

HUMAN MONOCYTE ARG-SERPIN cDNA  
Sequence, Chromosomal Assignment, and Homology to  
Plasminogen Activator-Inhibitor

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The phagocytic cells of the peripheral blood mononuclear population are a major source of serine proteases that play essential roles in the pathophysiology of tissue homeostasis, inflammation, and blood coagulation (1, 2). The neutral proteases secreted by stimulated monocytes and/or macrophages include: elastase (3), plasminogen activators (4-7), collagenase (8, 9), as well as components of both the coagulation (10) and complement pathways (11). In addition to contributing to the structural reorganization of tissue, particularly at inflammatory sites, some of these proteases also appear to be instrumental in the control of mononuclear cell and tumor cell migration (12-14), as well as both the cytolytic (15) and mitogenic activities of macrophages (16).

The mechanism universally used to regulate the destructive effects of serine proteases is the coordinated synthesis of specific endogenous inhibitors (1, 2, 17). These serine protease inhibitors constitute a family of closely related proteins (the Serpin superfamily [18]), with the common functional feature that they trap the protease by presenting a reactive site that provides an ideal pseudosubstrate (17). The target specificity of each Serpin (i.e., Met or Val for elastase; Leu for chymase; Arg for thrombin) is determined by the amino acid residue located at its reactive center. Normally this residue is the same amino acid found on the NH<sub>2</sub>-terminal side of the peptide bond to be cleaved by the protease in the legitimate substrate (17). This crucial amino acid forms the basis for a Serpin classification scheme (e.g., Met-Serpin, Arg-Serpin etc.) (18). The enzyme and its inhibitor bind tenaciously to form a complex that is typically stable to boiling in detergent (19), but is susceptible to neutrophilic cleavage with ammonium hydroxide (20). Failure or lack of regulation of these potent proteolytic enzymes is known to lead to severe pathological effects (18, 21). For example, a genetic

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or environmentally induced deficiency in the most prevalent serum Met-Serpin,  $\alpha_1$  protease-inhibitor (API),<sup>1</sup> has been demonstrated to account for the loss of pulmonary function associated with emphysema (22). Conversely, an overproduction of the Arg-Serpin that inhibits the action of the plasminogen activator (PA) found in plasma, has been correlated with human thromboembolic disease (23).

Here we report the cDNA cloning, sequence, and chromosomal assignment of an Arg-Serpin gene transcribed by LPS-stimulated human monocytes that is tentatively identified as the monocyte/macrophage-derived plasminogen activator-inhibitor (PAI-2; nomenclature suggested by the International Committee Thrombosis Haemostasis, Subcommittee on Fibrinolysis, June 8, 1986) activity reported by several laboratories (5-7, 24, 25). Our isolation of a monocyte-derived cDNA coding for a PAI different from the endothelial cell PAI (now designated PAI-1) clone recently reported (26-28) substantiates the previous conclusion, based upon biochemical and immunological criteria, that these two molecules are distinct from one another (5, 29, 30). However, our finding that mRNA for the monocytic PAI-2 is synthesized in substantial quantities by LPS-stimulated endothelial cells brings into question the assumption that PAI-1 secreted by endothelial cells is the primary PAI responsible for regulation of vascular fibrinolysis (31). As a naturally occurring regulator of urokinase-type plasminogen activator activity (u-PA), this monocyte-derived Arg-Serpin may have utility both as a substitute for  $\epsilon$ -aminocaproic acid (EACA) in the therapeutic modulation of fibrinolysis (32), as well as in the inhibition of tumor cell infiltration, metastasis, and growth (13, 14, 33).

### Materials and Methods

**Molecular Cloning of Human Monocyte cDNA Library.** The preparation of the cDNA library from poly(A)<sup>+</sup> RNA isolated from LPS-stimulated, human peripheral blood monocytes is described in detail elsewhere (34, 35). The clone pcD-1214 (Fig. 1) carried in the Okayama-Berg expression vector (36) was characterized as harboring an ~2 kb pair (kbp) cDNA insert coding for a stimulation-specific mRNA, by virtue of its hybridization exclusively to cDNA probes made from stimulated monocyte poly(A)<sup>+</sup> RNA (data not shown). This clone was used as a negative control during characterization of the cDNA for IL-1 $\beta$  from this same library (34).

**DNA Sequencing and Analysis.** Fragments of the pcD-1214 cDNA were subcloned into M13 vectors in preparation for DNA sequencing by the dideoxynucleotide chain termination method (37). Two different sequencing strategies were undertaken, both using  $\alpha$ -[<sup>35</sup>S]dATP (DuPont NEN Research Products, Boston, MA) as the isotopic tracer and a universal primer. The first approach used a 2.3-kbp Xho I fragment (Fig. 1), which was either subcloned intact into Sal I-digested M13mp9, or digested with a variety of restriction endonucleases to generate convenient subfragments for both sequencing and subcloning into appropriately digested M13mp8 or M13mp9 vectors. These subclones were carried in the *Escherichia coli* host JM103 (38).

