The anaphylatoxin C3a is a proinflammatory mediator generated during complement activation. The tight control of C3a receptor (C3aR) expression is crucial for the regulation of anaphylatoxin-mediated effects. Key factors regulating constitutive expression of the C3aR in the mast cell line HMC-1 and receptor induction by dibutyryl-cAMP in monomyeloblastic U937 cells were determined by functional characterization of the C3aR promoter. Nucleotides −18 to −285 upstream of the translational start site proved to be critical for promoter activity in HMC-1 cells. Binding sites for the transcription factors AP-1 and Ets could be located. Overexpressed c-Jun/c-Fos (AP-1) and Ets-1 led synergistically to increased promoter activity that was substantially reduced by site-directed mutagenesis of the corresponding elements within the C3aR promoter. In HMC-1 cells, Ets interacted directly with the predicted binding motif of the C3aR promoter as determined by electromobility shift assays. AP-1 binding to the C3aR promoter was augmented during C3aR induction in U937 cells. A retroviral gene transfer system was used to express a dominant negative mutant of Ets-1 in these cells. The resulting cells failed to up-regulate the C3aR after stimulation with dibutyryl-cAMP and showed decreased AP-1 binding, suggesting that Ets acts here indirectly. Thus, it was established that Ets and the AP-1 element mediates dibutyryl-cAMP induction of C3aR promoter activity, hence providing a mechanistic explanation of dibutyryl-cAMP-dependent up-regulation of C3aR expression. In conclusion, this study demonstrates an important role of AP-1 and a member of the Ets family in the transcriptional regulation of C3aR expression, a prerequisite for the ability of C3a to participate in immunomodulation and inflammation.

The complement system is activated in a variety of diseases (1–3) and leads to the generation of the anaphylatoxic peptide C3a. C3a is a proinflammatory mediator specific to its heptahelical receptor

8 This work was financed by Deutsche Forschungsgemeinschaft Grant SFB 566/A04. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed. Tel.: 49-511-532-4342; Fax: 49-511-532-4336; E-mail: Klos.Andreas@mhh-hannover.de

2 The abbreviations used are: C3a, anaphylatoxic peptide derived from complement factor C3; C3aR, C3a receptor; C5a, anaphylatoxic peptide derived from complement factor C5; C5aR, C5a receptor, CD88; AP-1, transcription factor activator protein-1; wt, wild type; AP-1wt, double-stranded consensus oligonucleotide representing the AP-1 wild type sequence and recognition motif; AP-1mut, double-stranded consensus oligonucleotide with a point mutation within its AP-1 binding motif; AP-1/C3aRwt, double-stranded oligonucleotide representing part of the C3aR promoter including the putative AP-1 wild type sequence and recognition motif; AP-1/C3aRmut, double-stranded oligonucleotide representing part of the C3aR promoter with a point mutation within its putative AP-1 binding motif; Bt,cAMP, dibutyryl-cAMP; EMSA, electromobility shift assay; Ets, transcription factor family E26 transcription-specific; Ets-1, transcription factor E26-transformation specific-1; Ets-1DN, dominant negative mutant of Ets-1; Ets-1wt, double-stranded consensus oligonucleotide representing the Ets-1 wild type sequence and recognition motif; Ets-1mut/ Ets-1mut2, double-stranded consensus oligonucleotide with point mutations within their Ets-1 binding motif; Ets-C3aRwt, double-stranded oligonucleotide representing part of the C3aR promoter including the putative Ets-1 wild type sequence and recognition motif; Ets-C3aRmut, double-stranded oligonucleotide representing part of the C3aR promoter with a point mutation within its putative Ets-1 binding motif; MfLP, formylmethionylleucylphenylalanine; Ha-Ras, small GTP-protein Harvey-Ras; HD293, human embryonic kidney cells; HMC-1, human mast cell line; NC, negative control oligonucleotide; TPA, phorbol ester 12-O-tetradecanoylphorbol 13-ace- tate; U937, human monocyt-like cell line; TIS, transcription initiation or start site; SEAP, secreted alkaline phosphatase; RT, reverse transcription; UTR, untranslated region; DN, dominant negative.
surrounding blood vessels (27). Finally, in experimental allergic encephalitis the C3aR was elevated on microglia, infiltrating monocytes/macrophages, and astrocytes (28). C3aR regulation can also be observed in other tissues: In ovalbumin- or lipopolysaccharide-challenged mice, C3aR expression increased on bronchial smooth muscle cells (29). In patients suffering from atopic dermatitis or allergic contact dermatitis, C3aR expression can be detected in CD4+ and CD8+ T-cell clones, cell types that normally do not express this receptor (6, 30). Corresponding to the regulation in situ, the C3aR can be induced in cell culture by Bt-cAMP or interferon-γ on monomyeloblastic cell lines such as U937 (31).

