Cloning and Expression of Murine Interleukin-1 Receptor Antagonist in Macrophages Stimulated by Colony-Stimulating Factor 1

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Colony-stimulating factor 1 (CSF-1) can act on mature macrophages to modulate their production of inflammatory cytokines. A cDNA encoding the interleukin-1 receptor antagonist (IL-1Ra) was cloned by subtractive hybridization from a CSF-1-stimulated murine macrophage cell line, sequenced, and expressed in mammalian and bacterial cells. Mouse IL-1Ra is a 22-Kd glycoprotein that is 76% identical to its human counterpart, shows considerably less similarity to IL-1α and IL-1β, and competes with IL-1α for binding to the type 1 IL-1 receptor normally expressed on T cells and fibroblasts. CSF-1 treatment of mouse bone marrow-derived macrophages led to a rapid and sustained increase in IL-1Ra mRNA during the G1 phase of the cell cycle as well as to increases in mRNAs encoding IL-1α and IL-1β. Cycloheximide inhibited CSF-1-induced IL-1α mRNA synthesis, but augmented IL-1β mRNA production and did not affect induction of IL-1Ra mRNA. No IL-1Ra mRNA was observed in CSF-1-stimulated mouse fibroblasts engineered to express CSF-1 receptors, demonstrating that its regulation depends on cell context and can be dissociated from the proliferative response. In agreement, bacterial lipopolysaccharide, a nonmitogenic activator, also induced IL-1Ra and IL-1 mRNAs in macrophages. Unlike IL-1α and β, IL-1Ra contains a signal peptide. The kinetics of its induction and its ability to gain access to the secretory compartment imply that IL-1Ra may be secreted more efficiently than IL-1, and suggest that macrophages both positively and negatively regulate the IL-1 response.

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MATERIALS AND METHODS

Cells and culture conditions. SV40-immortalized murine BAC1.2F5 macrophages depend on CSF-1 for their proliferation and survival in culture.26,27 They arrest in the early G1 phase of the cell cycle when deprived of CSF-1 and die within 36 hours in the absence of the growth factor. Like normal murine bone marrow-derived macrophages, G1-arrested BAC1.2F5 cells restimulated with mitogenic doses of CSF-1 progress synchronously into S phase 12 to 10 hours later, after which they can complete cell division in the absence of the growth factor.28 BAC1.2F5 cells were grown in Dulbecco’s modified Eagle’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine, and 25% L-cell-conditioned medium as a source of murine CSF-1.29 Mouse NIH-3T3 fibroblasts and C127 epithelial cells were maintained in the same medium without CSF-1 supplements. Chinese hamster ovary (CHO) cells were grown in Ham’s F12 medium supplemented with 10% FCS and 20 mmol/L HEPES buffer (Flow Laboratories, McLean, VA).

Molecular cloning of mouse IL-1Ra cDNA from CSF-1-stimulated macrophages. Polyadenylated RNA was extracted from BAC1.2F5 cells that had been starved for CSF-1 for 18 hours and then
restimulated with the growth factor for 3 hours. A cDNA library created in bacteriophage λgt10 and containing approximately 1 × 10^5 clones was screened with a subtracted radiolabeled probe enriched for sequences expressed in the induced cells. The probe was prepared in a reaction mix containing [32P]dCTP and actinomycin D by reverse transcription of polyadenylated RNA from induced cells, followed by three sequential cycles of hybridization of the negative strand cDNA to total RNA from uninduced BAC1.2F5 cells. All hybridization steps were performed for 20 hours at 60°C in phosphate buffer (pH 6.8) containing 1.0 mol/L Na^+ to a final C1 of 3.5 × 10^{-2} mol/L-sifier, and hybrids were removed by chromatography on hydroxyapatite using conditions of equivalent stringency. The resulting single-stranded cDNA, representing 2.6% of the initial radiolabeled transcripts, was used to screen the library.

Twenty-three clones identified by this procedure were plaque-purified and assayed for their ability to cross-hybridize to one another, thereby defining five unique families of clones, only one of which is discussed below. The DNA "inserts" of two phages contained partially overlapping sequences homologous to human IL-1Ra. All hybridization steps were performed for 1.7 kb expressed in CSF-1-stimulated BAC1.2F5 cells and murine bone marrow macrophages (see Results). The longest cDNA, representing approximately 1.7 kb, was recloned into a phagemid vector (Stratagene, La Jolla, CA) and subjected to nucleotide sequencing analysis using the dideoxy chain termination method. The sequence of this clone terminated at a polyadenylation signal and therefore appeared to represent the 3' 1.7 kb of IL-1Ra mRNA. Its 5' end corresponded to nucleotide 135 of the sequence shown in Fig 1A. A restriction fragment from the 5' end of this cDNA was labeled with random primers and used to reprobe the library, yielding three additional phages which were plaque-purified and subcloned. Two of these clones contained additional sequences at their 5' ends, beginning at nucleotides 1 and 80, respectively, in the sequence shown in Fig 1A.