The second approach used the deletion subcloning method of Dale et al. (39). Two M13mp9 subclones containing the entire pcD-1214 Xho I fragment cloned in opposite

<sup>1</sup> *Abbreviations used in this paper:* API,  $\alpha_1$ -protease inhibitor; ATIII, antithrombin III; EACA,  $\epsilon$ -aminocaproic acid; ETAF, epidermal thymocyte-activating factor; GTC, guanidinium isothiocyanate; MAS, monocyte Arg-Serpin; MDP, muramyl dipeptide; ORF, open reading frame; PA, plasminogen activator; PAI, plasminogen activator-inhibitor; TNF, tumor necrosis factor; t-PA, tissue-type plasminogen activator; TSST-1, toxic shock syndrome toxin; u-PA, urokinase-type plasminogen activator.

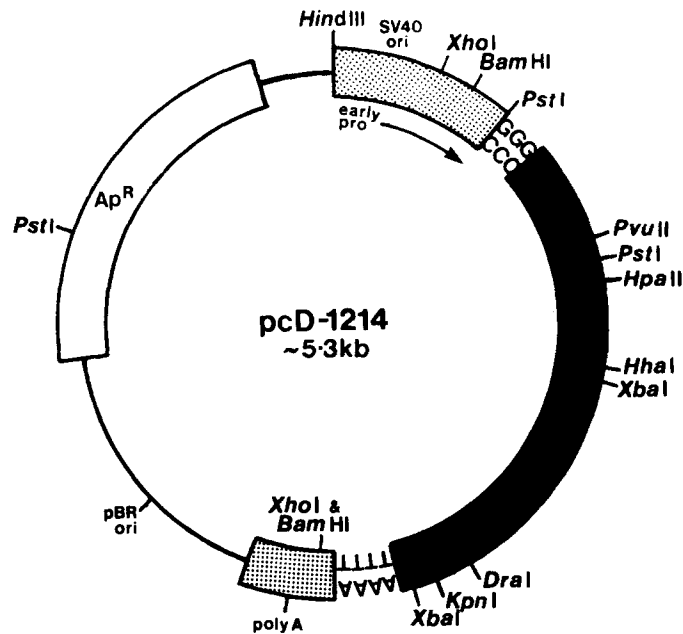


FIGURE 1. Restriction map (not to scale) of cDNA clone pcD-1214 isolated from an LPS-stimulated, human monocyte library constructed in the Okayama and Berg (36) expression vector. Only sites for restriction enzymes that cleave once or twice within the cDNA insert (solid area) are indicated. Portions of the shuttle vector derived from SV40 (shaded) are distinguished from the pBR322 segments (line and open area).

orientations were used to generate single-stranded phage DNA which was linearized by Eco RI digestion of the template annealed to a oligonucleotide (RD22 from International Biotechnologies Inc., New Haven, CT) spanning the Eco RI site in the M13mp9 polylinker region. A series of overlapping deletions on each strand of the pcD-1214 cDNA were created precisely as described in the IBI Cyclone Manual. Competent *E. coli* JM101 were transformed with pooled samples of deleted template DNA. Clones were selected for DNA sequencing based upon estimates of their insert size obtained by direct electrophoresis of phage template DNA on 0.7% agarose gels. Single-stranded DNA prepared from M13mp9 vector without an insert and M13mp9 containing an undeleted pcD-1214 Xho I fragment served as molecular weight markers.

Nucleotide sequence obtained from the M13 subclones generated as described above was assembled using computer programs (40). Subsequent computer analysis of the completed sequence was performed using a variety of software from both the University of Wisconsin (Madison, WI) Genetics Computer Group (UWGCG) (41) and National Biomedical Research Foundation (NBRF; Washington, DC) packages (42, 43).

**Northern Blot Analysis.** Poly(A)<sup>+</sup> RNA isolated from guanidinium isothiocyanate (GTC)-extracted human monocytes as described previously (34) was fractionated on 1.5% formaldehyde-agarose gels (44), electroblotted to Genescreen membranes (DuPont New Research Products), and hybridized in the presence of 50% formamide/10% dextran sulphate with a 1,256-bp Pst I–Dra I pcD-1214 fragment (Figs. 1 and 3B) according to the protocol developed for use with Genescreen by the manufacturer. The cDNA probe was isolated and nick-translated using established procedures. Samples (1 μg) of the Bethesda Research Laboratories (Bethesda, MD)–Gibco Laboratories (Grand Island, NY) RNA ladder were run in adjacent lanes on the same gel for use as molecular weight markers. These standards were visualized after blotting by staining the Genescreen strips with ferri-dye (45).

**Chromosomal Assignment.** The gene complementary to the pcD-1214 cDNA sequence

was assigned to a particular human chromosome by Southern blot analysis of mouse-human somatic cell hybrid genomic DNA as described previously (46). A panel of hybrid cell lines was obtained, selected, and evaluated for their human chromosomal content as published (47-49). Human, mouse, and hybrid genomic DNAs were digested with Bgl II, fractionated by electrophoresis in 0.7% agarose gels and blotted to Genescreen (DuPont NEN Research Products) membranes. These blots were also probed with the internal 1,256-bp Pst I-Dra I fragment isolated from the pcD-1214 plasmid and nick translated in the presence of  $\alpha$ -[ $^{32}\text{P}$ ]dCTP (DuPont NEN Research Products) to  $>10^8$  cpm/ $\mu\text{g}$  by conventional techniques. The conditions for membrane hybridization, washing, and autoradiography were all as described previously (46).

