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Enhancer-Mediated Control of Macrophage-Specific Arginase I Expression

Anne-Laure Pauleau, Robert Rutschman, Roland Lang, Alessandra Pernis, Stephanie S. Watowich, and Peter J. Murray

Arginase I expression in the liver must remain constant throughout life to eliminate excess nitrogen via the urea cycle. In contrast, arginase I expression in macrophages is silent until signals from Th2 cytokines such as IL-4 and IL-13 are received and the mRNA is then induced four to five orders of magnitude. Arginase I is hypothesized to play a regulatory and potentially pathogenic role in diseases such as asthma, parasitic, bacterial, and worm infections by modulating NO levels and promoting fibrosis. We show that Th2-inducible arginase I expression in mouse macrophages is controlled by an enhancer that lies ~3 kb from the basal promoter. PU.1, IL-4-induced STAT6, and C/EBPβ assemble at the enhancer and await the effect of another STAT6-regulated protein(s) that must be synthesized de novo. Identification of a powerful extrahepatic regulatory enhancer for arginase I provides potential to manipulate arginase I activity in immune cells while sparing liver urea cycle function. The Journal of Immunology, 2004, 172: 7565–7573.

A
arginase I is a central metabolic enzyme of liver function, catalyzing the nitrogen elimination step of the Krebs urea cycle (1). In this context, arginase I expression must remain constant throughout life. Genetic abnormalities in the genes that encode the urea cycle enzymes, including arginase I, are associated with a variety of pathological conditions related to the failure to eliminate nitrogen from the body (1). In contrast, arginase I expression is also found in nonhepatic cells (2–4) including cells of the hemopoietic system (2, 5), most significantly in macrophages (2, 5). The biological function of arginase I in macrophages is the subject of intense research, related predominantly to its anticipated role in regulating NO production from activated macrophages: arginine being the common substrate for both arginase I and the NO synthases (1, 6, 7). In vitro studies have established that arginase I can deplete arginine from macrophages and from the milieu, leaving none available for NO synthases to generate NO (8, 9). This process is complex and not strictly dependent on substrate competition but also involves translational control of NO synthase expression (10). The complexity of the competition between NO synthases and arginase I is speculated to have crucial in vivo roles in diseases where NO levels must be tightly controlled. Of further significance are the secondary metabolites of arginase I, primarily ornithine, that can feed into pathways favoring collagen biosynthesis, and hence fibrosis. Ornithine is also the precursor of polyamines, essential molecules that have a plethora of biological activities (1, 2).

We were interested in the regulatory steps that lead to arginase I expression in macrophages. The rationale for this investigation is linked to the development of future agonists or antagonists of arginase I in diseases where the role of the enzyme is implicated in pathogenesis. It would be unlikely that direct agonists or antagonists of arginase I would have any useful pharmacological role because of toxicity associated with inhibition or exacerbation of liver arginase I function. Therefore, we chose to investigate the upstream regulatory steps that control arginase I gene expression in macrophages with the concept that these pathways could eventually be regulated pharmacologically.

Unlike liver arginase I whose expression is constant throughout postnatal life (1, 11), macrophage arginase I expression is tightly regulated. In resting murine macrophages, arginase I levels are undetectable at the mRNA, protein, and enzymatic levels (5, 9). However, once exposed to cytokines that stimulate STAT6 activity (IL-4 and IL-13), arginase I mRNA, protein, and enzymatic levels are up-regulated four to five orders of magnitude (5, 9). The STAT6-mediated control of arginase I expression has also been revealed in diseases dominated by Th2 responses including helminth, parasitic infections, and asthma (12–17). The tight control over arginase I expression has led to the implication that it is crucially linked to pathologic sequelae in these diseases. We have previously shown that STAT6 is essential for IL-4/IL-13-mediated arginase I expression and that STAT6 regulates the expression of another gene(s) that is required for expression (9). In this study, we define the regulatory mechanisms involved in this process. We show that the regulation of arginase I in macrophages is controlled by a complex enhancer element located 3-kb upstream of the transcription start site. Surprisingly, the enhancer is regulated both directly and indirectly by STAT6 and a series of other transcription factors that assemble in a temporal manner to induce arginase I gene expression.

