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Characterization of the Murine C3a Receptor Enhancer-Promoter: Expression Control by an Activator Protein 1 Sequence and an Ets-Like Site

Carol B. Martin* and Brian K. Martin2*†‡

The complement system plays a critical role in innate immune defense and includes >30 serum and cell-associated proteins. Three different pathways can induce complement activation, including spontaneous fluid-phase turnover (alternative pathway), Ab-Ag complexes (classical pathway), and bacterial polysaccharides (mannan-binding lectin pathway) (for a review see Ref. 1). There are several effector functions mediated by complement, including opsonization, cell lysis, and inflammation.

Activation of complement through any of the three activation pathways leads to cleavage of C3 to C3a and C3b. C3b is a critical opsonin and is a central part of the C5 convertase enzyme complex.

The small, anaphylatoxin cleavage products of C3 and C5, C3a and C5a, mediate inflammation. These proteins act after binding to their receptors, members of the rhodopsin seven transmembrane-spanning G protein-coupled receptor family. Depending on the cell to which the proteins bind, a number of functions are activated by the anaphylatoxins. Traditionally, the receptors for these proteins have been thought to be predominantly expressed on myeloid cells and, to a lesser extent, endothelial cells (2). This correlates to the major functions induced by the anaphylatoxins, including chemotaxis, degranulation, and increased blood vessel permeability (2). However, over the last 10 years there have been many studies demonstrating expression of these receptors on a large number of other cell types, including parenchymal cells of the brain (oligodendrocytes (3), astrocytes (4–6), and neurons (7, 8)), liver (9, 10), and lung (11, 12). Collectively, these studies indicate that the cellular distribution of the anaphylatoxin receptors is broader than had been suspected previously. This suggests that cellular functions induced by anaphylatoxin proteins in nonmyeloid cells may be unique to those cell types.

Recent studies using the mouse model have found novel roles for C3a in lung biology, sepsis, and liver regeneration. Mice genetically deficient in C3aR are protected from asthma (13, 14), demonstrating that C3a directly contributes to this disease. Interestingly, experiments in C3aR knockout mice have demonstrated marked alteration in Th2 effector functions, possibly through modulation of APC activity (15, 16). It is not yet clear whether the effect in asthma is due to C3aR activities on parenchymal cells (11) or via changes in Th2 effector functions (11, 17). A recent study suggests that C3aR specifically contributes to lung inflammation in an asthma model (18). Genetic deletion of C3aR enhances endotoxin-mediated toxicity in sepsis, suggesting that this molecule can also function in an anti-inflammatory role (19). Recent data have demonstrated a novel role for C3a in liver regeneration (9, 10). C3aR knockout mice are less susceptible to experimental autoimmune encephalomyelitis (EAE)3, and ectopic C3a expression exacerbates disease (20). Collectively, these studies demonstrate that C3aR is important in a number of disease models and also suggest that modulation of C3aR expression may be critical.

In support of this hypothesis, C3aR expression is altered in a number of diseases. Ischemic injury in the nervous system leads to enhanced expression of C3aR at both the mRNA and protein level.

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Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; DMS, dimethyl sulfate.

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Interestingly, C3a is protective against N-methyl-D-aspartate-induced cell death of neurons, and this study suggested astrocyte-specific C3aR expression was necessary for this protection (23). Levels of C3aR are enhanced in multiple sclerosis (24), as well as in animal EAE (25). C3aR is expressed by endothelial cells and binding to C3a modulates cytokine expression (26), and endothelial cell expression is induced in the CNS during ischemic injury (21). Expression of C3aR on epithelial cells and smooth muscle is induced by LPS (11). These studies collectively suggest that an understanding of C3aR gene regulation should be studied.

Despite the importance of C3aR in disease, there is a distinct lack of studies examining the genetic regulatory mechanisms responsible for C3aR transcriptional control. To examine the enhancer-promoter organization of the C3aR gene, we cloned an ∼2 kb region upstream of the transcription start site for the gene. We demonstrate that a small region located at −72 to −35 bp is critical for C3aR gene regulation, and sequence analysis indicates consensus sites for AP-1, NF-κB, Ets, and GATA in this region. We demonstrate binding of Jun but not Fos to this site. In vivo footprinting demonstrates an expected DNA binding site at the AP-1 site, but the footprint does not suggest binding of an Ets family member. Indeed, the Ab to Ets-1/2 proteins is unable to supershift complexes from this site. Our data suggest cooperative interaction between these adjacent sites and also suggest the transcription factor is binding to a novel sequence. Although the control region for C3aR is contained in a small area, potential regulation could be complex due to the presence of several interacting transcription factors.

Materials and Methods

Cell culture

The murine macrophage cell line RAW 264.7 (ATCC TIB71) was obtained from the American Type Culture Collection. The B16 melanoma cell line was a gift from J. Yang (National Cancer Institute, Bethesda, MD). Cells were maintained in DMEM (Invitrogen Life Technologies) with 2 mM L-glutamine supplemented with 10% (v/v) FBS (HyClone) and penicillin-streptomycin (Invitrogen Life Technologies).