*In Vitro Translation.* The pcD-1214 cDNA insert was subcloned as a 3' truncated (pcD-1214 $\Delta$ Dx) 1.63-kbp Pst I-Dra I fragment (Figs. 1 and 3B) into the expression vector pSP64 (Promega Biotech, Madison, WI) polylinker region in preparation for in vitro transcription using Eco RI linearized template plasmid DNA and SP6 RNA polymerase (Promega Biotech) as described by the manufacturer. Uncapped RNA transcripts were translated in a rabbit reticulocyte lysate in the presence of [ $^{35}\text{S}$ ]methionine (DuPont NEN Research Products) and analyzed by SDS-PAGE as previously described (34).

## Results

*Characterization of the Stimulation-specific Clone pcD-1214.* Restriction enzyme analysis of pcD-1214 plasmid revealed a cDNA insert of  $\sim 2$  kb in length (data not shown). Characterization of the mRNA complementary to the pcD-1214 insert by Northern blot hybridization to monocyte poly(A) $^+$  RNA revealed a single species of mRNA  $\sim 2,000$  nucleotides (nt) in length, the expression of which is clearly initiated by stimulation (either adherence or LPS) of human monocytes (Fig. 2, lanes 1-4). From this initial analysis, it appeared that the pcD-1214 insert was probably a full-length copy of the template mRNA. A minor, higher molecular size RNA ( $\sim 6$  kb) that hybridizes to the pcD-1214 probe was seen in samples containing relatively more 2-kb mRNA (e.g., Fig. 2, lane 4). Since the poly(A) $^+$  RNA preparations used in this study were prepared from GTC cell lysates, this high-molecular-weight species may represent a stable splicing intermediate.

A preliminary survey of pcD-1214 gene expression in IL-1-producing cells by Northern blot analysis revealed that transcription of this gene is linked to cellular stimulation. For example, endothelial cells from human umbilical vein synthesize appreciable amounts of pcD-1214 mRNA only when stimulated with LPS (Fig. 2, lanes 7 and 8). Similarly, cells of the histiocytic lymphoma cell line U937 activate the pcD-1214 gene on exposure to toxic shock syndrome toxin (TSST-1) (50) (Fig. 2, lane 5), whereas the keratinocyte line Colo 16 (Fig. 2, lane 6), which is a constitutive producer of IL-1 (epidermal thymocyte-activating factor [ETAF]) (51), does not transcribe the pcD-1214 gene.

The precise kinetics of expression of the pcD-1214 gene in monocytic cells are presently under investigation, but preliminary data from nuclear run-off experiments in the monocytic leukemia line THP-1 (data not shown) indicate that, in common with the IL-1 $\beta$  gene, the pcD-1214 gene may undergo transient expression of the type found in association with many oncogenes and competence factors (52). This conclusion is consistent with the Northern blot data presented in Fig. 2, which indicate that the levels of pcD-1214 mRNA in peripheral blood monocytes 4 h after LPS stimulation (lane 4) are considerably higher than those seen at 12 h after stimulation (lane 1).

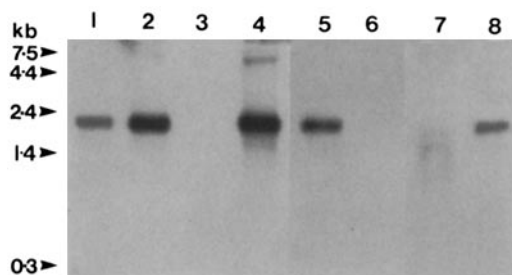


FIGURE 2. Northern blot analysis of expression of pcD-1214 mRNA by different cell types in response to stimulation. All RNA samples are poly(A)<sup>+</sup> with the exception of lanes 3 and 7. Lane 1, 100 ng 12-h LPS-stimulated monocyte; lane 2, 100 ng 4-h adhered monocyte; lane 3, 10  $\mu$ g unstimulated monocyte; lane 4, 100 ng 4-h LPS-stimulated monocyte; lane 5, 100 ng TSST-1-stimulated U937 cell; lane 6, 100 ng Colo16 keratinocyte cell; lane 7, 10  $\mu$ g unstimulated endothelial cell; lane 8, 30 ng LPS-stimulated endothelial cell. Nucleotide lengths indicated in left margin are derived from RNA ladder (Bethesda Research Laboratories-Gibco Laboratories) migrated in parallel lanes of same gel.

*Nucleotide Sequence of Monocyte cDNA pcD-1214.* Dideoxynucleotide sequencing of the pcD-1214 overlapping subclones generated a complete 1,900-bp sequence from both strands of the cDNA. The nucleotide sequence of the coding strand, together with its deduced amino acid sequence, are shown in Fig. 3A, and a compilation of significant structural features of both amino acid and nucleotide sequences are summarized on the restriction map shown in Fig. 3B.

*Open Reading Frames.* Analysis of the compiled nucleotide sequence revealed the longest open reading frame (ORF) to be 1,248 nt commencing with the ATG at position 73 and terminating at nucleotide 1,320 (TAA) of frame 1 as represented in Fig. 3A. The amino acid sequence coded for by this ORF conforms well to coding criteria such as codon utilization, amino acid constituency, and positional base preference as determined by analysis with several different algorithms (41, 53, 54). The initiator methionine bears a number of the features that seem to characterize the majority of eukaryotic mRNAs sequenced thus far. For example, it is the closest to the 5' end of the sequence, conforms perfectly at crucial positions (capitalized below) to the Kozak consensus (ccRccATGG), and is preceded by both in-frame (TAA at -27) and out-frame (TGA at -7) terminators (55).