Materials and Methods

Cells, Abs, and reagents

RAW macrophages were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 with 10% FBS, penicillin/streptomycin, and minimal nonessential amino acids (complete RPMI). Mouse IL-4 and IL-10 were purchased from BD Biosciences/
PharMingen (San Diego, CA) and resuspended to 1 µg/ml in complete RPMI before use. Final cytokine concentrations used are described in detail in Results specific for each experiment. Luciferase reporter constructs and reagents for the analysis of luciferase expression were purchased from Promega (Madison, WI). Abs were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA) and were used for formalin immunoprecipitation or EMSA supershift reactions at a 1/100 dilution. The Abs are as follows: anti-SfAT6 (M200 rabbit, S20 rabbit, M20 goat), anti-IFN regulatory factor-4 (IRF-4) (M17 goat), anti-PU.1 (T21 rabbit), anti-C/EBPβ (C19 rabbit), anti-C/EBPβ (A22 rabbit). Anti-acetylated H3, anti-acetylated H4, and anti-hyperacetylated H4 rabbit polyclonal Abs were from Upstate Biotechnology (Waltham, MA). An additional anti-SfAT6 rabbit polyclonal Ab (18) was a gift of Dr. J. Ihle (Department of Biochemistry, St. Jude Children’s Research Hospital). For immunoprecipitations with goat Abs, a rabbit anti-goat Ab (Pierce, Rockford, IL) was added at a final dilution of 1/1000 before capture of Ig conjugates with protein A.

RNA isolation, Northern blotting, and real-time RT-PCR
Total RNA was isolated from macrophages using TRizol (Invitrogen, Carlsbad, CA) as described (19, 20). RNA was separated on formaldehyde agarose gels and blotted to Hybond N membranes (Amersham Pharmacia Biotech, Piscataway, NJ) for Northern analysis as described (9). Real-time RT-PCR was performed as described in detail using Superscript II (Invitrogen) for reverse transcription and FAM-labeled probes (20) listed in Table I.

PAC isolation, promoter cloning, oligonucleotide definition and location, site-directed mutagenesis
Probes from the proximal region of the arginase I promoter were used to screen a mouse 129 PAC library supplied by the Human Genome Mapping Project Resource Centre (Hinxton, U.K.). Four clones (510-F22, 513-G17, 522-M2, and 526-J2) were identified. Bacteria were grown in 25 µg/ml kanamycin according to the supplier’s instructions. Plasmid DNA was isolated according to the supplier’s instructions. The presence of the arginase I promoter was confirmed by PCR using oligonucleotides specific for regions within the promoter. Oligonucleotides to amplify the promoter fragments, introduce mutations, and perform EMSA are detailed in Table I including the position of each oligonucleotide relative to the A residue in the initiation codon of the arginase I gene. The detailed description of the murine arginase I locus can be found at: www.ensembl.org/Mus_musculus/geneview?gene=ENSMUSG00000019987.

Promoter fragments were cloned into the pGL3-basic vector (Promega) using the restriction sites detailed in Table I. Site-directed mutagenesis was performed using the Stratagene (La Jolla, CA) Quick-Change procedure according to the manufacturer’s instructions. All mutations were made in the −313 to −3810 construct. In all cases, each mutation was constructed and analyzed completely independently twice.

Transfection of RAW cells and reporter assays
RAW cells were grown in complete RPMI. Cells were harvested by gentle scraping, centrifuged (200 × g, 5 min) and washed once with PBS. Cells were resuspended in OptiMEM (Invitrogen) at a density of 1 × 10^6 cells per ml. Cells (0.5 ml) were gently mixed with 10 µg of plasmid DNA and electroporated at 250 V, 975 µF in 0.4-cm cuvettes. Each transfection was made up to 6 ml with complete RPMI and plated at 1 ml/well in 12-well plates. Following overnight incubation at 37°C, the medium was replaced (1 ml) and the cells were allowed to rest for 2–3 h. Cells (duplicate wells) were left untreated or stimulated with IL-4 or IL-4 and IL-10 for 16–20 h. Reporter activity was performed with the Promega reagents according to the manufacturer’s instructions and luciferase activity was measured using a luminometer set to a 10-s measurement time.