The aim of the current study was to obtain a deeper understanding of the regulation of the human C3aR, in particular, its transcriptional control, which has never been investigated before. Constitutive expression of the C3aR in the mast cell line HMC-1 (32) and receptor induction by Bt-cAMP (and TPA) in monomyeloblastic U937 cells (33) were investigated. The transcription initiation site was identified. The essential part of the C3aR promoter was narrowed down by 5’ and 3’ deletion analysis. Detailed characterization by various methods revealed a crucial role for the transcription factors activator protein-1 (AP-1) and E26-transformation specific (Ets) and their corresponding binding sites within the C3aR promoter. Ets seems to act directly on the C3aR promoter but also indirectly when receptor expression is induced.

MATERIALS AND METHODS

All reagents were obtained from Sigma if not otherwise indicated.

Cell Lines and Cell Culture Conditions—The human mast cell line HMC-1 (kindly provided by J. H. Butterfield) and human monomyeloblastic U937 cells (ATCC CRL 1593) were cultured in RPMI 1640 medium (Biochrom AG, Berlin, Germany) at 37 °C, 5% CO2. The human embryonic kidney cell line HEK293 (ATCC CRL 1573) (4) was cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (Biochrom AG). Both media were supplemented with 10% (v/v) fetal calf serum (Sigma).

Transient Transfection of HMC-1 Cells and Reporter Gene Assay—Human C3aR promoter fragments were generated by PCR using the pDP1 plasmid as template (34) and subcloned in between the Nhel and HindIII site of pSEAP2-E (Clontech, Heidelberg, Germany). Primer sequences can be obtained from the corresponding author. Point mutations were introduced using the QuikChange mutagenesis protocol (Stratagene, La Jolla, CA): TATAAA to GCTAAA for the TATA box, CAGGAAG to CAAGAAG within the C3aR promoter. Ets seems to act directly on the C3aR promoter but also indirectly when receptor expression is induced.

Generation of a Dominant Negative Mutant of Ets-1 and Its Transduction into U937 Cells—A retroviral gene transfer system was applied to generate U937 cells expressing a dominant negative mutant of Ets-1. It consists of amino acids 306–441 of chicken Ets-1 (accession number M22462) and is, thus, lacking the transactivation domain (35).

RNA Preparation and cDNA Synthesis—Total RNA was isolated from cell cultures using TRIzolTM reagent (Invitrogen) according to the manufacturer’s protocol. Five µg of total RNA was transcribed by SuperScript II RNase H− reverse transcriptase from Invitrogen using an oligo(dT) primer (Stratagene).

Detection of the Transcription Initiation Site by RT-PCR—cDNA (2 of 60 µl) was generated using a reverse antisense primer (CTGCATCT-TCAAGCCAGGC) hybridizing to the coding region of the C3aR for C3aR synthesis. The resulting cDNA was applied as template in PCR reactions, combining this reverse antisense primer with a whole set of sense primers that anneal to the C3aR 5’-UTR. The amplified genomic DNA fragments could be excluded because they would additionally contain the 6-kilobase intron. The construct pSEAP2-E308-hC3aR-234, artificially combining part of the promoter region (308 bp) with part of the coding region of the C3aR, was used as a positive control. The primer positions upstream from the start codon were: 104–85 for P1, 141–121 for P2, 159–141 for P3, 190–172 for P4, 215–196 for P5, 230–211 for P6, 240–221 for P7, 253–233 for P8, 269–249 for P9, and 286–267 for P10.

Nuclease Protection Assay—3, 10, and 30 µg of RNA were used in the nuclease protection assay (Ambion Inc., Austin, TX). Thirty µg of yeast RNA served as the negative control. A probe representing part of exon 1 of the C3aR (position 444 to 426) was generated by PCR using a [α-32P]dATP-labeled oligonucleotide (CTGCCTGTCTGGTCCCCACCACTTAG; 426 to 264) as the reverse antisense primer and a biotinylated sense primer (444 to 425). Genomic sequence (ligated into the plasmid DP1) served as the template. The sense primer was removed using streptavidin-coupled Dynabeads (Dynal/Invitrogen). The RNA was hybridized overnight at 43 °C followed by nuclease treatment according to the manufacturer’s instruction. After digestion, the protected fragments were separated in a 6% denaturing polyacrylamide gel followed by 3 weeks of exposure of the x-ray film (Kodak X-Omat Ar35 × 43).