RACES

The spleen from a C57BL/6 mouse was harvested and the cells dissociated by mechanical disruption between frosted glass slides. The cells were washed once in PBS and the pellet resuspended in TRIZol (Invitrogen Life Technologies). RNA was extracted as per the manufacturer’s instructions. The RACE reaction was performed using the Smart RACE kit (BD Biosciences), as per the manufacturer’s instructions. The oligonucleotide used to create the C3aR cDNA is shown in Table I. The RACE reaction resulted in a prominent species of ∼850 bp, and this product was polished to generate blunt ends and isolated from the agarose gel. This cDNA product was cloned into the pCRScript-Amp vector (Stratagene). Six clones were selected and sequenced. The 5′-terminal sequence from these clones is presented in Fig. 1B.

Construction of reporter plasmids

The sequences for the murine C3aR gene promoter were retrieved from the Ensembl mouse genome server (http://www.ensembl.org/Mus_musculus/), and PCR primers were designed just 5′ of the translation start site (see Table I) and at a site 2040 bp upstream. Note that these primers incorporated restriction enzyme sites into each end to facilitate cloning. PCR was performed using genomic DNA isolated from C57BL/6 splenocytes and the Expand Long Template PCR system (Roche). The product was cloned into pGL2-basic (Promega). Sequencing was performed on this construct, and 500-bp regions at each end were confirmed identical to the sequence reported by Ensembl. The human C3aR promoter region was obtained from the Ensembl human genome server, and the murine-human sequences were aligned using the Lalign program (http://fasta.bioch.virginia.edu/ fasta/www/lalign.html). Deletions were generated using native restriction enzyme sites for every construct. These included AccI (∼1111), NdeI (∼829), and NcoI (∼87). The −34 bp construct was generated using PCR with the oligonucleotide shown in Table I. This oligonucleotide incorporated a 5′ KpnI site for cloning purposes. Constructs used in the consensus site mutation studies were made using the QuikChange Site-Directed Mutagenesis kit (Stratagene) in the context of the full-length promoter, according to the manufacturer’s instructions. The sequences modified by mutagenesis are presented in Table I, and each was confirmed by sequencing.

Transfection and reporter gene assays

All plasmids used for transfection were purified using the Qiagen Plasmid Maxi kit (Qiagen) with yield assessed by spectrophotometry. The transfection studies were done using at least two independent plasmid preparations, and each transfection was repeated at least three times. All cell lines were cotransfected in triplicate with 333 ng (for full-length constructs) or smaller constructs were transfected in equimolar ratios relative to the full length construct of luciferase reporter plasmid and 83 ng of pRL-null vector (Promega) per well in 24-well dishes. Transfection was performed using the transfection reagent ExGen 500 (Fermentas), according to the manufacturer’s instructions. pRL-null was used to normalize transfection and cell lysis efficiency. After incubation at 37°C for 18 h, the cells were washed with PBS. Cell lysis and determination of luciferase activity were performed using the Dual-Luciferase Reporter Assay System (Promega) using a Packard 96-well scintillation counter set to read luminescence. All luciferase data are presented as a percentage of the full-length promoter.

Table I. Oligonucleotides used

<table>
<thead>
<tr>
<th>Oligonucleotides used</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>C3aR RACE</td>
<td>AGGAGATGTTAGAGATGGCCTG</td>
</tr>
<tr>
<td>C3aRp cloning 5′</td>
<td>AGCGTATTCCACACCATTAGCCCTGAC</td>
</tr>
<tr>
<td>C3aRp cloning 3′</td>
<td>CTTAGAAGCCTTGGATAGAAATCCAAAGG</td>
</tr>
<tr>
<td>C3aRp-34</td>
<td>AGCTGATCAGTCATGACTGTGTCCT</td>
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<tr>
<td>GATA EMSA</td>
<td>GCTTGACGTCAGATAGCAGTATG</td>
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<tr>
<td>AP-1 EMSA</td>
<td>AGTTTAAAGGGTGATCTATGAAAC</td>
</tr>
<tr>
<td>NF-κB-Ets EMSA</td>
<td>CCTATGGAAGGGCTCTCTGAGGAT</td>
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<tr>
<td>NF-Etsb-Ets EMSA</td>
<td>CTATCGGGCCGGCTCTGAGGAT</td>
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<tr>
<td>NF-Bm-Ets EMSA</td>
<td>CTATCGGGCCGGCTCTGAGGAT</td>
</tr>
<tr>
<td>Footprinting sense 1</td>
<td>CTATCGGGCCGGCTCTGAGGAT</td>
</tr>
<tr>
<td>Footprinting sense 2</td>
<td>TTGAAGCACAGACAGCCCT</td>
</tr>
<tr>
<td>Footprinting sense 3</td>
<td>TCAAGCATCTCTCTCTGAAGTAGAGACAG</td>
</tr>
<tr>
<td>Footprinting antisense 1</td>
<td>CAGGATGTTAGAGATGGCCTG</td>
</tr>
<tr>
<td>Footprinting antisense 2</td>
<td>TGGAGATGTTAGAGATGGCCTG</td>
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<tr>
<td>Footprinting antisense 3</td>
<td>TGGAGATGTTAGAGATGGCCTG</td>
</tr>
<tr>
<td>RT-PCR C3aR sense</td>
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<tr>
<td>RT-PCR C3aR antisense</td>
<td>TGGAGATGTTAGAGATGGCCTG</td>
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<tr>
<td>RT-PCR GAPDH sense</td>
<td>GCGATTTGTGGAGGGCTCAAGT</td>
</tr>
<tr>
<td>RT-PCR GAPDH antisense</td>
<td>TGGAGATGTTAGAGATGGCCTG</td>
</tr>
</tbody>
</table>
RT-PCR