Stable RAW cell lines containing luciferase reporter constructs were derived by linearizing each plasmid with EcoRI and electroporating into the murine arginase I 5′ genomic fragments. These plasmids were co-transfected by electroporation as described above with Xhol-linearized pCDNA1 at a 10:1 ratio. After selection in 250 µg/ml G418 over a 2-wk period, lines were plated and stimulated with IL-4 or IL-4 and IL-10 and assayed for luciferase activity 14 h later.

Chromatin immunoprecipitations (ChiP)
ChiP was performed as described by the manufacturer of the ChiP reagents (Upstate Biotechnology), with the following modifications. RAW cells were grown in complete RPMI on 10-cm plates until there were ~4 × 10^6 cells per plate. Two plates were assigned per time point and stimulation condition. Cells attached to the plate were stimulated with IL-4 (10 ng/ml) for various times (typically 0, 1, 2, and 6 h). One plate was retained for accurate counting of cell numbers. Cells were fixed by incubation in 1% formaldehyde for 10 min at room temperature and then washed twice with PBS containing PMSF as described in the Upstate Biotechnology protocol. Cells were scraped from the plates and pelleted by centrifugation (200 × g, 10 min) and then resuspended in SDS lysis buffer (Upstate Biotechnology) and protein-DNA complexes fragmented by sonication using a Misonix Sonicator 3000 (Farmingdale, NY) set at 80% power, 6°C constant temperature for 8 × 30 s sonication cycles. These conditions empirically estimated denaturation of DNA with a mean length of 500–800 bp. Insoluble material wasremoved by centrifugation at 15,000 × g for 10 min. The lysate was then precleared with salmon sperm-saturated protein A-agarose slurry (Upstate Biotechnology) at a ratio of 50 µl per 2 × 10^6 cell equivalents for 2 h at 4°C. The agarose conjugates were removed by centrifugation and supernatants were incubated with polyclonal Abs to transcription factors as detailed in Materials and Methods. For anti-SFAT6 immunoprecipitations, each Abs listed in the Materials and Methods was tested and the M200 Ab was found to be superior in these assays. Accordingly, the M200 Ab was used for all subsequent experiments. Following overnight incubation at 4°C, salmon sperm-saturated protein A-agarose was added at a ratio of 30 µl/μg lysate and incubated at 4°C with gentle rocking for 2 h. Immunoprecipitated material was washed and eluted exactly according to the Upstate Biotechnology protocol. DNA cross-links were reversed by incubation with 20 µl of 5 M NaCl/500 µl eluted material for 4 h at 65°C. DNA was extracted with phenol/chloroform and then precipitated. DNA from each immunoprecipitation was resuspended in 50 µl of 10 mM Tris, pH 8.0, 1 mM EDTA and subjected to PCR analysis for the arginase I enhancer using primers 391 and 379 (Table I) using conditions empirically determined to amplify the amplicons before reaching the plateau (generally 25–26 cycles). Negative control reactions for background were performed with primers specific for the IL-12p40 and KC promoters that do not recruit STAT6 (Table I). Positive control reactions were performed using the “input” samples to the immunoprecipitation reactions that had their cross-links reversed according to the Upstate Biotechnology protocol.

EMSA
EMSA reactions were performed as described in detail (21, 22) using 5 µg of nuclear extract and oligonucleotide probes detailed in Table I and the Fig. 5 legend. Supershift reactions were performed using 1 µg of each Ab as detailed in Materials and Methods. Complexes were resolved on 1% Tris-borate-EDTA acrylamide gels.