*5' and 3' Untranslated Regions.* Although we have no direct evidence (e.g., from primer extension) to indicate that the 5' noncoding sequence represented by the 72 bp of the cDNA shown in Fig. 3A is complete, both the size of the complementary mRNA on Northern blots (see Fig. 2) and the fact that the cDNA sequence in pcD-1214 begins with a purine argue in favor of a full-length clone. The 3' non-translated sequence of 580 bp contains four AATAAA (underlined in Fig. 3A) consensus polyadenylation signals (56), in addition to the overlapping pair 17-26 bp proximal to the poly(A) tail in this clone. It appears that certain of these upstream polyadenylation signals in the pcD-1214 gene transcripts may be used by some cells as sites for alternate processing of mRNA. Northern blots of THP-1 cell RNA indicate that the processed mRNA for this gene in these monocytic-like cells is ~500 nt shorter than the 2-kb species found in monocytes and U937 cells (data not shown). Interestingly, there is a poly(A) addition signal located at position 1,456-1,461 in the pcD-1214 nucleotide

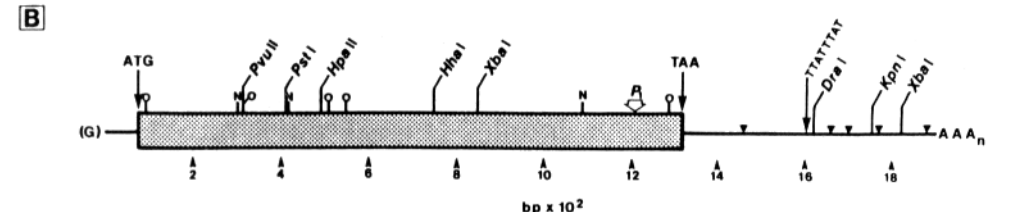
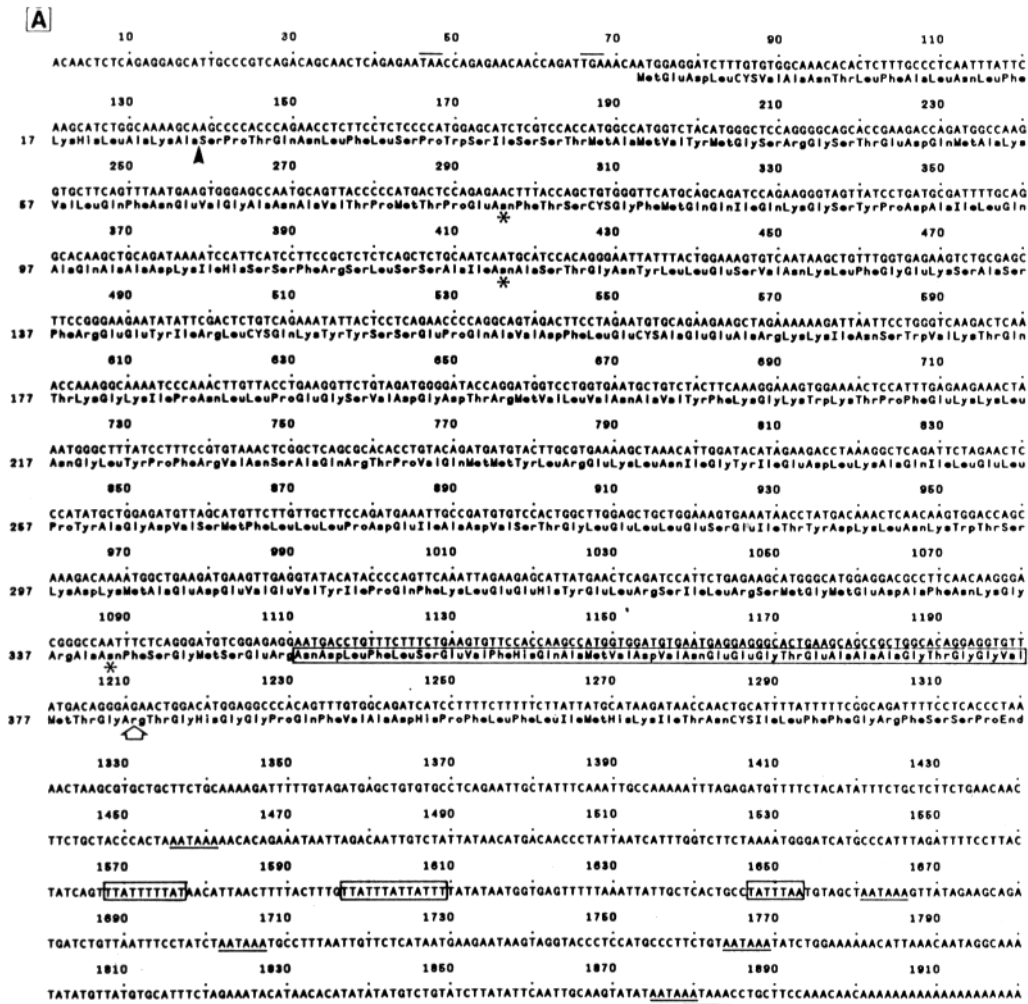