C3aR Real-time RT-PCR—TaqMan real-time RT-PCR was performed using a C3aR-specific TaqMan® gene expression assay (HS00377780-m1) in combination with the TaqMan® Universal PCR Master Mix and the 7000 Sequence Detection System for analysis (all Applied Biosystems, Weiterstadt, Germany). For normalization, the housekeeping gene RPS9 was used. Standard curves were generated using serially diluted cDNA. For each sample, the -fold induction was calculated as the ratio of the normalized volume equivalent of the transduced cells to the normalized volume equivalent of the corresponding mock-transfected cells.

Competitive 125I-Labeled C3a and C5a Binding Studies—Competitive binding studies with human 125I-labeled C3a or C5a (PerkinElmer Life Sciences) as tracer and increasing concentrations of cold C3a or C5a were performed as previously described (6).

Fura2 Assay for the Determination of Free Cytosolic Ca2+ in Bt-cAMP-induced U937 Cells—This assay was performed exactly as previously described (6, 31).

Electromobility Shift Assay (EMSA)—All oligonucleotides were annealed in equimolar amounts and end-labeled by T4 polynucleotide kinase with [γ-32P]ATP followed by desalting through G-50 Micro Columns (Amersham Biosciences). The following oligonucleotides were used for gel shift experiments: AP-1wt/AP-1mut, Gelshift AP-1 family kit (Active Motif, Rixensart, Belgium); AP-1-C3aRwt (position −233 to 41).
reverse antisense primer hybridizing to the coding region of the C3aR (Fig. 1, lower panel). Strong amplified fragments (P1–P4 in Fig. 1) became apparent using primer combinations with sense primers binding within 185 bp of the 5′-UTR relative to the start codon. Primer combinations with sense primers annealing further upstream resulted in weaker but still visible PCR products (P5–P10 in Fig. 1), suggesting an additional minor transcription start site located upstream of position −280. A vector containing the C3aR 5′-UTR (cloned from genomic DNA) in combination with part of the coding region of the C3aR served as positive control. As expected, amplified fragments resulted with all primer pairs (not shown).

Identification of Regulatory Regions by 5′ and 3′ Deletion of the C3aR 5′-UTR in HMC-1 Cells—To gain further insights into the modes of regulation of the C3aR, the minimal transcriptional unit of the C3aR promoter was mapped, and key regulatory elements within this promoter directing basal gene expression were identified. HMC-1 cells constitutively expressing the C3aR (and thus, exhibiting all necessary cofactors) were used in this deletion analysis. The gene of the human C3aR is located on chromosome 12p13. A single 6-kilobase intron is present 17 bp upstream of the ATG initiation codon of exon 2 (34). A 2-kilobase region of the C3aR 5′-UTR located directly upstream of the intron was analyzed by SEAP reporter gene assay (Fig. 2a). For this purpose, a whole set of overlapping fragments of the C3aR 5′-UTR was generated by PCR and cloned into the pSEAP2-Enhancer vector, as indicated in the corresponding figures (Fig. 2, b–e). In all figures and throughout the paper, all nucleotide positions are given relative to the ATG start codon. The names of the constructs indicate the lengths of the analyzed fragments. In all experiments pCMV-β expressing β-galactosidase was cotransfected and used for normalization. All reporter gene experiments were performed at least n = 3 times and repeated with an independent clone of each construct, leading essentially to the same results.

The long C3aR reporter gene constructs showed elevated SEAP activity compared with the pSEAP2-E control vector without insert. Progressive 5′ deletions of the C3aR 5′-UTR from position −1921 to position −285 (with a common 3′-end at position −18) did not significantly affect SEAP expression in HMC-1 cells (Fig. 2b). Additionally, a whole set of similar constructs with a 5′-end in between position −1921 and −285 was analyzed, demonstrating similar promoter activity (data not shown). Thus, although in principle possible, it is unlikely that there are positive and negative regulatory elements within this region whose effects can cancel each other. Hence, the region upstream of position −285 does not most likely contribute to promoter activity in HMC-1 cells. However, different results might be obtained in other cell lines or if other stimuli are present.