DNase I hypersensitivity
RAW cells were stimulated or non-stimulated with 10 ng/ml IL-4. Cells were seeded at ~250 × 10^6 cells by resuspension in lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl2, 1 mM PMSF, 0.05% Nonidet P-40). Additional Nonidet P-40 was added to the lysis buffer to a final concentration of 0.1% to achieve cell membrane lysis. The nuclei were then gently resuspended in DNase I digestion buffer (40 mM Tris, pH 7.9, 10 mM NaCl, 6 mM MgCl2, 10 mM CaCl2) to ~2 × 10^6 nuclei/ml. Aliquots of nuclei were incubated with a dilution range of highly purified DNase I (Amersham Pharmacia Biotech) over 0–2000 U/ml for 20 min at 37°C. DNA was isolated by first digesting nuclei and associated proteins with proteinase K (100 µg/ml) in SDS lysis buffer (100 mM Tris, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) at 55°C for 2 h. DNA was then precipitated with isopropanol and resuspended in 10 mM Tris, pH 8.0, 1 mM EDTA to a final concentration of 1 µg/µl. Ten micrograms were digested to completion overnight with EcoRI and EcoRV. Samples were resolved on a 0.7% agarose gel, transferred to Hybond N membranes and hybridized with a 1.5-kb probe from the arginase I promoter encompassing the −2389 to −3386 region that contains the enhancer element. Southern blots were washed at high stringency (0.1% SDS, 0.1× SSC, 65°C) for 3 h and exposed to film.

Results
Characterization of a transfected system to study arginase I regulation
To study the regulation of arginase I gene expression in macrophages through techniques such as promoter dissection, a transfec-table system was essential. Primary macrophages are unsuitable for this purpose because they cannot be readily transfected. Therefore, we turned to the RAW macrophage cell line as a model system. Using Northern blotting and real-time RT-PCR, we first asked

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3 Abbreviations used in this paper: IRF, IFN regulatory factor; ChIP, chromatin immunoprecipitation; CBP, CREB binding protein.
Table 1. Oligonucleotides used in this study

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whether IL-4 and IL-13 induced arginase I mRNA expression as we had found previously in primary macrophages (9). The results show that arginase I mRNA was strongly induced in a time-dependent manner (Fig. 1). Neither IL-10 nor LPS induced arginase I mRNA alone but IL-10 was synergistic with both IL-4 and IL-13 in inducing the mRNA, precisely as reported in primary macrophages (5, 9). Importantly, IL-4 or IL-13 induced a four to five orders-of-magnitude increase in arginase I mRNA levels with the same kinetics as in primary macrophages (5, 9). Taken together, the results argue that RAW cells were a suitable model to search for STAT6-responsive regulatory regions within the arginase I promoter.

Isolation of the IL-4-responsive region of the arginase I locus

Extensive studies have characterized the rat and human arginase I promoters to define the regulatory elements that control the precise, coordinate expression of the urea cycle encoding genes (11, 23–26). However, little is known about the mouse arginase I promoter. Preliminary studies established that regions immediately upstream of the anticipated transcriptional start site were constitutively active in luciferase reporter assays in HepG2 cells, a human hepatoma cell line (A.-L. Pauleau, unpublished data). However, none of these constructs was responsive to IL-4 when transfected into RAW cells suggesting that the IL-4-responsive region was not located in the proximal promoter region (data not shown).

We next cloned the mouse arginase I promoter region from mouse PAC clones containing the entire arginase I locus. Fragments encompassing ~6 kb relative to the initiation codon were cloned upstream of a luciferase reporter vector and transfected into RAW cells to test their response to IL-4, and, synergistically with IL-1. Constructs containing ~4 or ~6 kb regions upstream of the transcriptional start site were strongly responsive to IL-4 or IL-4 and IL-10 (Fig. 2). The -31/-3810 construct induced a 42-fold increase above background luciferase activity that was further augmented by IL-10. These results suggested that the IL-4 responsive element was present within this sequence. Systematic dissection of
this region led to a 159-bp sequence sufficient to induce reporter activity in response to IL-4 (Fig. 2). In these constructs, putative IL-4-responsive regions were fused to basal arginase I promoter fragments represented by the −31/−657 and −31/−2356 regions of the promoter. These constructs by themselves were unresponsive to IL-4 until fused to fragments containing the 159-bp sequence. Qualitatively similar results were obtained using stable RAW cell lines that were generated by cotransflecting linearized versions of these plasmids and then stimulating with IL-4 or IL-4 and IL-10 for 16–20 h and assaying for luciferase activity as described in Materials and Methods. Fold induction relative to transfected cells left untreated is shown on the ordinate. Note that enhancer activity in the pGL3-promoter vector a is lower because the basal activity of this plasmid is extremely high as a result of the strong SV40 promoter element.