FIGURE 3. (A) Nucleotide sequence of plasmid pcD-1214 cDNA 1,900-bp insert (nucleotides numbered above the lines). The 415-amino-acid polypeptide encoded by this sequence is indicated by three-letter code (amino acids numbered down left margin). Cysteine residues are capitalized (CYS) and potential N-linked glycosylation sites are shown by an asterisk. The solid arrowhead between Ala<sub>22</sub> and Ser<sub>23</sub> represents the proposed cleavage site for the signal peptide, and the putative reactive center (P<sub>1</sub> residue) Arg<sub>380</sub> for this Serpin is indicated with a broad, open arrow. The 30 carboxyl amino acids (residues 347–376) that match the recently published (30) sequence of the tryptic peptide from PAI-2 are boxed. Nucleotide sequences of interest are highlighted as follows: 5' translational terminators, overlined; polyadenylation signals, underlined; 3' AT-rich repeats, boxed.<sup>1</sup> (B) Restriction map of pcD-1214 cDNA indicating relative positions of rare restriction enzyme sites, the ORF (shaded box), cysteine residues (O), asparagine-linked glycosylation sites (N), putative Serpin P<sub>1</sub> reactive center (↓), polyadenylation signal sequences (▼) and conserved 3' untranslated AT-rich sequence (TTATTTAT).

<sup>1</sup> These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00630.

sequence (see Fig. 3A) that could generate a 1.5-kb mRNA similar to that seen in THP-1 cells.

Another feature of particular interest within the 3'-untranslated region of this clone (positions 1,597-1,604) is an 8-nt sequence (TTATTTAT) recently reported (57) to be conserved in genes encoding inflammatory mediators, some oncogenes, and growth factors. In fact, the degree of homology to tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) mRNA extends to a perfect match over a stretch of 13 nt and there are four flanking, partial repeats (>6 nt homology) of the 8-mer consensus sequence within the 3'-untranslated region of the pcD-1214 cDNA (Fig. 3A). The precise significance of this conserved 3' sequence is not yet clear, although recent work by Shaw and Kamen (58) suggests that it may be related to mRNA lability and possibly provide a target for a specific RNase involved in rapid message turnover.

*Properties of the Predicted Protein.* The nucleotide sequence shown in Fig. 3A can code for a 415-amino-acid long polypeptide of 46,596 molecular weight. This polypeptide has a hydrophobic NH<sub>2</sub>-terminus (14 of the first 20 amino acids are nonpolar), and therefore probably represents the precursor to a secreted protein. A comparison of the NH<sub>2</sub>-terminal sequence of the predicted protein with both the consensus sequences for known signal peptides (59) and a putative signal peptidase recognition site (60), suggests that removal of the leader sequence would occur between the Ala<sub>22</sub> and Ser<sub>23</sub> residues of the pcD-1214 polypeptide (filled arrowhead in Fig. 3A). This posttranslational processing of the precursor polypeptide would yield an extracellular protein with a molecular weight around 43,000 containing three potential sites for N-linked glycosylation (Asn-X-Ser/Thr, where X is not Pro [61]) and four of the five cysteine residues found within the 415-amino-acid long precursor polypeptide (see Fig. 3, A and B). The net charge of both the predicted precursor and mature polypeptides is -9. Therefore, in the absence of glycosylation, this protein would have an acidic isoelectric point.

*Homology of the Predicted Protein.* A search of the NBRF protein sequence database using the Lipman and Pearson (62) algorithm FASTP revealed that the polypeptide encoded by the pcD-1214 clone contained substantial homology to members of the serine protease inhibitor (Serpins) superfamily (63). The degree of amino acid homology between the amino acid sequence predicted from the pcD-1214 clone and the Serpins indicated by this preliminary analysis (data not shown) was sufficiently strong (e.g., antithrombin III (ATIII), 33%; OVA 39%; API, 31%) to suggest a tentative identification of the pcD-1214 polypeptide as a monocyte-derived Serpin. A more stringent assessment of the degree of relatedness between this monocyte Serpin and other human protease inhibitors was obtained by use of the NBRF program ALIGN. Fig. 4 shows the optimized alignments of the carboxyl segments of three human Serpins against the monocyte Serpin. Unlike FASTP, ALIGN takes into account all potential conservative replacements in aligning sequences. Boxed regions in Fig. 4 contain structurally similar amino acids grouped according to the classification of Toh et al. (64). The degree of sequence relatedness is scored as the number of standard deviation (SD) units the alignment is displaced from an alignment of two sequences after amino acid randomization. The align scores of 16-23 SD obtained (see table in





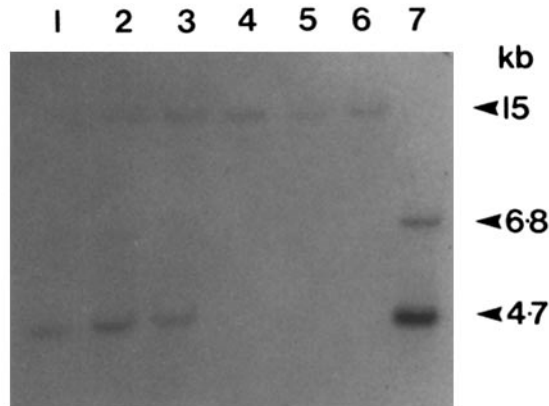


FIGURE 5. Southern blot analysis of somatic cell hybrids used in chromosomal assignment of monocyte Arg-Serpin gene. Lanes 1-5, somatic cell hybrid DNAs; lane 6, mouse genomic DNA; lane 7, human genomic DNA. Molecular size markers (right margin) are determined from a  $\lambda$ -Hind III digest run in parallel on the same gel.

high degree of perfect homology around the known reactive center residues in several of these Serpins allowed for classification of the monocyte antiprotease as an Arg-Serpin (MAS) (18).