Expression of cloned mouse IL-1Ra in eukaryotic cells. An EcoRI fragment containing the complete long open reading frame of mouse IL-1Ra (nucleotides 80 to 844, Fig 1A) was treated with the Klenow fragment of DNA polymerase I, ligated to BamHI linkers, and cloned into the unique BamHI site of a felvine sarcoma virus-based expression plasmid. The DNA was cotransfected into NIH-3T3, c127, and CHO cells together with a second plasmid expression vectop and introduced into CHO cells. Bacterial extracts were prepared by disrupting cells in 50 mmol/L Tris HCl, pH 7.5, containing 0.15 mol/L NaCl to a final C1 of 50 mmol/L KCl. Fractions, serially diluted in 25 mmol/L HEPES buffer, pH 7.2, containing 1% bovine serum albumin, were assayed for their ability to compete with [3H]-labeled IL-1α for receptor binding as described above.

Production of antiseraum to murine IL-1Ra. An EcoRI fragment containing nucleotides 135 to 1951 of the IL-1Ra coding sequence (Fig 1) was fused in frame to a glutathione S-transferase gene in an expression vector and introduced into Escherichia coli. Bacterial extracts were prepared by disrupting cells in a French press at 16,000 to 18,000 psi at 4°C, clarifying the supernatant by centrifugation at 10,000 g for 20 minutes, and filtering the soluble extract through 0.45-μm nitrocellulose. Extracts in 50 mmol/L Tris HCl, pH 7.5, containing 0.15 mol/L NaCl were fractionated on glutathione Sepharose 4B (Pharmacia, Piscataway, NJ), and the 46-Kd fusion protein was eluted in 50 mmol/L Tris HCl buffer, pH 7.5, containing 5 mmol/L glutathione. For immunization, the purified fusion protein in 50 mmol/L Tris HCl, pH 7.5, containing 100 mmol/L NaCl and 1 mmol/L CdCl₂ was digested with factor X, followed by injection into rabbits. Rabbits were immunized by
intramuscular injection of 10 μg of purified protein emulsified in complete Freund’s adjuvant and were boosted at 3-week intervals with 2 μg protein in incomplete adjuvant. The animals were bled 1 week after each boost, and antisera were tested for their ability to immunoprecipitate metabolically labeled IL-1Ra.

Metabolic labeling. Cells were metabolically labeled for various times with 150 μCi/mL of [35S]cysteine (600 Ci/mmol) and [35S]methionine (800 Ci/mmol) (Amersham), lysed with detergent, and the lysates immunoprecipitated with rabbit antiserum to IL-1Ra or preimmune serum as described. Radiolabeled proteins were separated on denaturing 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS), and the proteins were detected by autoradiography of the dried gels.

RESULTS

Polyadenylated RNA, isolated from murine BAC1.2F5 macrophages that had been growth-arrested in early G1 by CSF-1 deprivation and then restimulated with the growth factor for 3 hours, was used as a template to construct a λgt10 bacteriophage library. Five families of clones were derived using a subtracted cDNA probe enriched for sequences expressed in induced cells, one of which encoded murine IL-1Ra (see below). The sequence of this clone (Fig 1A) is 1,951 nucleotides in length and includes a single long open reading frame beginning with an ATG codon at residues 98 through 100 and terminating with a TAG codon at nucleotides 632 through 634. The putative 5′ untranslated sequence of 97 nucleotides includes two additional ATG codons in an alternative reading frame (nucleotides 18 through 20 and 57 through 59) followed by a TAA stop codon at nucleotides 129 through 131. None of the three ATG codons exactly conform to consensus sequences for translation initiation (PuCCATGG), although the second and third ATGs are more closely matched than the first. Therefore, initiation at the two upstream sites might decrease the rate of translational initiation at the third ATG. The open reading frame is followed by an untranslated sequence of 1,320 nucleotides, which included a signal for polyadenylation (residues 1921 through 1926) followed by a poly(A) sequence. AUUUA-rich motifs found in the 3′ untranslated segments of many other cytokine mRNAs, and that are presumed to be important in targeting them for degradation, were not identified within the 3′ untranslated region.