In subsequent experiments the pSEAP2-E267 construct representing nucleotide −285 to −18 of the C3aR 5′-UTR was usually used as internal standard. For better comparison, the promoter activity of this construct was defined as 100% (usually, the corresponding SEAP activity was in the range of 50,000–200,000 cps, and the activity of β-galactosidase used for normalization was in the range of 0.15–0.25 A950 nm). Further truncation of the 5′-end down to position −180 represented by the construct pSEAP2-E162 yielded a drastic reduction of promoter activity (Fig. 2c). Therefore, the region distinguishing the constructs 267 and 162, i.e., position −285 to −180, must exhibit positive regulatory promoter function.

To localize and identify positive regulatory elements positioned in this region, bioinformatic analysis using the MatInspector® Professional Software (Version 6.2) was performed. It led to the prediction of consensus transcription factor binding sites, including a putative AP-1

### RESULTS

C3aR Transcription Initiation Site Determined by Nuclease Protection Assay and RT-PCR—To localize the transcription initiation site (TIS in Fig. 1 and 2a) of the C3aR promoter, total RNA from HMC-1 cells was analyzed by nuclease protection assay. A single band at position −185 relative to the start codon became visible in the radiography (Fig. 1, upper panel), indicating the (major) transcription initiation site.

The RNA was additionally analyzed by RT-PCR, combining a whole set of sense primers annealing to the C3aR 5′-UTR with one common

![Diagram](image-url)
element centered at position −225 and an adjacent Ets site centered at position −207. Hence, one additional construct was generated excluding the AP-1 site but still including the Ets binding motif (pSEAP2-E197). Its activity was drastically reduced. However, promoter activity of this fragment containing the putative Ets site was still higher than those of pSEAP2-E162 (Fig. 2c). These data suggest that the AP-1 and the Ets binding motifs play a prominent role in C3aR regulation.

Promoter activity also decreased due to stepwise 3′ deletion (pSEAP2-E-206, -142, and -125 in Fig. 2d). Thus, the region from position −160 to −18 also exhibits positive regulatory elements and participates in constitutive C3aR expression. The construct pSEAP2-E162 had no promoter activity (Fig. 2c). Hence, most likely the positive regulatory elements spanning −160 to −18 can only act in the context of additional promoter regions further upstream, present in pSEAP2-E-267, -206, -142, or -125, respectively.

**TATA-less Promoter**—The promoters of G-protein-coupled receptors are often TATA-less. Two putative TATA boxes are located close to the major transcription initiation site in the C3aR promoter (position −185; see above). To clarify experimentally whether these TATAA sequences represent active boxes or not, reporter gene constructs with

---

**FIGURE 2.** Deletion analysis of the C3aR promoter (b–d) and mutations of putative TATA-boxes (e), analyzed by SEAP reporter gene assays in HMC-1 cells, indicate positive regulatory promoter elements and a TATA-less promoter. The genomic organization of the human C3aR (hC3aR), the predicted cis-acting elements (MatInspector®Software) for AP-1 and Ets, and two putative TATA-boxes are depicted in the upper part of this figure (a). A ~2-kilobase region of the promoter located in exon one was stepwise deleted from the 5′-end (b and c), and the 3′-end (d). Two putative TATA boxes, TATA1 centered at −275 and TATA2 centered at −351, were deleted either as separate mutations (mutTATA1 and mutTATA2) or combined (mutTATA1/2) (e). Nucleotide positions are given relative to the start codon (+1). The horizontal lines at the left represent graphically the part of the C3aR promoter that has been ligated into the pSEAP2-E reporter gene vector. The names of the constructs indicate the length of the analyzed part of the C3aR promoter. For normalization, a plasmid expressing β-galactosidase under the control of the CMV promoter was cotransfected. On the right side of b–e, the normalized SEAP activity determined in the supernatant 3 days after transient transfection is depicted as the mean ± S.D. of n = 3 (b–d) and n = 4 (e) independent experiments (and duplicates within each experiment), respectively. For better comparison, the C3aR promoter activity of construct pSEAP2-E267 was defined as 100%. ORF, open reading frame; TIS, transcription initiation site; kb, kilobases.
induced by AP-1 or Ets-1 alone (Fig. 3) with the presence of Ha-Ras) yielded higher SEAP activity than the activity struct. The simultaneous cotransfection of c-Jun, c-Fos, and Ets-1 (in HMC-1 cells were transfected with the pSEAP2-E267 wild type construct. As demonstrated above, overexpressed Ets-1 did not increase promoter activity of the wild type C3aR promoter or mutants of the predicted AP-1 and Ets site, centered at position −225 and −207, respectively. The depicted results are the mean ± S.D. of n = 3 independent experiments (and duplicates within each experiment).