Total RNA was harvested from the endothelial cell lines and the RAW264.7 murine macrophage cell line using TRizol (Invitrogen Life Technologies). The RNA was reverse transcribed and analyzed using the Superscript One-Step RT-PCR kit (Invitrogen Life Technologies) according to the manufacturer’s instructions. PCR was performed using a thermocycler (Techne Genius; Midwest Scientific) under the following conditions: 94°C (5 s), 55°C (10 s), and 70°C (30 s), and samples of products were taken at the cycle number indicated in each figure. The primers used for amplification are shown in Table I. C5aR sense, 5′-CCATTAGTC CGACCCGGTTC-3′; C5aR antisense, 5′-AAGAGACGAGGAT TCAAGTTG-3′; GAPDH sense, 5′-GGTACCTGAGGAGCT TGCAG-3′; and GAPDH antisense, 5′-TGGGATGGTCT GTGAAGTCG-3′. The RT-PCR products were separated on a 1.0% agarose gel and visualized by ethidium bromide staining.

EMSA

RAW 264.7 nuclear extracts were prepared from 90% confluent cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce), according to the manufacturer’s instructions. Double-stranded oligonucleotides were synthesized (IDT). These single-stranded oligonucleotides were labeled with 5 μl of 5 μM biotin-14-dCTP (Invitrogen Life Technologies) using TdT (Promega) at 37°C for 45 min. Reactions were stopped with 0.5 M EDTA, and labeled oligonucleotides were purified by chloroform/isoamyl alcohol (24:1) extractions. Labeled oligonucleotides were annealed by heating to 94°C, cooled to room temperature on the bench top, and incubated at 4°C overnight to obtain double-stranded nucleotides. Unlabeled competitor probes were similarly prepared (see Table I) and used at 500-fold molar excess relative to the labeled probe.

For EMSA, the binding reactions were performed using the Lightshift Chemiluminescent EMSA kit (Pierce), according to the manufacturer’s instructions. Binding reactions were conducted for 20 min at 25°C in 20 μl of reaction buffer containing 1 μg of poly (dI-dC), 40 fmol labeled probe, and 5 μl (5 μg μl) of nuclear extract. For competition and supershift assays, unlabeled probes in 500-fold excess or Ab (Santa Cruz Biotechnology) were added to the reaction mixture for 20 min at 25°C before the addition of the biotin-dCTP labeled probe. The DNA-protein complexes were separated on 4% nondenaturing polyacrylamide gel in 0.5 × Tris-borate/EDTA at 4°C and transferred to a Hybond-N+ transfer membrane (Amersham Biosciences) using an Owl Separation Systems Semi-Dry Electroblotting system at 300 mA for 1 h. Membranes were analyzed using the Phototope-Star Detection kit (New England Biolabs), followed by exposure to blue-sensitive x-ray film (MidSci).

In vivo DNA footprinting was essentially as described previously (27). Briefly, cells in log phase of growth were treated with dimethyl sulfate (DMS) and DNA prepared. DNA binding proteins were stripped using phenol-chloroform extraction. One set of DNA was then treated with DMS as a control to visualize the DNA ladder with no proteins bound. The DNA was cleaved using 1 M piperidine. Ligation-mediated PCR was used to amplify the region of the C3aR promoter. The sequences of the primers used for these analyses are shown in Table I. The DNA fragments were separated on a 7% sequencing gel and transferred to a Hybond-N+ transfer membrane (Amersham Biosciences) using semidy electroblotting. The products were visualized by chemiluminescence using the Phototope-Star detection kit and developed using x-ray film.

Results

Cloning of the C3aR enhancer-promoter and determination of transcriptional start site

Three separate groups independently published the human C3aR gene sequence in 1996 (28–30), while two groups reported the mouse gene sequence in 1997 (31, 32). However, the promoter enhancer organization of the human or mouse genes was not examined. Description of the mouse genome by the Ensembl group (33) allowed us to use sequences upstream of the murine 5′ untranslated region to design PCR primers to clone this region. The Ensembl group presented sequences up to 80 bp upstream of the ATG translation start site but did not present information on the transcription start site. There were no gaps reported in sequences extending 2500 bp upstream of the translation initiation site. Using this region as a reference, we designed a 3′ PCR primer that began 17 bp upstream of the translation initiation sequence, while the 5′ PCR primer was 2040 bp upstream of the 3′ oligonucleotide. Using a high-fidelity PCR system, we observed a product of predicted size, and cloned this region into the pGL-2Basic vector. We sequenced this region to determine whether there were any sequences that varied from those reported by Ensembl, no differences in the first 400 bp (Fig. IA and data not shown).

To be sure that we were working in the region of the promoter, we needed to precisely identify this site. We used RACE to establish the sequences at the start site. As shown in Fig. 1B, of the six RACE products cloned and sequenced, three terminated in the ACTTC sequence and another extended 2 bp further upstream. The remaining two products terminated 28 and 30 bp upstream of the ACTTC sequence. It is not clear whether the multiple start sites in the C3aR promoter are due to the reverse transcriptase not efficiently extending to the end of the mRNA or if there are indeed several start sites (as is often the case in promoters without TATA boxes (34)). These data demonstrated that we had cloned a region upstream of the transcription initiation site.