Comparison of the nucleotide sequence shown in Fig 1A with others in the GenBank Database (release 63; Los Alamos National Laboratory, Los Alamos, NM) showed homology to those of human IL-1α and β. This prompted us to compare the sequence with that of human IL-1Ra, which had been recently cloned from monocytes and, independently, from U937 leukemia cells. The predicted 178-amino acid sequence encoded by the murine cDNA was found to be 76% identical to human IL-1Ra (Fig 1B). In contrast, the predicted sequence of human IL-1Ra shows only 26% identity to human IL-1β and 19% to human IL-1α, which are identical to each other at only 26% of their residues. Both the human and murine IL-1Ra clones are predicted to contain hydrophobic signal sequences at their aminotermini, but the mouse precursor contains 26 rather than 25 N-terminal hydrophobic residues and is therefore one amino acid longer than its human counterpart (Fig 1B). Cleavage of the human polypeptide by leader peptidase between residues 25 and 26 (arrow, Fig 1B) results in a mature 152-amino acid polypeptide of 17.1 Kd with an N-terminal sequence corresponding to that of the purified monocyte-derived receptor antagonist. The mouse and human IL-1Ra polypeptides each contain a single canonical site for addition of an asparagine-linked oligosaccharide chain (star, Fig 1B). Glycosylation of the human polypeptide results in the production of a mature extracellular protein of 22 Kd.

To demonstrate that the murine cDNA encoded a biologically active IL-1 antagonist, we cloned it into a mammalian expression vector and introduced the gene into mouse C127 epithelial cells and CHO cells. Because the 5′ cloning site used to insert the IL-1Ra cDNA into the retroviral vector corresponded to nucleotide 80 (Fig 1), neither of the two upstream ATGs was included. When used to compete for the binding of radiolabeled human IL-1α to the cloned murine IL-1 receptor expressed on CHO cells, specific inhibition of IL-1 binding was observed using conditioned medium from vector-infected, but not control, cultures (Fig 2A). Partial purification of the competing activity by gel filtration indicated that the protein in the active fractions had an apparent molecular weight of about 20 Kd (Fig 2B), consistent with the predicted mass of the monomeric, extracellular glycoprotein. We also expressed the IL-1Ra cDNA as a bacterial fusion protein and prepared antiserum to the engineered polypeptide. Metabolic labeling of mammalian cells engineered to produce IL-1Ra followed by immunoprecipitation of cell lysates using this antiserum confirmed the presence of a specifically precipitable polypeptide of 22 Kd (Fig 2C). However, the undenatured bacterial fusion protein was not biologically active in competing with IL-1α for binding to its receptor on mammalian cells. It is unlikely that this was due to its lack of carbohydrate, because enzymatically deglycosylated human IL-1Ra retains biologic activity, so we suspect that the glutathione S-transferase moiety inhibits the binding.

To study the kinetics of induction of IL-1Ra in macrophages, starved BAC1.2F5 cells were stimulated with CSF-1 for various times, and their RNA was isolated and subjected to Northern blotting analysis using the radiolabeled murine IL-1Ra cDNA as a probe. Figure 3A shows that IL-1Ra transcripts of about 2.1 kb were detected within 10 minutes of CSF-1 stimulation, and their level continued to increase for 5 hours. By contrast, expression of c-fms (encoding CSF-1R) remained relatively constant throughout the same period. BAC1.2F5 cells stimulated for 3 hours with CSF-1 in the presence of cycloheximide showed a similar elevation in their level of IL-1Ra transcripts (Fig 3A). The fact that new protein synthesis was not required shows that induction of IL-1Ra is part of the immediate early response to CSF-1 in these cells.

The kinetics of induction of IL-1Ra were also studied in normal bone marrow-derived macrophages. Cells harvested from the long bones of Balb/c mice were propagated for 10 days in serum-containing medium containing CSF-1 as the only exogenous hematopoietin. Under these conditions,
A B