*Chromosomal Assignment of the Monocyte Arg-Serpin Gene.* Genomic DNA extracted from a series of human-mouse somatic cell hybrids and digested with Bgl II was analyzed by Southern blotting for the segregation of the monocyte Arg-Serpin gene with human chromosomes. The blots were probed with the 1,256-bp Pst I-Dra I fragment from pcD-1214. Fig. 5 shows that this probe hybridized strongly to 4.7- and 6.8-kbp Bgl II fragments of human DNA (lane 7) and crosshybridized weakly to a 15-kbp mouse fragment (lane 6). Hybrid DNAs that contained both mouse and human sequences complementary to the Arg-Serpin probe (Fig. 5, lanes 1-3, 5) were readily distinguishable in this analysis from genomic DNA that contained only the mouse gene (lane 4). Fig. 6 presents the data obtained from Southern blot analysis of 32 independent hybrid lines with respect to the segregation of the Arg-Serpin probe and specific human chromosomes. These results allowed the unambiguous assignment of the monocyte Arg-Serpin gene to human chromosome 18, since in every instance, the presence or absence of the human fragment pattern correlated with the retention and loss, respectively, of this chromosome.

*In Vitro Synthesis of Monocyte Arg-Serpin Polypeptide.* To confirm the fidelity of the deduced ORF postulated for the monocyte Arg-Serpin encoded within the pcD-1214 cDNA, a subclone containing the SP6 promoter was used to generate in vitro transcripts which were subsequently translated in reticulocyte lysates containing [ $^{35}$ S]methionine. This resulted in the synthesis of a predominant, radiolabeled polypeptide with an  $M_r$  of approximately 42,000 when analyzed by SDS-PAGE (Fig. 7). The size of this protein corresponds well to that predicted from the nucleotide sequence (46,000 mol wt) and is within the accepted limits of variability for SDS-PAGE analysis. The minor species of labeled polypeptides with a smaller  $M_r$  seen in this analysis (Fig. 7) probably represent translation products derived from initiation at internal AUG codons with a strong Kozak consensus sequence (-3 purine and G at +4) (55). There are six such positions (around residues 39, 41, 196, 327, 329, 359) within the pcD-1214

Hybrid	pcD-1214	Human Chromosomes																						Translocations	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		X
ATR-13	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-	-	t	5/X	
DUA-3BSAGA	-	-	+	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	
DUA-5BSAGA	+	-	+	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	
DUM-13	+	+	+	-	+	+	-	-	+	+	-	+	+	-	t	+	+	+	+	+	+	+	+	t	X/15 15/X
GAR-1	-	-	-	+	-	+	-	+	-	+	-	+	-	+	+	+	+	-	-	+	-	-	-	+	
ICL-15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
JSR-2	-	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	
JSR-14	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	
MSL-16	+	-	-	+	+	+	-	+	+	t	+	-	+	-	+	+	+	+	+	+	+	+	+	-	17/9
REN-7	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
REN-8D	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
REN-10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
REN-11	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	
REX-11BSAgB	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	
REX-11BSHF	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	t	t	22/X
SIR-8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
SIR-11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
TSL-1	+	-	-	+	-	-	-	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	-	
TSL-2	+	-	+	t	+	+	-	-	+	-	-	+	-	-	-	-	-	t	+	-	+	+	+	+	
VTL-8	-	-	-	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
VTL-8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
VTL-17	-	-	-	-	+	-	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	
WIL-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
WIL-5	+	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
WIL-6	-	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	
WIL-8X	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
WIL-8Y	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
WIL-13	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
WIL-14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
XER-11	+	+	+	+	+	+	+	+	+	+	t	+	-	+	+	+	+	+	+	+	+	+	+	t	11/X X/11
XTR-2	+	-	-	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	t	3/X
XTR-3BSAgH	-	+	-	+	+	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	t	3X 10q-

Chromosome	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
Concordant # (+/+)	6	6	14	9	12	7	9	10	4	15	8	10	7	13	8	8	13	17	7	10	14	6	7
(-/-)	14	11	10	8	10	12	7	7	14	9	11	9	8	8	10	13	5	15	13	5	4	10	5
Discordant # (+/-)	11	11	1	8	5	10	8	7	12	2	8	7	10	4	8	9	3	0	10	7	3	10	5
(-/+)	1	4	5	8	5	3	8	8	1	6	4	8	7	7	5	2	10	0	2	10	11	5	9
% discordancy	38	47	20	44	31	41	50	47	42	25	38	41	53	34	42	34	42	0	37	53	44	48	54