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Sequence of the murine and human C3aR promoter region. A. Mouse and human C3aR enhancer-promoter alignment. The mouse C3aR promoter enhancer region sequence was cloned using PCR. Sequences conserved between mouse and human are indicated using dashed lines. Boxes indicate consensus transcriptional control regions and are labeled accordingly. The open triangle at −87 and closed triangle at −34 indicate the sites of the corresponding luciferase constructs. The horizontal arrowheads indicate transcriptional start sites as determined by RACE. B. RACE products. The sequences of six different RACE products are presented. Although most terminate in the downstream area, the presence of the longer transcripts indicate that some begin further upstream.
Identification of potential transcriptional regulatory elements

Two common methods used to identify potential transcription regulatory elements are phylogenetic conservation and database searches for common regulatory motifs; thus, we next compared the sequence of the genomic regions from the human and mouse genes to determine which regions of the promoter were conserved. As shown in Fig. 1A, there are local regions with strong conservation between the promoters, but these analyses alone could not identify possible regulatory motifs. To identify which conserved regions might be responsible for transcription factor binding, we used the web program TFSearch to locate consensus binding sites. As shown in Fig. 1A, the C3aR promoter has a number of conserved elements, including sites for AP-1, NF-κB, Ets, and GATA factors. Of these sites, the AP-1, Ets, and GATA were conserved between the mouse and human sequences. However, the NF-κB site was not phylogenetically conserved. These data suggested that these transcription regulatory elements might play a critical role in C3aR gene regulation, but functional assays must be used to conclusively establish this relationship.

Deletion analyses of the murine C3aR enhancer-promoter

Sequences upstream of the transcription start site typically mediate control of gene expression, and to establish the importance of upstream regions, we cloned our 2040-bp fragment into a luciferase reporter vector. We first needed to establish that we had cell-specific expression of the full-length construct. In all transfection experiments, we used a Renilla luciferase control vector to normalize transfection efficiency for every construct and cell line. We transfected the reporter vector into B16 melanoma cells (which have no evidence of C3aR mRNA) and RAW 264.7 macrophages (which express high levels of C3aR mRNA; data not shown). As shown in Fig. 2A, the full-length construct (extending 1972 bp upstream of the most 5′ transcription start site) directed expression of luciferase at least 100-fold higher than the empty reporter construct. Most critically, the full-length construct did not mediate expression in B16 cells, indicating that our construct contained cell-specific regulatory motifs. Specifically, the full-length C3aR construct (−1972) in B16 cells was only 2.4-fold more active than empty vector, and this activity was 140-fold less than the activity in RAW 264.7 cells. These data demonstrated that our full length C3aR enhancer-promoter vector was fully capable of directing cell-specific reporter gene expression in these two cell types.

Although the most common location for gene regulatory motifs is in the area 5′ of the transcriptional start sites, these regions can be located in many different places, including introns and 3′ of the gene. There is precedent for transcriptional control regions in the introns of complement genes, including the human C5aR gene (35) and in the murine CD21 gene (36). The majority of G protein-coupled receptor genes (including anaphylatoxin receptor genes) have a single intron separating the 5′ untranslated region from an exon that contains the entire coding sequence (37). To examine any potential contribution of the murine C3aR exon, we cloned this ~4720 bp region using PCR and primers designed using Ensembl sequences. We cloned this region directly to our −1972 bp construct, such that the structure of this reporter construct was exactly that of the endogenous C3aR gene, except that the luciferase gene now took the place of the coding region for C3aR. These constructs were transfected in equimolar ratios into RAW 264.7 macrophages and B16 melanoma cells. As shown in Fig. 2B, the presence of the intron does not significantly change luciferase activity in RAW 264.7 cells, nor did it change expression in B16 cells; thus, we conclude that the C3aR intron does not appreciably contribute to C3aR expression in these cell types. However, we cannot preclude a function for this region in other cell types.