Characterization of AP-1 and Ets Binding Sites within the C3aR Promoter Determined in HMC-1 Cells—In the following experiments the role of the above mentioned putative cis-acting elements for AP-1 and Ets was elucidated. First, we overexpressed the corresponding transcription factors in HMC-1 cells by transient transfection and analyzed them by SEAP reporter gene assay. SEAP expression in the absence of exogenous transcription factors and Ha-Ras was defined as 100%. The small GTP-binding protein Ha-Ras, which is needed for the activation of AP-1 and Ets, was partially coexpressed, as indicated in Fig. 3, a–c. Moreover, the AP-1 and Ets binding motifs in the reporter gene construct pSEAP2-E267 were disrupted by site-directed mutagenesis. Simultaneous coexpression of the pSEAP2-E267 wild type promoter construct with c-Jun, c-Fos, and Ha-Ras yielded a strong, more than 10-fold increase of SEAP expression (Fig. 3a). In the same setting, the construct with the mutated AP-1 binding site showed only a slight increase of reporter gene activity. The individual transcription factor subunits of AP-1 (c-Jun and c-Fos in this study), just as Ha-Ras alone, increased the promoter activity ∼3-fold. c-Jun, but not c-Fos, can form homodimers. Accordingly, the combined overexpression of c-Jun and Ha-Ras yielded a 5-fold enhancement of promoter activity. Hence, these data clearly show that c-Jun/c-Fos (or other members of the AP-1 family) can induce C3aR promoter activity and demonstrate the position of the consensus AP-1 element.

Similar experiments were performed for the Ets cis-acting element within the C3aR promoter. Therefore, Ets-1 was overexpressed as a prototypical member of the large Ets transcription factor family exhibiting the unique winged helix-turn-helix motif known as the Ets domain. As depicted in Fig. 3b, coexpression of Ets-1 and Ha-Ras strongly elevated promoter activity of the pSEAP2-E267 wild type construct. As expected, expression of Ets-1 or Ha-Ras alone was much less effective. The corresponding reporter gene construct with a mutation in the Ets binding motif showed decreased basal activity, which only modestly increased in the presence of overexpressed Ha-Ras and/or Ets-1. These data demonstrate and localize in HMC-1 cells a cis-acting element for an Ets transcription factor within the C3aR promoter.

To check whether AP-1 and Ets cooperate in C3aR regulation, HMC-1 cells were transfected with the pSEAP2-E267 wild type construct. The simultaneous cotransfection of c-Jun, c-Fos, and Ets-1 (in the presence of Ha-Ras) yielded higher SEAP activity than the activity induced by AP-1 or Ets-1 alone (Fig. 3c). For a detailed analysis of this cooperation, the mutants of the Ets and of the AP-1 binding motif, respectively, were additionally examined. Similar to other figures depicting results of reporter gene assays, 100% reporter activity is also defined here by the SEAP expression of the pSEAP2-E267 wild type construct. As demonstrated above, overexpressed Ets-1 did not increase promoter activity of the Ets binding site mutant, and overexpression of AP-1 did not result in higher promoter activity of the mutant with the destroyed AP-1 motif. Intriguiningly, overexpression of Ets-1 caused only a slight increase in promoter activity of the AP-1 binding site mutant and vice versa. Overexpressed AP-1 resulted in an augmentation of enzyme activity, which was much smaller than that which can be observed on the wild type C3aR reporter gene construct.

Hence, we have identified a consensus AP-1 transcription factor binding site centered at position −225 and a consensus Ets binding element centered at position −207. Our data strongly suggest that the two corresponding transcription factors cooperate in the activation of the C3aR promoter.

Up-regulation of C3aR Transcription in HEK293 Cells Transiently Transfected with AP-1 and Ets-1—We wanted to clarify whether AP-1 and Ets do not only up-regulate promoter activity but demonstrate that

![Figure 3](image-url)
they can actually increase C3aR transcription. Hence, C3aR-mRNA was quantified when the two transcription factors were coexpressed in cells that normally do not express the C3aR. In these experiments on HEK293 cells, Ha-Ras was always cotransfected. On day 3, mRNA was isolated. The level of C3aR-mRNA was determined by TaqMan® realtime RT-PCR; the housekeeping gene RPS9 was used for normalization.

