Characterization of the Murine IgG Fc Receptor III and IIB Gene Promoters

A SINGLE TWO-NUCLEOTIDE DIFFERENCE DETERMINES THEIR INVERSE RESPONSIVENESS TO C5a*

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The two low affinity IgG Fc receptors (FcγR), FcγRIII and FcγRIIB, are coexpressed on myeloid effector cells, and their genes, as reported here, are positively and negatively regulated by both C5a and interferon-γ through different signaling mechanisms. Two 48- and 43-bp sequences (C5a-inductive region (CIR) and C5a-suppressive region (CSR)) in the FcγRIII and FcγRIIB 5′-flanking regions that are necessary for C5a induction and suppression, respectively, are defined. Sequence analysis of the CIR and CSR, which localize apart from the interferon-γ-responsive regions in each gene, revealed the presence of a novel element that differs by two nucleotides between FcγRIII and FcγRIIB. Mutation analysis of the CIR and CSR showed that this small difference determines inverse responsiveness in an FcγR gene context-dependent manner. Our study suggests that C5a uses similar DNA motifs (defined as GTGAXXTCCA) in both pathways of transcriptional induction and suppression of FcγRIII and FcγRIIB.

Activated complement component C5a is a pleiotropic molecule that regulates the activity of many cell types, with a broad range of biological functions in the immune system (1). C5a binds to at least two seven-transmembrane domain receptors, the C5a anaphylatoxin receptor (C5aR); CD88) and C5L2, expressed on a variety of immune cells, including circulating leukocytes, mast cells, basophils, macrophages, and many others. C5aR-dependent activation of these cells by C5a results in inflammatory mediator release and granule secretion, which in turn alter vascular permeability, induce smooth muscle contraction, and promote inflammatory cell migration (2). It is well established that this C5a-triggered cascade of events contributes to the pathogenesis of various diseases in humans, including myocardial ischemia/reperfusion injury and respiratory distress syndrome (3–5). In addition, genetic deletion of C5aR is very effective in preventing inflammation in animal models of erosive arthritis, anti-phospholipid syndrome, and antibody-dependent type II autoimmunity (6–8).

Complement activation occurs through multiple pathways (classical, alternative, and lectin-binding) in the circulation, each of which produces C5a. Interestingly, C5a is also formed within the extravascular tissue compartments through activation of resident innate immune cells such as tissue macrophages and requires the presence of receptors for the Fc portion of IgG (FcγR) (reviewed in Ref. 9). The FcγR exert their function through paired expression of activating (FcγRI, FcγRIIa, and FcγRIIB) and inhibitory (FcγRIIB) receptors (reviewed in Refs. 10 and 11). Compelling evidence suggests that the ratio of the opposing signaling FcγR is critical in setting the cellular thresholds for the pathogenic activity of autoantibodies (reviewed in Refs. 10 –12) and that C5a regulates this ratio, thus amplifying inflammatory FcγR responses in autoimmunity (8, 13).

Although C5a and FcγR likely cooperate in the context of immunological diseases, the molecular mechanisms of transcriptional regulation of C5a-responsive genes, such as those encoding FcγRIII and FcγRIIB, remain to be elucidated. FcγRIII expression on myeloid cells is enhanced by exposure to C5a, and this reflects an increase in the rate of FcγRIII and FcγR transcription (8, 13). In contrast, C5a rapidly down-regulates FcγRIIB both in vitro and in vivo (13, 14). FcγRIII and FcγRIIB thus represent a prototypic C5a-responsive gene pair whose simultaneous induction and suppression result in augmentation of the innate immune response.

In this work, we present the first characterization of cis-acting DNA elements in the FcγRIII and FcγRIIB 5′-flanking regions that are responsible for gene activation and inhibition by C5a and compare the data with those obtained for interferon-γ (IFN-γ), a lymphokine that has previously been established to induce and suppress gene transcription of the same two low affinity FcγR (15). We found that inverse FcγR gene regulation differs between C5a and IFN-γ with respect to time kinetics, signaling requirements, and promoter localizations. Two positive and negative acting regions, termed the C5a-inductive region (CIR) and the C5a-suppressive region (CSR), are shown to be responsible for FcγRIII induction and FcγRIIB suppression. Both the CIR and CSR contain homologous GTGAXXTCCA sequences, indicating that similar DNA motifs are involved in the transcriptional activation and inhibition of the functionally distinct FcγR.