FIGURE 4. Mutagenesis analysis of the arginase I enhancer. Mutations were introduced into the −31/−3810 plasmid using site-directed mutagenesis. Mutations, labeled A through G, were constructed as shown (a). Each mutant was made twice, independently, and the results from one series of experiments are shown. Mutants were transfected into RAW cells, stimulated with IL-4 or IL-4 + IL-10 and assayed for luciferase activity (b). Fold induction relative to transfected cells left untreated is shown on the ordinate. The position of the EMSA probes used in Fig. 5 are shown underlined.
FIGURE 5. (Legend continues)
transcription start site previously mapped by Mori and colleagues (data not shown, and Ref. 25). This suggests that the IL-4 responsive region was unlikely to act as an alternative promoter in macrophages.

The IL-4-responsive region behaves as a classical enhancer

While creating the constructs to characterize the IL-4-responsive region, we noticed that the element could be cloned into reporter constructs in either orientation or independent of the location from the start site of the reporter. This suggested that the 159-bp fragment may act as a classically defined enhancer element. To experimentally test this possibility, we cloned the 159-bp fragment downstream of the polyadenylation site in luciferase constructs driven by the strong SV40 promoter, or two non-IL-4-responsive arginase I proximal promoter fragments (Fig. 3). When these constructs were transfected into RAW cells and stimulated with IL-4, the 159-bp fragment increased IL-4 responsiveness. This was also true for enhancing the activity of the SV40 promoter, whose basal activity is extremely high, 3- to 6-fold (Fig. 3a). For the $31/−657$ or $31/−2365$ constructs, basal activity of these promoters is low and augmented slightly by the addition of IL-4 but robustly induced when the 159-bp fragment is cloned downstream. These results define the IL-4-responsive element of the arginase I promoter as an enhancer.

In silico analysis of the enhancer using programs designed to predict transcription factor binding sites (e.g., TransFac) suggested the presence of multiple putative binding sites for transcription factors including C/EBPβ, PU.1, and STAT6 (Fig. 4a). To ascertain the importance of these sites, we initially attempted to further reduce the size of the enhancer by creating fragments progressively reduced in size from either end. Surprisingly, none of these fragments displayed IL-4 responsiveness suggesting that the enhancer could not be minimized in this manner (data not shown).

We then created plasmids containing mutations in each putative transcription factor binding site or adjacent sequences in the case of the putative PU.1 site with the rationale that PU.1 forms tight complexes with proteins such as IRF-4 that we had identified in microarray screens and considered potentially important in regulation of the enhancer (see Discussion). All mutations were made in the $−31/−3810$ plasmid that contains the enhancer, the basal promoter, and the transcription start site. Each mutant lost its ability to induce reporter expression in response to IL-4 or IL-4 and IL-10 although mutations in the putative STAT6 binding site completely abrogated reporter activity while mutations at other sites (e.g., mutants B and E) retained some inducibility (Fig. 4b). Similar results were found using stable RAW cell lines generated by transfecting linearized versions of these plasmids. This data, combined with the inability to reduce the overall size of the enhancer suggested that IL-4-mediated regulation of arginase I gene regulation was more complex than expected and required the assembly of a variety of transcription factors to the enhancer.