Compared with HEK293 cells, which only expressed Ha-Ras, a significant increase of C3aR-mRNA was observed when Ets-1 was additionally transfected or when Ets-1 and AP-1 were additionally coexpressed (Fig. 4). Moreover, the increase of C3aR-mRNA was significantly higher when AP-1 and Ets-1 were cotransfected as compared with the expression of only one of these two transcription factors. Additionally, in accordance with the data obtained in the reporter gene assays, an up-regulatory effect of AP-1 on C3aR transcription could only be seen when Ets-1 was simultaneously expressed. Thus, AP-1 and Ets can up-regulate the level of C3aR-mRNA, i.e. C3aR transcription.

Investigation of C3aR Induction and Signaling in U937 Cells Expressing a Dominant Negative Mutant of Ets-1—To elucidate the role of Ets in C3aR induction, the up-regulation of the anaphylatoxin receptor was investigated when this transcription factor was functionally inactivated by a dominant negative mutant (Ets-1DN) lacking the transactivation domain. A retroviral gene transfer and expression system (RetroX, Clontech®) had to be used for that purpose because U937 cells cannot be transiently transfected. Using the retroviral expression vector pQCXIN, Ets-1DN under the control of a CMV-promoter, and the neomycin resistance gene were cotranslated in mammalian cells via the internal ribosomal entry site (IRES) from a bicistronic message (Fig. 5a). Control virus was obtained from the same construct, lacking Ets-1DN.

After selection with G418, a transduction/expression efficiency of 15-fold down to 2-fold (data not shown).

As expected, 3 days of Bt2cAMP treatment led to a strong increase of C3aR expression in control vector-transduced U937 cells. In contrast, in three independent competitive binding studies, specific 125I-labeled C3a binding was almost completely diminished in Bt2cAMP-treated U937 cells stably expressing Ets-1DN. The residual binding was only slightly higher than the minimal one, which could be observed in non-treated cells (Fig. 5b). Corresponding to that, only a slight increase of Ca2+ signaling became apparent in the Bt2cAMP-treated U937—Ets-1DN cells when 40 nM C3a were applied (Fig. 5d). Control cells transduced with pQCXIN and induced with Bt2cAMP showed a normal C3a response in this assay.

The increase of C3aR-mRNA determined by TaqMan® real-time RT-PCR after 72 h of induction with Bt2cAMP is in good accordance with the results of the binding studies and the functional Ca2+ assay (demonstrating an inhibition of C3aR up-regulation). Expression of the dominant negative mutant (Ets-1DN) strongly diminished C3aR transcription in U937 cells in 3 experiments from −15-fold down to −2-fold (data not shown).

To check how specific the effects of the Ets-1 dominant negative mutant on receptor regulation are, the C5a receptor (C5aR, CD88) and fMLP receptor were also analyzed (37). These G-protein-coupled receptors are closely related to each other and to the C3aR, exhibiting overlapping functions. Both receptors can also be induced by Bt2cAMP on U937 cells. Their activation causes a strong Ca2+ response. In contrast to the observations on the C3aR, there was almost no difference in the Ca2+ response of Bt2cAMP-induced U937 cells due to the expression of Ets-1DN applying C5a or fMLP as stimulus (Fig. 5, e and f). In accordance with that, 125I-labeled C5a binding of Bt2cAMP-treated U937 cells decreased only partially in the presence of Ets-1DN (Fig. 5c). Most likely, the remaining number of C5aR binding sites is still sufficient to reach an almost normal Ca2+ response. Hence, our results clearly show that induction of C3aR expression in U937 cells by Bt2cAMP is strictly dependent on Ets. Additionally, our data suggest that the regulation of the C5aR or the fMLP receptor is at least partially different from that of the C3aR, i.e. the induction of these receptors is less dependent on the Ets transcription factor family.

Binding of AP-1 to the C3aR Promoter; Determined by Electromobility Shift Assay—To confirm the presence of nuclear/transcription factors capable of binding to the AP-1 site centered at position −225, EMSAs were carried out using nuclear extracts prepared from U937 cells and a radiolabeled double-stranded oligonucleotide probe spanning nucleotides −213 to −233 of the C3aR promoter (AP-1–C3aRwt). Additionally, a commercial double-stranded AP-1 control oligonucleotide served also as radiolabeled probe (AP-1wt). For competition the same nonlabeled oligonucleotides and their derivatives with specifically mutated AP-1 binding motifs were coinubicated (AP-1–C3aRmut and AP-1mut).