FIGURE 6. Segregation of Arg-Serpin probe (pcD-1214) with human chromosomes in human-mouse cell hybrids. The pcD-1214 probe was hybridized to Southern blots containing Bgl II-digested DNA from the human-mouse cell hybrids listed in the Figure. (t) Indicates a translocated piece (see translocations for details) of the chromosome is present, but not an intact chromosome. The translocations (t) were not used in calculating the percent discordancy. The Arg-Serpin segregation was determined by scoring the presence (+) or absence (-) of human bands in the hybrids on the blots. The data are compiled from 32 somatic cell hybrids between 13 unrelated human, and 4 mouse cell lines. The hybrids were characterized by chromosome analysis, mapped enzyme markers, and partly by mapped DNA probes. Concordant hybrids either retained or lost the Arg-Serpin gene, together with a specific human chromosome. Discordant hybrids either retained the gene, but not a specific chromosome, or the reverse. Percent discordancy indicates the degree of discordant segregation for a marker and a chromosome. A 0% discordancy is the basis for chromosome assignment.

transcripts. Internal initiation of this type has been described elsewhere for eukaryotic translation systems (65, 66).

### Discussion

Nucleotide sequencing of the stimulation-specific clone pcD-1214 isolated from an LPS-stimulated, human monocyte cDNA library (34, 35) revealed an ORF

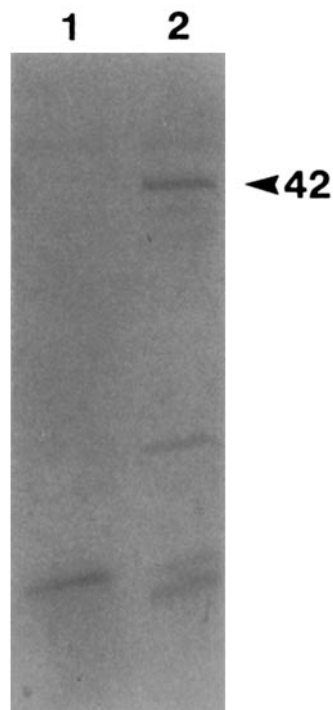


FIGURE 7. In vitro translation of monocyte Arg-Serpin. RNA transcripts of pcD-1214 made in vitro with SP6 polymerase were added to reticulocyte lysate containing [<sup>35</sup>S]methionine. The translation products were visualized by fluorography after fractionation on a 17.5% polyacrylamide gel. The position of barley  $\alpha$ -amylase (42,000) migrated in an adjacent lane provides a molecular weight marker. Lane 1, distilled water control; lane 2, 750 ng pcD-1214 $\Delta$ DX RNA transcript.

coding for the precursor to a secreted polypeptide of 415 amino acids with a calculated molecular weight of 46,596 and a 22 residue signal peptide sequence. The legitimacy of this reading frame was established by reticulocyte lysate translation of SP6-generated cDNA transcripts to yield a polypeptide with an apparent  $M_r$  of 42,000 in SDS-polyacrylamide gels (Fig. 7). Alignment of the predicted amino acid sequence with the sequences of other proteins recorded in the NBRF database or others recently published (27, 28) identified this human monocyte product as being a member of the Serpin (antiprotease) superfamily (63). The highest degree of homology between the Serpins is generally found within the hydrophobic regions located at the COOH-termini, particularly around the reaction center that determines target specificity (21). Taking advantage of this homology to optimize alignment, the monocyte Serpin was found to possess an arginine (Arg<sub>380</sub>) residue within the reactive center at the crucial P<sub>1</sub> position. This feature places this monocyte antiprotease within the Arg-Serpin subgroup, which includes ATIII and the PAI-1. In common with all Serpins sequenced to-date, the hinge residue at P<sub>17</sub> is glutamic acid (18). Both overall amino acid homology and alignment scores (see Fig. 4) suggest that the monocyte Arg-Serpin is marginally more closely related to ATIII than to PAI-1.

A recently published (30) partial amino acid sequence (30 carboxyl residues) of monocyte-derived PAI-2 was found to exactly match residues 347–376 (boxed in Fig. 3A) of the amino acid sequence deduced from our clone pcD-1214. Perfect homology over 30 amino acid residues strongly suggests that the monocyte Arg-Serpin identified in this is the same protein isolated and characterized biochemically from U937 cells by Kruithof et al. (30) and now classified as PAI-2. Northern blots of mRNA from stimulated U937 cells probed with the

monocyte Arg-Serpin clone confirm transcriptional activity of this gene in these cells (Fig. 2, lane 5). Definitive confirmation that our monocyte cDNA clone pcD-1214 codes for PAI-2 will await functional assays of the recombinant protein (e.g., PA substrate binding assay by SDS-PAGE analysis of substrate-inhibitor complex; kinetic analysis of PA substrate inhibition). However, a brief report appeared recently (67) on the isolation of PAI-2 clones from a human placental cDNA library. This placental cDNA also codes for a 415-amino-acid Arg-Serpin and publication of the complete sequence will allow its identity to our pcD-1214 clone to be verified.