Factors that assemble at the enhancer

ChIP and EMSA reactions were performed to characterize the factors that can bind to the enhancer. In untreated RAW cells, STAT6, and C/EBPβ were weakly detected at the enhancer (Fig. 5a). Following IL-4 treatment, however, STAT6, C/EBPβ, and the coactivator CREB binding protein were recruited to the enhancer in a time-dependent manner. Correlative data from EMSA binding reactions also showed that IL-4-inducible complexes bound to oligonucleotide probes encompassing the putative STAT6 and C/EBPβ sites (Fig. 5f). Most interestingly, ChIP experiments showed that STAT6 itself is recruited to the enhancer which suggests that this factor plays two distinct functions in arginase I regulation: STAT6 directly binds the enhancer as shown here, but also directs the regulation of one or more other genes that are crucial for arginase expression (9). This indirect role of STAT6 was revealed in our previous work showing that cycloheximide blocks arginase I expression induced by IL-4. ChIP analysis also showed
that PU.1 was constitutively bound to the enhancer (Fig. 5b). Within the enhancer, the most likely binding sites for PU.1 are the GGAA motifs (Fig. 4). Site-directed mutagenesis of these sites substantially reduced enhancer activity (Fig. 4). Thus, PU.1 could serve as a factor that coordinates factor assembly at the enhancer. The ChIP data also correlated with supershift EMSA results definitively showing that PU.1 bound to an oligonucleotide probe encompassing the putative PU.1 binding sites (Fig. 5c). Finally, we performed additional ChIP experiments to ask whether chromatin at the enhancer was in a closed or open configuration based on the levels of acetylated histones (Fig. 5c). The results showed that acetylated and hyperacetylated histone H4 and acetylated H3 were readily immunoprecipitated from unstimulated cells, indicating that the arginase I enhancer is most likely in an open conformation readily accessible to the IL-4-receptor signaling pathway. Overall, it appears that multiple factors form a complex at the enhancer and likely assemble in the correct temporal order. Thus, STAT6 directly binds, along with PU.1, CBP, C/EBPβ, and possibly other components, and this complex awaits the STAT6-regulated production of another factor that establishes transcriptional regulation of the locus.

To search for the STAT6-regulated factor(s), we have used two types of microarray approaches. The first, an Affymetrix screen (Santa Clara, CA), was designed to focus on the identification of IL-4-regulated genes. In this pool of genes, we expected to identify transcription factors that could then be tested as candidate factors for regulating the enhancer. In this group, two mRNAs were identified, Krox-20 and IRF-4, whose induction was confirmed by Northern and immunoblotting analysis. Krox-20 had previously been identified as IL-4 regulated in B cells (29). However, transfection experiments failed to confirm any role for this factor in arginase I regulation (data not shown). IRF-4 was appealing because it forms a complex with PU.1 that is understood in atomic detail (30), and plays an important role in regulating IL-4 responses (31–34). However, macrophages from IRF-4−/− mice had completely normal IL-4-mediated induction of arginase I (data not shown). We also designed a microarray screen taking advantage of the fact that cycloheximide blocks induction of arginase I mRNA when macrophages are stimulated with IL-4. Macrophages were treated with IL-4 with or without cycloheximide for various times, and mRNA expression was measured on M20 arrays that contain ∼20,000 cDNAs. Here, we were searching for genes that were induced by IL-4 in the presence or absence of cycloheximide. Genes that require new protein synthesis to be induced (e.g., arginase I) would not be present in the samples treated with cycloheximide. In this screen, we identified a candidate transcription factor, ATF1. However, the induction of this protein was weak and not dependent on STAT6 (data not shown). At this stage, it is not possible to definitively determine whether STAT6 induces a transcription factor. It is possible that STAT6 regulates other processes required for transcription of the arginase I gene, including chromatin-dependent factors. In addition, we cannot definitively exclude the possibility that cycloheximide regulates the degradation of a factor(s) that assembles at the enhancer. This effect would preclude indirect effects of STAT6 on other genes.

Direct pharmacological manipulation of arginase I in diseases where macrophage activity is implicated will most likely be impossible because of liver toxicity associated with the disruption of urea cycle. For example, if arginase I activity promotes fibrosis in schistosomiasis (12, 14, 35) or accentuates asthmatic reactions (13), then our ability to regulate arginase I activity only in macrophages would be an appealing target. This report defines the basis for macrophage specificity and suggests that the control of the regulation of the arginase I enhancer could be targeted while sparing constitutive expression of arginase I in the liver.
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