As depicted in Fig. 6a, incubation of the radiolabeled AP-1–C3aRwt probe with nuclear extracts from native (0 h) and induced U937 cells (2, 6, 24, or 48 h of Bt2cAMP) resulted in the formation of radiolabeled nuclear factor-DNA complexes. At least three bands could be distinguished, most likely representing the binding of dimers consisting of different monomers of the Fos, Jun, or activating transcription factor subfamilies. This phenomenon is well known for AP-1 binding (38). The nuclear factor-DNA complexes could be efficiently competed by an excess of nonlabeled double-stranded AP-1wt. The formation of the radiolabeled complexes could not be competed by the oligonucleotide AP-1mut, demonstrating the specificity of the observed AP-1 binding to the C3aR promoter (left part of Fig. 6a). In three independent experiments, the signal intensity of the radiolabeled nuclear factor-DNA complexes clearly increased during the time course of the experiment, as
FIGURE 5. a, plasmid map of the retroviral expression vector pQCXIN-Ets-1DN. Shown are competitive binding studies with $^{125}$I-labeled C3a (b) and $^{125}$I-labeled C5a (c) and determination of the Ca$^{2+}$ response to C3a (d), C5a (e), or FMLP (f) in the Fura2-AM assay on native and Bt$_2$cAMP-induced U937 cells expressing a dominant negative mutant of Ets-1 (Ets-1DN). α, the dominant negative mutant of Ets-1 was ligated downstream from a CMV promoter into a NotI restriction site. In eukaryotic cells, a neomycin resistance gene for G418 selection was cotransfected from a bicistronic message via the internal ribosomal entry site (IRES). α+ indicates the packaging signal. The long terminal repeats (LTRs) are derived from hybrids of CMV/murine sarcoma virus (MSV) or the Moloney murine leukemia virus (MoMuLV). Specifically, b–f, human monomyeloblastic U937 cells that were either transduced with pQCXIN control vector or pQCXIN-Ets-1DN were incubated for 3 days with 500 μM Bt$_2$cAMP or buffer, respectively. α + c, competitive binding curves were obtained applying tracer amounts of $^{125}$I-labeled C3a or $^{125}$I-labeled C5a and competing with increasing amounts of nonlabeled C3a or C5a, respectively. d–f, the increase of free cytosolic Ca$^{2+}$ as response to 40 nM C3a, 20 nM C5a, or 1 μM FMLP was determined using the fluorescence indicator Fura2. The result of one representative of n = 3 independent experiments (and duplicates (b + c) and quadruplicates (d–f) within the experiment, respectively) with similar outcome is depicted.

FIGURE 6. EMSA demonstrating the up-regulation of functional AP-1 and binding to the C3αR promoter in U937 cells during induction with Bt$_2$cAMP (a) or with the phorbol ester TPA (b) as well as the binding of AP-1 to the C3αR promoter in HMC-1 cells (c). a, b, and c, nuclear extracts (NE) of U937 cells induced with Bt$_2$cAMP for 0, 2, 6, 24, and 48 h (a) or TPA (48 h, 10 nM) and native cells (b), was incubated with a radiolabeled double-stranded oligonucleotide representing part of the C3αR promoter, in particular an AP-1 binding motif centered at position −225 (c$^{32}$P-labeled AP-1-C3αRwt). Nuclear factor-DNA complex formation was competed with an excess of nonlabeled double-stranded oligonucleotides as indicated: AP-1wt, AP-1mut (both of them 500-fold), AP-1-C3αRwt, and AP-1-C3αRmut (5-, 50-, and 500-fold) for α, and AP-1-C3αRwt as well as AP-1-C3αRmut (500-fold) for b. α, the shift of radioactivity (i.e., binding of AP-1 to the labeled probe) was measured using phosphorimaging and quantified by computer analysis. After subtraction of the background, the relative band intensities without competition for 0, 2, 6, 24, and 48 h were 1, 3, 7, 8.5, and 6.1. c, nuclear extract from HMC-1 cells was incubated either with c$^{32}$P-labeled AP-1-C3αRwt or with a radiolabeled consensus AP-1 oligonucleotide (AP-1wt) as probe. Nuclear factor-DNA complex formation was competed with a 500-fold excess of nonlabeled double-stranded oligonucleotides as indicated: AP-1wt, AP-1mut, AP-1-C3αRwt. In all three EMSA, the oligonucleotides exhibiting the AP-1 consensus motif could not compete, demonstrating the specificity of the interaction. The results of one of two independent experiments are depicted.
AP-1 and Ets as Regulators of C3a Receptor Expression
determined by phosphorimaging and subsequent computer analysis (Quantity One 4.5.0 software, Bio-Rad).