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EXPERIMENTAL PROCEDURES

Cells and Reagents—MH-S and RAW 264.7 macrophage cell lines were cultured in RPMI 1640 medium (Biochrom AG, Berlin, Germany) supplemented with 10% fetal calf serum at 37 °C and 5% CO₂. Recombinant human C5a was from Sigma. Recombinant mouse IFN-γ/H9253 was from Roche Applied Science (Penzberg, Germany). Pharmacological inhibitors such as pertussis toxin, phosphatidylinositol 3-kinase-/H9253 (PI3K/H9253) inhibitor, PP2, and Akt inhibitor IV were obtained from Calbiochem.

Plasmid Construction—Mouse FcγRIII and FcγRIIB gene promoter fragments were amplified by PCR using FcγRIII and FcγRIIB genomic clones (kindly provided by Dr. Toshiyuki Takai, Department of Experimental Immunology, Tohoku University, Sendai, Japan) as templates and subcloned into the NheI and HindIII sites of the luciferase reporter vector pGL3-Basic (Promega Corp.). The PCR primers used for cloning of the FcγRIIIB promoter (positions 729 to +585) were 5'-ATAGCTAGCGCCCTCATAGAGAAGC-3' (sense) and 5'-CTTAAGCTTTGTGACGGAGCAGAAGTAC-3' (antisense). The PCR primers used for cloning of the FcγRIII promoter (positions -1117 to +18) were 5'-ATAGCTAGGGCCCTCATAGGAGAAGC-3' (sense) and 5'-CTTAAGCTTTGTGACGGAGCAGAAGTAC-3' (antisense). 5'- and 3'-end deletions were introduced by standard PCR mutagenesis. Point mutations were introduced using the QuikChange mutagenesis protocol (Stratagene, La Jolla, CA): GTGAAGTCCA to GTGAGTTCCA for the negative C5a-responsive region of FcγRIIB and GTGAGGTCCA to GTGAAAGTCCA for the positive C5a-responsive region of FcγRIII. All constructs were confirmed by direct DNA sequencing.

Transient Transfection and Luciferase Reporter Gene Assays—All FcγRIII and FcγRIIB promoter-reporter plasmids (0.7 µg) were cotransfected with 0.3 µg of the reference plasmid pRL-CMV into 5 × 10⁵ RAW 264.7 cells in 12-well plates using 1.6 µl of Lipofectamine TM (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions. The cells were recovered after 24 h, cultured for 24 h in 1% fetal calf serum-containing RPMI 1640 medium, and treated with 4.6 nM C5a (50 ng of recombinant human C5a) or 300 units/ml recombinant mouse IFN-γ or left unstimulated for an additional 0.5–4 h. In some experiments, cells were pretreated for 1 h with pertussis toxin (200 ng/ml) to block Gi-dependent C5aR activity (14). In additional inhibition experiments, the following reagents were used: PP2 (10 µM; inhibitor of Src kinases), PI3Kγ inhibitor (10 nM), and Akt inhibitor IV (1.25 µM). The cells were then lysed and measured for luciferase activities using the Dual-Luciferase reporter assay system (Promega Corp.). The firefly luciferase activity was normalized to Renilla luciferase activity to yield the relative promoter activity.