**FIGURE 2.** C3aR reporter gene assays using deletions and intron containing vectors. A. Deletion analyses of the C3aR 5′ region. Using endogenous restriction sites, we created a series of deletions at the indicated sites. These luciferase constructs were transfected in equimolar amounts into RAW 264.7 cells, and reporter activity was determined. Only deletion of sequence between −87 and −34 strongly affected reporter gene activity. None had significant reporter activity in nonexpressing B16 melanoma cells. B. Intron containing reporters do not have significantly altered reporter activity. The intron separating the 5′ untranslated region of C3aR from the coding sequence was cloned using PCR and cloned exactly as in the normal C3aR gene, with the luciferase reporter taking the place of the coding region for C3aR. The constructs were transfected into RAW 264.7 and B16 melanoma cells. All values are normalized to the full-length construct, and the mean ± SD of triplicate samples is presented. In all cases, the reporter activity was normalized to a Renilla luciferase control. *, *p = 0.016; **, *p < 0.0001 relative to C3a Rp −1972. C. RT-PCR analysis of C3aR in RAW 264 macrophage cells. Messenger RNA was isolated from untreated RAW 264.7 cells or cells treated with 10 ng/ml LPS. Reverse transcription and PCR were conducted using one-step RT-PCR using the oligonucleotides shown in Table I, and products were removed at the indicated cycle number. D. RT-PCR analysis of C3aR in B16 cells.
We next wanted to determine which sequences in the 1972 bp 5’ of the transcription start site were critical for expression in macrophages and whether deletion of these sequences altered expression in cells. Using endogenous restriction sites, we generated a series of deletion constructs, deleting 861 bp (−1111 construct), 1143 bp (−829 construct), 1885 bp (−87 construct), and 1938 bp (−34 construct) of the sequence. Again, we used cotransfection with Renilla luciferase under the control of a ubiquitous promoter and normalized values to the Renilla luciferase activity. All constructs were transfected in equimolar ratios. As shown in Fig. 2A, constructs −1111 and −829 had no change in reporter activity relative to the full-length −1972 construct, while the −87 construct had a loss of only 25% activity (which was statistically significant). However, deletion of an additional 53 bp to create the −34 construct (▲ in Fig. 1A) resulted in complete loss of transcriptional activity (<3% activity relative to the full-length construct in RAW 264.7 cells; p < 0.0001). None of the deletions significantly altered the activity of the reporter in B16 cells (Fig. 2A). These data suggested that the majority of the transcriptional control activity of the C3aR promoter enhancer in macrophages resides within a 53-bp region from −87 to −34. Interestingly, this corresponded to the region identified in our transcription factor database searches, including AP-1, NF-κB, Ets, and GATA (Fig. 1A). Although our deletion analyses identified the region critical for C3aR expression, these studies did not uncover the relative contribution of potential regulatory motifs.

Next we sought to confirm the luciferase analyses at the RNA level. We used RT-PCR to amplify C3aR message and used comparison to GAPDH to determine relative expression. As shown in Fig. 2C, RAW 264.7 cells expressed significant levels of C3aR message. Expression was increased greatly when the cells were treated with 10 ng/ml LPS. In contrast, mRNA expression for C3aR could not be detected in the B16 cell line in either unstimulated or LPS-stimulated cells (Fig. 2D).

Site-directed mutagenesis of the C3aR regulatory region

To determine the significance of the putative regulatory motifs in the −87 to −34 region, we used mutagenesis to alter each site and examined promoter activity. In each case, a 3-bp mutation was generated in nucleotides that are involved in transcription factor binding for the protein in question (Table II). All mutations were generated in the context of the full-length −1972 enhancer-promoter. Mutation of the NF-κB core sequence did not significantly alter the activity of the C3aR reporter construct (Fig. 3). This was not unexpected because the NF-κB site is not conserved between species (Fig. 1A); thus, it is unlikely that this molecule functions in C3aR regulation. However, we cannot exclude the possibility that this site is important in other cells or during modulation of gene expression. Mutation of the GATA sequence resulted in a reproducibly decrease in promoter activity of ~75%. These data suggest that the GATA sequence functions as a negative regulatory element in RAW 264.7 cells.

We next mutated the AP-1 sequence. AP-1 motifs are found in a large number of genes and facilitate both basal and induced gene expression by binding to several protein families, including Jun, Fos, and ATF (38). Mutation in the AP-1 site drastically altered luciferase activity, decreasing 4-fold (Fig. 3). The Ets family of transcription factors binds to a common GGAAC core motif and are also found in a large number of genes (39) (including complement genes such as Crry (40)). Mutation of the C3aR Ets consensus region resulted in a 60% decrease in activity. Interestingly, it is well known that Ets and AP-1 can cooperate in facilitating gene expression (41); thus, we examined cooperativity between the two regions by mutating both the AP-1 and ETS sites. As shown in Fig. 3, mutation of both sites resulted in a 20-fold decrease in promoter activity, establishing a cooperative role for these sequences in C3aR gene regulation. These experiments demonstrated that the AP-1 and Ets sites are critical for positive regulation of the C3aR promoter in RAW 264.7 macrophage cells, and the GATA site functions as a negative regulatory element.

<p>| Table II. Consensus transcription factor binding sequences in the murine C3aR gene and mutations generated for luciferase and EMSAs |</p>
<table>
<thead>
<tr>
<th>C3a Promoter Sequence</th>
<th>AP-1</th>
<th>NF-κB</th>
<th>Ets</th>
<th>GATA</th>
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</thead>
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<tr>
<td>Consensus Mutations</td>
<td>−71 to −65</td>
<td>−54 to −63</td>
<td>−47 to −56</td>
<td>−40 to −33</td>
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<td>TGA CTC A</td>
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<td>GGA T A T</td>
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<td>−100–98/−81–79</td>
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Figure 3. Luciferase reporter gene assays with C3aR promoter site-directed mutants. Mutations were generated in each of the four predicted transcriptional control regions to assess the contribution of each site to promoter regulation. In each case, 3 bp at the core of the predicted consensus sequence were mutated, except in the AP-1/Etsm construct in which both the AP-1 and Ets sites were mutated. The constructs were transfected into RAW 264.7 and B16 melanoma cells. All values are normalized to the full-length construct, and the mean ± SD of triplicate samples is presented. In all cases, the reporter activity was normalized to a Renilla luciferase control. *, p < 0.05; **, p < 0.005; and ***., p < 0.001.
a strong complex (Fig. 5A, lane 2; see arrow), and this complex was effectively competed with self probe (lane 3). Mutation of the NF-κB site did not decrease the ability of unlabeled probe to compete for binding to the protein (Fig. 5, lane 4). However, mutation of the Ets site or mutation of both Ets and NF-κB completely abolished the ability of unlabeled oligonucleotides to compete in the formation of the DNA-protein complex (Fig. 5, lanes 5 and 6). These data indicate that nuclear proteins form a complex at the Ets site, as hypothesized based on data from the luciferase experiments.

