I and AMCF-II gene regulation and/or mRNA degradation may have different regulatory control mechanisms. In vitro, non-LPS-stimulated alveolar macrophages also transcribe the genes for AMCF-I and AMCF-II. This may be an effect of in vitro activation of the macrophages (e.g., by adherence) as reported by others (Kasahara et al., 1990). Further studies will be needed to determine the in vivo relevance of this phenomenon.

We have found that LPS-stimulated alveolar macrophages make two potent and effective PMN chemoattractants: AMCF-I, which appears to be the porcine homologue of human NAP-1/IL-8; and AMCF-II, which may be the porcine homologue of a member of the GRO subfamily of chemoattractants, or the recently described protein ENA-78. This may explain why Sylvester and associates (Sylvester et al., 1990) found that the neutrophil chemotactic activity present in conditioned media from human alveolar macrophages is only partially inhibitable by an antibody to IL-8. Our finding suggests that strategies designed to limit inflammatory reactions in the lung must be aimed at both of these PMN chemoattractants.

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