Fig 2. Characterization of the mouse IL-1Ra protein. (A) Competition of IL-1Ra and $^{125}$I-IL-1α for receptor binding. CHO cells expressing the mouse type I IL-1 receptor were incubated with $^{125}$I-IL-1α (approximately 1 ng) in the presence of purified IL-1 (○) at the concentrations shown. Conditioned media from control CHO cells (□) or CHO cells engineered to produce murine IL-1Ra (●) were concentrated 50-fold and tested at different dilutions for competing activity. Log 1 indicates competition obtained with the undiluted 50X concentrate that was diluted through a 10-fold range (to log 0). (B) Conditioned medium from control CHO cells (□) or from those engineered to produce murine IL-1Ra activity (●) was fractionated by molecular sieving on an HPLC column, and fractions were tested at equal dilution for competition with $^{125}$I-IL-1α for receptor binding. The peak competing activity was obtained for proteins of approximately 20 Kd. (C) Control C127 cells (lanes 1 through 3) or those engineered to express murine IL-1Ra (lanes 4 through 6) were metabolically labeled for 30 minutes (lanes 1, 2, 4, and 5) or 2 hours (lanes 3 and 6), lysed, and immunoprecipitated with either preimmune (lanes 1 and 4) or immune (lanes 2, 3, 5, and 6) serum. Radiolabeled proteins were separated on denaturing polyacrylamide gels containing SDS and detected by autoradiography (4-day exposure) of the dried slab gel. The position of specifically precipitated IL-1Ra is indicated by the arrow at the right margin; the positions of proteins of known molecular weight are shown at the left.

pure populations of mononuclear phagocytes are obtained. As expected, these cells expressed typical markers of the mononuclear phagocyte lineage, including CSF-1R (Fig 3B). When starved of CSF-1 for 18 hours and restimulated for various times, IL-1Ra transcripts were induced within 15 minutes, reached a maximum at 10 hours (approximating the G1/S transition), and declined. The initial rapid phase of induction could involve mRNA stabilization, but transcriptional regulation would logically contribute to the relatively long duration of the response. However, we have not analyzed the process using nuclear run-on experiments and therefore cannot gauge the relative contributions of transcriptional and posttranscriptional mechanisms to IL-1Ra mRNA induction.

IL-1 mRNAs were also induced by CSF-1, with peak levels of IL-1α (Fig 3B) and IL-1β (not shown) mRNAs detected 6 hours after stimulation. Bacterial lipopolysaccharide (LPS) treatment of CSF-1-starved cells also augmented the levels of IL-1Ra and IL-1 mRNAs, but in this case the relative amounts of the IL-1 mRNAs exceeded that of IL-1Ra (Fig 3B). Induction of IL-1α mRNA was inhibited by cycloheximide, whereas the levels of IL-1β mRNA were further increased by treatment of cells with the protein synthesis inhibitor (data not shown). Thus, induction of the IL-1β mRNA, like IL-1Ra, occurs as part of the immediate early response to CSF-1, whereas induction of IL-1α requires new protein synthesis. The different patterns of IL-1α, IL-1β, and IL-1Ra mRNA expression in response to cycloheximide treatment indicate that their rates of mRNA turnover are independently regulated.

DISCUSSION

IL-1 affects a broad range of local and systemic hematologic, immunologic, neurologic, and metabolic activities. During the inflammatory response, the two structurally distinct forms of IL-1, IL-1α and IL-1β, are synthesized by mononuclear phagocytes in response to endotoxin and TNF-α, and despite their limited sequence homology to one another, they act on the same target cells by binding to common receptors. The spectrum of biologic responses to IL-1α and β depends on the nature of the responding cells. For example, both IL-1 species induce the production of IL-2 and upregulate expression of the IL-2 receptor in T cells. They each stimulate the antifibrinolytic and adhesive activities of endothelium and augment the synthesis of prostaglandins and proteases by fibroblasts and chondrocytes. The activities of IL-1 on T cells and fibroblasts are
mediated by the type I IL-1 receptor,\textsuperscript{33,35} but a second class of IL-1 receptors (type II) is expressed on both B lymphocytes and macrophages.\textsuperscript{40,49}

The apparent complexity of the cytokine networks that regulate IL-1 production by macrophages has been compounded by the recent identification of the human IL-1 receptor antagonist.\textsuperscript{20,22} IL-1Ra synthesis is rapidly induced in human monocytes or in U937 leukemia cells stimulated by aggregated IgG, granulocyte-macrophage CSF (GM-CSF), LPS, or phorbol esters. The secreted 22-Kd glycoprotein binds to type I IL-1 receptors with the same affinity as either IL-1\(\alpha\) or \(\beta\). Although human IL-1Ra does not bind to murine B cells or neutrophils,\textsuperscript{21,23} it can compete with IL-1 for binding to human type II IL-1 receptors. Therefore, we assume that murine IL-1Ra can bind in a similar species-specific manner to IL-1 receptors expressed on mouse B cells and macrophages.