There are a large number of Ets proteins that include those that are ubiquitous as well as cell type-specific proteins (39). The TFSearch program used to identify transcription factor binding sites in the C3aR promoter identified the Ets as a potential Ets-1 binding sequence. We used supershift analysis to examine this possibility. Abs to c-Jun, c-Fos, or Ets-1/2 did not result in a shifted complex, even when the gel was overexposed to increase sensitivity (Fig. 5B). These data suggested that either the C3aR Ets site binds to a more cell-specific Ets factor (such as PU.1) or binds to an as yet unidentified factor.

Our luciferase mutation analyses demonstrated that the GATA site is a negative regulatory element, and we hypothesized that we would observe a DNA-protein complex using this site as a probe. As shown in Fig. 6, there was a specific complex that could be

**EMSA analyses of C3aR promoter regulatory elements**

Our experiments in RAW 264.7 cells demonstrated that the AP-1 sequence is critical for C3aR promoter regulation, but those experiments did not establish which AP-1 partners were responsible for promoter activity. The AP-1 sequence is a binding target for several different factors, including members of the Jun, Fos, and ATF families (38, 42). We used EMSA analyses to examine the proteins involved in AP-1 binding in RAW 264.7 cells. An oligonucleotide probe encompassing the C3aR AP-1 site formed at least two DNA-protein complexes on a native polyacrylamide gel (see arrows, Fig. 4A). The complexes were effectively competed using self probe but did not compete with irrelevant GATA probe (compare Fig. 4A, lanes 3 and 4).

We used supershift assays to determine the identity of the proteins in the shifted complex. As shown in Fig. 4B, addition of Ab to c-Jun resulted in the appearance of a slower migrating complex, indicating that c-Jun was part of this complex (lane 3). Abs to either c-Fos (an AP-1 binding protein) did not result in either a diminution of the intensity of shifted complex or a supershifted band (lane 4), indicating that this protein is not part of the complex at the AP-1 site in RAW264.7 cells. Abs to Ets-1/2 did result in a slight loss of signal intensity but no shifted bands (lane 5), suggesting that Ets-1 proteins may play a small role in the DNA binding complexes at this site.

We next wanted to examine potential proteins binding to the Ets site in the C3aR promoter. The caveat of these experiments is that the NF-κB and Ets sites overlap in the murine C3aR promoter (Fig. 1A); thus, we had to examine each sequence in turn to determine which regions were responsible for formation of DNA-protein complexes. A probe encompassing the NF-κB and Ets sites formed...
competed with a self-probe (lane 3) but not a probe from the AP-1 site (lane 4). We should note that this is a relatively weak complex because it took long autoradiograph exposures to see this complex. These data suggest a member of the GATA family of transcriptional regulators plays a role in C3aR regulation in the RAW 264.7 macrophage cell line.

We have extended our analyses of the shifted complexes in primary macrophages. We see no difference in the pattern of shifted complexes between RAW 264.7 cells and primary macrophages (data not shown), suggesting a similar regulatory mechanism between these two lines. One difference is in expression, such that primary macrophages have significantly higher levels of C3aR mRNA relative to RAW cells.

**DNA binding proteins in B16 nuclear extracts**

Luciferase experiments described above demonstrate that the AP-1, Ets, and GATA sites are important in reporter gene assays, and EMSAs demonstrate nuclear proteins binding to these sites. We hypothesized that lack of protein binding to the positive sites (AP-1 or Ets) or increased protein binding to the negative site (GATA) would mediate the low transcriptional activity of the C3aR promoter in B16 melanoma cells. Using the AP-1 site as a probe, we were unable to demonstrate a specific DNA binding complex in B16 nuclear extracts (Fig. 7A). Because the self-probe did not compete for binding in any shifted complex (lane 6) nor could Abs to c-Jun, c-Fos, or Ets1/2 supershift any bands (lanes 3–5), all these DNA–protein complexes were nonspecific. The Ets site did specifically shift a strong complex (Fig. 7B, lane 2), and it could be competed with self probe (lane 3), albeit not as well as in RAW264.7 experiments (Fig. 5). There was a single band (△) that competed with all oligonucleotides, suggesting there may be a B16 protein binding outside the tested regions. Finally, the GATA site also did not shift a complex that could be competed with self probe (compare Fig. 7C, lanes 2 and 3). Collectively, these data suggest that the AP-1, Ets, and GATA sites do not form the strong complexes seen in RAW264.7 nuclear extracts. The lack of binding at the AP-1 site is likely a critical factor that leads to low C3aR promoter activity in these melanoma cells.