Statistical Analysis—Data for comparison of mean values among samples were analyzed by a two-sided unpaired Student’s t test.
RESULTS

Different Time Kinetics and Signaling Pathways in the Inverse Gene Regulation of FcγRIIB and FcγRIII by IFN-γ and C5α—To compare the promoter regions of murine FcγRIII and FcγRIIB, two reporter plasmids were constructed using FcγRIII upstream sequences from positions −1117 to +18 and FcγRIIB upstream sequences from positions −729 to +585 cloned in front of the promoterless luciferase gene. These two constructs, FcγRIII p(−1117/+18)Luc and FcγRIIB p(−729/+585)Luc, were transfected into alveolar (MH-S) and peritoneal (RAW 264.7) macrophage cell lines of mouse origin. The representative experiments in RAW 264.7 cells are shown in Fig. 1. In both RAW 264.7 and MH-S cells, FcγRIII p(−1117)Luc was induced by C5α and IFN-γ, whereas FcγRIIB p(−729)Luc was reduced by each stimulus. Optimal responses of enhanced versus suppressed FcγRIII and FcγRIIB promoter activities in transfected cells were obtained by stimulating them with C5α for 1–2 h and with IFN-γ for 4 h (Fig. 1). These data indicate that both C5α and IFN-γ activate, by different kinetics, inverse FcγR regulation in macrophages, thus confirming previous findings in FcγR mRNA and protein detection assays (13–15).

C5α and IFN-γ were also analyzed for their signaling requirements in the regulation of FcγRIII p(−1117)Luc and FcγRIIB p(−729)Luc promoter activities. In RAW 264.7 cells, the simultaneous positive and negative FcγRIII and FcγRIIB regulation by C5α, but not by IFN-γ (which, in contrast to C5α, was inhibited by the Src kinase inhibitor PP2), was sensitive to pharmacological inhibition with pertussis toxin (a general inhibitor of Gα proteins) and the PI3K- and Akt-specific inhibitors (Fig. 2). These results indicate that FcγR regulation by C5α involves the same Gα/P13K-Akt signaling complex that is activated by C5αR for triggering inflammatory cell migration and suppression of interleukin-12/23 production (14, 16). They also suggest that IFN-γ and C5α control inverse FcγR expression through different pathways.

Localization of the Positive CIR in the FcγRIII Promoter—Various 5′-deletion mutants of FcγRIII p(−1117/+18)Luc were tested for their ability to respond to C5α in comparison with IFN-γ. In RAW 264.7 cells, the property of induction by IFN-γ was constantly seen for all deletion mutants containing at least 92 bp of 5′-flanking sequence and the first 18 bp of the 5UT1/S1 exon (Fig. 3). In contrast to IFN-γ, however, induction by C5α, which was significantly detectable in FcγRIII p(−1117)Luc, was lost when an additional 570 bp of 5′-flanking sequence was deleted (Fig. 3A). These results suggest that the promoter elements that confer IFN-γ induction lie within a sequence from positions −92 to +18, the previously established region that constitutes the basal promoter of mouse FcγRIII (17, 18). They also suggest that the C5α-responsive DNA elements localize to more upstream sequences in a region...
between positions −1117 and −548. As shown in Fig. 3B, functional characterization of additional deletion mutants identified C5a responsiveness within a 48-bp sequence at positions −808 to −761 in the FcyRIII gene promoter, which we have termed the CIR.

Localization of the Negative CSR in the FcyRIIB Promoter—In both Bal17 B cells and P388D1 macrophages, it has been shown that a sequence at positions +75 to +585 is required to drive full reporter gene activity in stable FcyRIIB promoter transfectants (19). This observation, combined with the finding that FcyRIIB p(+729/+585)Luc but not FcyRIIB p(+729/+75)Luc responded to C5a and IFN-γ, prompted us to examine the presence of regulatory elements in region +75 to +585 that may contribute to FcyRIIB suppression. Several 3′-deletion mutants of FcyRIIB p(−729/+585)Luc were analyzed for their ability to negatively respond to C5a in comparison with IFN-γ. Suppression of FcyRIIB gene activity by C5a was seen in p(−729/+585)Luc and p(−729/+484)Luc, but not in the p(−729/+442)Luc deletion mutant (Fig. 4), thus indicating that C5a suppression is contained within the 43-bp sequence at positions +442 to +484, which we have termed the CSR. In contrast to C5a, suppression by IFN-γ remained detectable in

![Diagram](image)
p(−729/+75)Luc and the additional FcγRIIB p(−729/+585)Luc deletion mutant (Fig. 4). These results suggest that the regulatory elements that confer transcriptional repression by IFN-γ are differently located compared with those that mediate FcγRIIB suppression by C5a.