Several studies (5-7) have identified stimulated monocytes/macrophages as a major source of u-PA. Urokinase is synthesized as a prepropeptide (68), but is secreted as a single-chain, zymogenic prourokinase (69). Proteolysis yields a two-chain, active u-PA, the NH<sub>2</sub>-terminal portion of which binds to specific monocyte-membrane receptors, while the catalytic COOH-terminal domain protrudes from the cell surface to facilitate localized proteolysis (70). The control of tissue proteolysis brought about by monocyte secretion of u-PA appears to be regulated by a corresponding monocyte-derived PAI (PAI-2) that has most recently been characterized as having a *pI* around 5, and an *M<sub>r</sub>* on SDS-PAGE of 47,000 (30). Human monocytes/macrophages secrete their specific PAI-2 after stimulation with either LPS (7), PMA (5), or muramyl dipeptide (MDP) (71).

The primary role of PA is to mediate the conversion of the plasma zymogen plasminogen to produce plasmin, an endopeptidase that degrades fibrin (69). Endotoxin-stimulated macrophages are a source of plasmin and have been shown to hydrolyze fibrin (6), probably more effectively by the action of prourokinase rather than mature u-PA (72). However, the tissue-type plasminogen activator (t-PA) and its corresponding fast-acting inhibitor produced predominantly by endothelial cells (PAI-1) have traditionally been considered to be the primary molecules in the homeostatic control of the fibrinolytic system (32, 73). In addition to its potential role in fibrinolysis, u-PA activity has been specifically implicated in the destruction of connective tissue that is a prerequisite to both normal macrophage interstitial migration (74), as well as the analogous process involved in tumor metastasis (13, 14) and stroma production (33).

The assignment of the monocyte Arg-Serpin gene to human chromosome 18 provides a useful marker on a relatively poorly mapped chromosome in the genome and establishes a firm genetic basis for the study of inherited deficiencies in this gene that might be manifested clinically as various fibrinolytic, thrombolytic, inflammatory, and even metastatic conditions (75) resulting from unrestricted release of plasmin by PA. Recently the PAI-1 gene was reported to be located on human chromosome 7 (28). The other Serpins mapped to-date include: ATIII to the long arm of chromosome 1 (1q23-q25) (76, 77), API and  $\alpha_1$ -antichymotrypsin to the long arm of chromosome 14 (14q31-q32) (78, 79), and the recently recognized antiprotease thyroxine-binding globulin, on the long arm of the X-chromosome (80). The u-PA gene has been localized to 10q24-pter (81), whereas the t-PA gene is on chromosome 8p12 (81, 82).

Perhaps the most striking observation from the present study is the demonstration that the monocytic Arg-Serpin gene appears to be very active in endo-

thelial cells after stimulation with LPS (Fig. 2, lanes 7 and 8). This is in contrast to the PAI-1 which has been found to be synthesized constitutively by these cells (26, 29, 83, 84). The possibility that PAI-1 and PAI-2 are both synthesized by endothelial cells may warrant a reassessment of the relative importance of PAI-1 and PAI-2 in endothelial cell control of fibrinolysis and localized vasculitis associated with tumor cell penetration. In some respects PAI-2 could be considered a physiologically more viable candidate for modulation of intravascular PA activity than PAI-1. For example, PAI-1 is secreted by endothelial cells as a latent inhibitor that can only be activated *in vitro* by treatment with SDS (85) and presumably also requires some cofactor *in vivo* to achieve full biological activity. The extent to which PAI-2 gene expression is under similar mechanisms of control in both monocytic and endothelial cells is now a major focus of our attention.

### Summary

An LPS-stimulated, human monocyte cDNA library was screened for stimulation-specific clones. One clone (pcD-1214) contained a 1.9-kb pair insert that hybridized to a 2,000-nucleotide mRNA expressed by peripheral blood monocytes, the histiocytic lymphoma cell line U937, and umbilical cord endothelial cells. The 415-amino-acid precursor polypeptide predicted from the cDNA (46,596 molecular weight) has a putative 22-residue signal peptide and ~35% homology with members of the serine protease inhibitor (Serp) superfamily. On the basis of amino acid homology and alignment of COOH-terminal residues within the Serpin-reactive center, the clone pcD-1214 was identified as coding for an Arg-Serpin. Southern blot analysis of human-mouse somatic cell hybrid DNA locates the Arg-Serpin gene on human chromosome 18. A perfect match between amino acid residues 347-376 in this Arg-Serpin and the published sequence of a 30-residue, tryptic peptide from the COOH-terminus of a monocyte plasminogen activator-inhibitor (PAI-2), strongly suggests that the Arg-Serpin encoded by pcD-1214 is PAI-2.

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*Note added in proof:* After the submission of this article for publication, the complete nucleotide sequence of human placental PAI-2 cDNA was reported (86). The near-perfect homology between these two sequences confirms the conclusion that our clone pcD-1214 codes for human PAI-2. Apart from an additional 17 nucleotides in the 5' untranslated region of the monocyte sequence reported here, the two nucleotide sequences differ at only 8 positions out of 1,900. Four of these differences are located within the coding region, although only three result in alterations of the deduced amino acid sequence. These differences are as follows: residue 120, placenta Asp(GAT), monocyte Asn(AAT); residue 393, placenta Pro(CCG), monocyte Pro(CCT); residue 404, placenta Lys(AAG), monocyte Asn(AAC); residue 413, placenta Cys(TGC), monocyte Ser(TCC).

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