**In vivo DNA footprinting of the C3aR promoter**

The experiments described above establish that the AP-1 and Ets sites are critical for C3aR promoter regulation, but we don’t know how these factors bind to the promoter. One way to determine whether these sites are bound by typical transcription factors is to examine the DNA footprint formed in this region. Traditional DNA footprinting involves either the addition of a purified transcription factor to the DNA of interest or isolation of bound probe from EMSAs. Methylation of specific nucleotides interferes with the ability of transcription factors to bind their respective sites, and comparison of bound vs unbound probe can be used to determine which bases are critical for DNA binding. However, these assays do not reveal how base modification may alter DNA binding in vivo. With in vivo DNA footprinting, proteins bound to DNA inside the cell block methylation by DMS. The DNA is chemically cleaved, and ligation-mediated PCR is used to amplify the region of interest. The DNA products are resolved on an acrylamide gel, and the pattern of bands from in vivo treated DNA is compared with DNA treated with DMS in vitro after removal of all proteins.

We designed primers for in vivo footprinting of both the sense and antisense strands of the C3aR promoter region. Two characteristics of the chemistry should be noted. First, DMS coupled with 1 M piperidine cleavage results primarily in cleavage at deoxyguanosine residues. The sense PCR primers extend using the antisense primer as a template; thus, when the antisense strand terminates at a cleaved deoxyguanosine residue, the sense strand

**FIGURE 6.** EMSA analyses of the C3aR promoter GATA site. Nuclear extracts from RAW264.7 cells were bound to a double-stranded oligonucleotide probe encompassing the C3aR GATA site. A single band was present (arrow) that competed with the self-probe (lane 3) but not the irrelevant probe (lane 4).

**FIGURE 7.** EMSA analyses of the C3aR promoter using B16 extracts. Nuclear extracts from B16 melanoma cells were bound to a double-stranded oligonucleotide probe encompassing the C3aR AP-1 site (A), the NF-κB/Ets site (B), or the GATA site (C).
being synthesized will be visualized as a stop at a deoxycytosine residue. Fig. 8 shows the in vivo DNA footprint of the C3aR promoter. In the sense strand, we saw a footprint at the AP-1 site as expected. Surprisingly, we did not see the expected footprint at the Ets site. The typical consensus Ets sequence is GGAAG, a sequence present in the C3aR Ets site. We expected a footprint including the first two Gs of this sequence; however, we did not see protection in this region and instead saw an enhancement just downstream. Enhancements are indicative of the binding of DNA near sites of protein binding. We also observed an enhancement 16 bp upstream of the transcription start site, likely due to binding of the basal transcription machinery at the start site. The antisense strand had a similar pattern. Once again, we observed an expected protection encompassing the AP-1 site, but the core of the Ets site showed an enhancement. However, we did observe protected bases just upstream of the predicted Ets site. Transcription factor searches for the sequence at the observed footprint did not suggest any obvious candidates for regulation at this site. These data are not consistent with an Ets transcription factor and suggest that the AP-1 site is cooperating with an as yet unknown factor.

Induced C3aR expression is due to cooperativity between AP-1 and Ets sites

All data presented this far were generated using RAW 264.7 cells that were not stimulated; thus, the expression of C3aR was at a basal state. Experiments have shown that LPS is capable of inducing expression of C3aR (11); therefore, we hypothesized that C3aR induction would be mediated through the previously identified transcription factor sites. Luciferase constructs were transfected into RAW 264.7 cells, then the cells were treated with 10 ng/ml LPS for 18 h. As shown in Fig. 9, LPS treatment resulted in a 6-fold increase in the activity of the promoter. Interestingly, there appears to be higher induction at the mRNA level (Fig. 2), suggesting mechanisms other than transcription induction modulate mRNA levels of C3aR in RAW 264.7 cells. Mutation in the NF-xB site did not alter basal activity or induction by LPS. Similarly, mutation in GATA leads to an increase in basal activity, but the ability to be induced by LPS was unaltered by this mutation. Mutations in both AP-1 and Ets sites decreased basal activity, but there was an increase of 5.7- and 4.0-fold activity with LPS treatment (differences were not statistically significant), indicating that individually, these mutations did not alter the ability to respond to LPS. Mutation of both sites altered LPS induction, such that treatment increased expression only 2.4-fold over untreated cells (p = 0.021). These data indicate that either the AP-1 or Ets sites can mediate induction of C3aR activity by bacterial LPS, but when both sites are mutated, there is significant loss of promoter inducibility, most likely an additive effect. The changes in expression observed in these experiments suggested that we might observe altered protein-DNA complexes in EMSA analyses, although we found no apparent differences when LPS-treated extracts were used in these assays (data not shown). These data suggest that

**FIGURE 8.** In vivo DNA footprinting of the C3aR promoter. Cells were treated with DMS and genomic DNA prepared. This DNA (or control DNA) was treated with piperidine to cleave predominantly at deoxyguanosine residues. Ligation-mediated PCR was used to amplify the DNA, and sequence-specific probes were used to determine either protected residues (△) or residues with enhanced cleavage (▲). Because PCR is used for amplification, the opposite strand residues are visualized (i.e., deoxycytosines rather than deoxyguanosines).

**FIGURE 9.** Luciferase reporter gene assays in response to LPS treatment with C3aR promoter site-directed mutants. The indicated reporter gene site-directed mutants were transfected into RAW 264.7 cells and then treated with 10 ng/ml LPS. LPS induced approximately a 6-fold induction in C3aR promoter expression. Only mutation in both the AP-1 and Ets sites together significantly reduced the ability of LPS to induce promoter expression. The constructs were transfected into RAW 264.7 and B16 melanoma cells. All values are normalized to the full-length construct, and the mean ± the SEM of triplicate samples is presented. In all cases, the reporter activity was normalized to a Renilla luciferase control.
either the new complexes that bind to the site have the same size as those that bind in untreated cells (thus, there is no difference in migration) or the activation induced by LPS is dependent upon an activator that does not directly bind to DNA.