A Two-nucleotide Difference in the CIR and CSR Determines Induction Versus Suppression of FcγRIIB by C5a—Sequence analysis of the CSR of FcγRIIB showed no obvious homology to previously defined consensus sites of known transcription factors. Comparison with the CIR, however, revealed the existence of a DNA motif (GTGAGTCCA; here named the C5a homology element (CHE)) in the FcγRIIB CSR that is similar to the GTGAGTCCA sequence of the FcγRIII CIR with a two-nucleotide difference (Fig. 5A). To examine whether this difference in the two CHE determines the opposite functionalities of the CSR and CIR, the FcγRIII-related CHE was introduced into the FcγRIIB gene and vice versa, creating FcγRIIB p(CHE-RIII)Luc and FcγRIIB p(CHE-RIIB)Luc. When transfected into RAW 264.7 cells, both constructs achieved almost normal levels of constitutive expression, but lost any C5a responsiveness (Fig. 5B), indicating that an intact GTGAXYCCA CHE motif is critical for both activation and repression by C5a. In addition, it appears that the two CHE act in an FcγR gene context-specific manner because they are active in transferring neither induction nor suppression from one gene to the other.

DISCUSSION

Our analysis of the murine FcγRIII and FcγRIIB gene regulatory regions suggests different mechanisms of transcriptional control by IFN-γ and C5a. Within the promoter of the FcγRIII gene, we have confirmed that the 92 bp upstream from the transcription initiation site are sufficient to drive reporter gene activity in macrophage cell lines and to mediate PP2-sensitive IFN-γ activation. Furthermore, we have defined a distal region responsible for C5a induction as the 48 bp at position −808 to −761, called the CIR. The CIR acts as a transcriptional activator in the presence of C5a, but not IFN-γ. The 5′-flanking region of FcγRIIB contains, in addition to upstream sequences from the start of transcription, two noncoding 5UT1 and 5UT2 exons. Constitutive FcγRIIB activity in macrophages depends on intronic sequences downstream of the 5UT2 exons. We define the region responsible for C5a suppression, the CSR, as the 43 bp within this intronic domain, from positions +442 to +484. Moreover, the CSR localizes apart from the negative IFN-γ-responsive region, and transcriptional repression of FcγRIIB by C5a involves the Gα-PI3K-Akt pathway of C5aR signaling.

Previous work on the intracellular signaling mechanisms of C5aR suggested that, in addition to the Gα-PI3K-Akt cascade, other kinase pathways that lead to activation of NF-κB, cAMP-responsive element-binding protein (CREB), and STAT3 (signal transducer and activator of transcription)
can be activated by C5aR (20–23). Although the CSR acts as a transcriptional repressor in the presence of C5a, it appears not to involve binding of NF-κB, CREB, and STAT3. This is suggested by the lack of any consensus sites for these transcription factors within the CSR. Examination of the CSR reveals, however, the presence of a DNA motif that is similar to the CHE in the CIR of FcγRIII. For the FcγRIIB and FcγRIII promoters, we have found that a single two-nucleo-
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otide difference in the two CHE is responsible for the selectivity of induction versus suppression by C5a.

Recent studies elucidating the synergy by which complement and FcγR trigger inflammatory processes and immunopathological events provide us with a better understanding of the role of C5a as a critical amplifier of FcγR-mediated responses through induction of FcγRIII and suppression of FcγRIIB (7–9, 13, 14, 24–27). The results from our study suggest that C5a uses similar DNA motifs (defined as GTGAXXTCCA, the CHE) in both pathways of transcriptional induction and suppression of FcγRIII and FcγRIIB. To our knowledge, this is the first work that examines the molecular events of both the positive and negative gene regulatory capacity of C5a at the promoter level. It may thus form the basis for future work on the isolation and characterization of the relevant CHE-binding factors that, as indicated here, are likely not related to the NF-κB and CREB transcription factors.

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