Induction of IL-1Ra mRNA is part of the immediate early response of quiescent mouse macrophages brought into cycle by CSF-1. CSF-1 induced a rapid and prolonged increase in IL-1Ra mRNA levels that peaked 10 hours later during the late G1 phase of the cell cycle. Murine IL-1Ra is 76% identical to its human counterpart but shows considerably less sequence homology to either IL-1\(\alpha\) or \(\beta\), and, as predicted, was able to compete with radiolabeled IL-1\(\alpha\) for binding to CHO cells engineered to express type I mouse IL-1 receptors. Molecular sieving analysis showed that the soluble IL-1 competing activity produced by mammalian cells expressing murine IL-1Ra cDNA was similar in molecular weight to the protein engineered in bacteria, indicating that murine IL-1Ra is biologically active in its monomeric 22-Kd form. Although the CSF-1-induced production of IL-1Ra was associated with the release of macrophages from growth arrest, IL-1Ra mRNA was also induced by LPS, a macrophage activator that inhibits CSF-1-induced mitogenesis by triggering the proteolytic cleavage of CSF-1R from the cell surface.\textsuperscript{50} Conversely, whereas transduction of the human \(c-fms\) gene into mouse NIH-3T3 fibroblasts enables them to proliferate in response to human recombinant CSF-1,\textsuperscript{50} treatment of such cells with CSF-1 did not induce IL-1Ra mRNA (negative data not shown) under conditions where \(c-fos\) and \(junB\) are rapidly induced.\textsuperscript{51} Thus, the regulation of IL-1Ra by CSF-1 depends on cell context and appears to reflect a macro-
phage effector function rather than the proliferative response to the growth factor.

IL-1Ra contains an aminoterminal signal peptide and must be transported through the secretory pathway because it undergoes addition of a single asparagine-linked oligosaccharide chain. Despite their numerous systemic activities, however, both the α and β forms of IL-1 lack signal sequences and are synthesized from larger 31- to 34-KD precursors whose intracellular transport and proteolytic processing to mature 17-KD secreted forms are determined by as yet unexplained mechanisms. IL-1 precursors do not gain access to the endoplasmic reticulum. Indeed, fibroblasts stably expressing cloned IL-1α or β cDNAs fail to secrete processed 17-KD IL-1 into the culture medium, although their precursors accumulate in the cytoplasm. Even in activated human monocytes, the half-life for secretion of IL-1 is comparatively slow, measured at about 3 hours for IL-1β and considerably longer for IL-1α. Several lines of evidence suggest that IL-1α can be expressed as a bioactive membrane-bound form at the cell surface, which is accessible to cleavage by extracellular proteases and blocked in its activity by appropriate antibodies, but mechanisms governing membrane association have not been defined. Unglycosylated IL-1 precursors may be transported to the cell surface through an alternative vesicular pathway and cleaved just before release. Although processing and release are not obligatory steps, they are triggered by treatment of cells with calcium ionophores, heat shock, and inhibitors of either protein synthesis or oxidative phosphorylation. Moreover, drugs such as blefeldin A and monensin, which block protein transport within the classical secretory pathway and inhibit the production of cytokines such as IL-6 and TNF-α, accelerate the proteolytic processing and release of IL-1β.

CSF-1 induction of IL-1Ra and IL-1β mRNA in mouse macrophages is not inhibited by cycloheximide, whereas production of IL-1α is contingent on new protein synthesis. Although both the IL-1Ra and IL-1β genes participate in the immediate early response to CSF-1, monocytic cell lines such as U-937 that secrete IL-1Ra after induction, synthesize but neither process nor secrete IL-1β. Taken together, these results imply that the CSF-1–induced synthesis of IL-1Ra in macrophages temporarily precedes that of IL-1α, whereas its secretion is likely to occur more efficiently than either form of IL-1. The functional contribution of macrophages in elaborating both classes of cytokines in response to stimulation by hematopoietins and inflammatory activators therefore poses a paradox. One possibility is that macrophage production of IL-1Ra might block binding of IL-1 to macrophage receptors, thereby preventing their autocrine stimulation. Alternatively, IL-1Ra might bind to yet another class of receptors and exhibit as yet undetermined agonistic activity on other target cells.

NOTE ADDED IN PROOF

The nucleotide sequence of murine IL-2Ra has been filed in GenBank (Los Alamos National Laboratory, Los Alamos, NM) under accession number M64404.

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Cloning and expression of murine interleukin-1 receptor antagonist in macrophages stimulated by colony-stimulating factor 1

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