Discussion

In this report, we have demonstrated that a 2040-bp fragment of the C3aR promoter possesses the ability to direct strong reporter gene expression in a macrophage cell line while not allowing expression in a non-C3aR-expressing melanoma cell line. Although some other complement receptor genes have been shown to have transcription control sequences in the first intron (35, 36, 43–45), the first (and only) intron in the C3aR gene does not confer regulatory function in the cells tested.

We used deletion analyses to localize the major control region for the C3aR gene to a small 53-bp region just 5′ of the transcription start site. Included in this region are consensus AP-1, NF-kB, Ets, and GATA sites. Four lines of evidence exclude NF-κB as contributing to C3aR regulation in RAW 264.7 macrophage cells. First, the NF-κB site is not conserved in the human C3aR promoter (Fig. 1A), and second, mutation of this site does not alter reporter gene activity in macrophages or melanoma cells (Fig. 3). Mutation of this sequence did not alter the ability of probes from this region to form protein DNA complexes in EMSA (Fig. 5). Finally, there was no visible footprint in this region (Fig. 8). Collectively, these data demonstrate no role for this sequence in RAW 264.7 macrophages nor in B16 melanoma cells. We cannot at this time exclude a role for NF-kB-mediated C3aR regulation in other cell types.

The GATA site showed interesting negative regulatory features in RAW 264.7 cells. Mutation of the site resulted in a 75% increase in reporter gene activity in macrophages. It remains to be seen whether this site has negative regulatory activity in other C3aR-expressing cells or under conditions in which the expression of the gene is down-modulated.

The most important regulatory elements found in the C3aR promoter associated with macrophage expression are the AP-1 and Ets sites. The AP-1 site is found in a large number of enhancer-promoters. AP-1 sites can be characterized as either 12-O-tetradecanoylphorbol-13-acetate response elements (5′-TGAG/CTCA-3′) or cAMP response elements (5′-TGACGTCA-3′) (42). The C3aR AP-1 exactly matches a cAMP response element site (Fig. 1). AP-1 is not a single molecule but instead can be composed of a number of different proteins, including members of the Jun, Fos, Maf, and ATF protein families (42). The c-Jun molecule can form heterodimers that activate transcription, and interaction with c-Fos (which does not form homodimers) can further activate AP-1 element-containing genes (46). JunB, in contrast, negatively regulates genes containing AP-1 elements (47, 48). The C3aR AP-1 site is conserved in the human and murine C3aR promoters (Fig. 1A) and is critical for transcriptional activity (Fig. 3). Interestingly, we showed that c-Jun (and not c-Fos) forms DNA-protein complexes in extracts from RAW 264.7 cells (Fig. 4B). There are a large number of individual proteins that can form AP-1 complexes, although we have only scratched the surface regarding potential AP-1 binding partners in macrophage cells.

Ets proteins are important transcription regulators in a wide variety of genes (39). Usually, these proteins bind to a GGAAG consensus sequence, and this sequence is found in both the human and mouse C3aR promoters (antisense strand, Fig. 1). Early data strongly suggested that a typical Ets family member was functioning at this site. The TFSearch website algorithm suggested that the C3aR Ets site contained the consensus binding sequence for Ets-1. Mutation of the GGAAG site to CTCAG decreased promoter activity by 60% (Fig. 3), and unlabeled oligonucleotides containing this same mutation could not compete for binding in EMSA analyses (Fig. 5A). These data implicated Ets factors and possibly Ets-1. However, other data suggested that this site does not bind to a typical Ets family member. First, Ab against Ets-1 and Ets-2 does not result in a supershifted EMSA complex (Fig. 5B). Second, in vivo footprinting for an Ets site should show protection of all the deoxyguanosines in the GGAAG sequence, but none of these nucleotides were significantly protected when methylated (Fig. 8). A number of studies have shown that the expected protection at this site includes all the deoxyguanosines in the GGAAG sequence (49–53). These data suggest alternate possibilities for this site. First, there could be a novel Ets family member binding to this region. Second, one of the less studied Ets family members could be binding to this site in RAW 264.7 cells and producing a novel in vivo footprint. Finally, it may be that the protein binding to this site is not a member of the Ets family.

There is a large body of literature describing interaction of Ets family proteins with AP-1 molecules. For instance, RANTES (54), urokinase-type plasminogen activator (55), matrix metalloproteinase (56), and osteopontin (57) are all regulated through Ets/AP-1 interactions. The proximity of the Ets site to the AP-1 site is one clue as to their potential for interaction. Usually, spacing between adjacent cooperative sites is critical in their functionality, as has been demonstrated for AP-1 and the Ets family protein PEA3 (58). Interestingly, a change in two nucleotides in different areas of the murine and human C3aR promoters maintains spacing between the AP-1 and Ets sites (see the area between the sites in Fig. 1). These data suggest that spacing between the AP-1 and Ets sites may be critical for promoter activity. Collectively, we predict that the AP-1 and Ets sites in the C3aR promoter will be critical for promoter activity in diverse cell types.

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Disclosures

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References