To further characterize the interaction of the C3aR promoter with AP-1, the radiolabeled AP-1-C3aRwt probe was also competed with the corresponding nonlabeled oligonucleotide and its mutated counterpart using nuclear extracts from U937 cells stimulated for 48 h with Bt$_2$cAMP (right part of Fig. 6a). As expected, the radiolabeled nuclear factor-DNA complexes could be specifically competed by the nonlabeled AP-1-C3aRwt oligonucleotide but not by the otherwise identical oligonucleotide harboring the mutated AP-1 site (AP-1-C3aRmut).

Similar results were obtained using the radiolabeled AP-1 probe (AP-1wt) and, as competitor, AP-1wt, AP-1mut, AP-1-C3aRwt, or an irrelevant double-stranded oligonucleotide serving as negative control. The results confirmed the up-regulation of a nuclear factor binding to the AP-1 cis-acting element in U937 cells due to Bt$_2$cAMP treatment and the ability of this factor to bind specifically to the C3aR promoter (data not shown).

In HMC-1 cells, which constitutively express the C3aR, a similar nuclear factor-DNA complex resulted when the radiolabeled AP-1-C3aRwt probe was incubated with nuclear extract. This complex could be specifically competed by the nonlabeled AP-1wt oligonucleotide (and the nonlabeled AP-1-C3aRwt oligonucleotide) but not by AP-1mut (left part of Fig. 6c). The same pattern resulted when AP-1wt served as the radiolabeled probe (right part of Fig. 6c). Intriguingly, in HMC-1 cells, the nuclear factor-DNA complex consisted of one prominent band that showed a different electrophoretic mobility compared with the prominent bands observed in U937 cells. This observation suggests that different members of the AP-1 family bind to the C3aR promoter in the context of HMC-1 or U937 cells, respectively.

AP-1 was also found as a transcription factor that mediates gene induction by the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA or PMA) and, hence, the alternative name TPA response element for the recognition site of AP-1. TPA is an activator of protein kinase C. Moreover, it induces up-regulation of the C3aR-mRNA in U937 cells as demonstrated by Northern blot analysis (27). As expected, when radiolabeled AP-1-C3aRwt was applied as probe, one main nuclear factor-DNA complex could be detected in the EMSA using nuclear extract (abbreviated as NE in the figure) from TPA induced U937 cells (right part of Fig. 6b). The complex could be efficiently competed by an excess of nonlabeled AP-1-C3aRwt oligonucleotide but not with the corresponding oligonucleotide exhibiting a mutation in its AP-1 site (AP-1-C3aRmut). Using the same amount of nuclear extract from native U937 cells, such a complex was not detectable (left part of Fig. 6b).

As described above, C3aR induction was suppressed in U937 cells by the transduced Ets-1DN. To check whether the up-regulation of functional AP-1 is indirectly affected by the dominant negative mutant of Ets-1, EMSAs were performed incubating radiolabeled AP-1-C3aRwt as probe with nuclear extract from the corresponding U937 cells. In this experiment two different modes of induction were compared; 48 h of incubation with Bt$_2$cAMP (right part of Fig. 6a). As expected, the radiolabeled nuclear factor-DNA complexes were specifically competed by the nonlabeled AP-1-C3aRwt oligonucleotide but not by the otherwise identical oligonucleotide harboring the mutated AP-1 site (AP-1-C3aRmut).

Similar results were obtained using the radiolabeled AP-1 probe (AP-1wt) and, as competitor, AP-1wt, AP-1mut, AP-1-C3aRwt, or an irrelevant double-stranded oligonucleotide serving as negative control. The results confirmed the up-regulation of a nuclear factor binding to the AP-1 cis-acting element in U937 cells due to Bt$_2$cAMP treatment and the ability of this factor to bind specifically to the C3aR promoter (data not shown).

In contrast to the strong band caused by Ets-1DN, the intensity of the weak bands was the same in U937 cells transduced with pQCXIN-Ets-1DN or with the negative control virus (left two thin arrows of Fig. 7a). These nuclear factor-DNA complexes could reflect the participation of endogenous transcription factors. Indeed, the Ets-1DN-DNA complex as well as the endogenous nuclear factor-DNA complexes bound to the radiolabeled double-stranded Ets-1wt probe could be efficiently competed by a 50-fold excess of nonlabeled double-stranded Ets-1wt oligonucleotide. A double-stranded oligonucleotide probe spanning nucleotides 196 to 219 of the C3aR promoter including the predicted Ets site (Ets-C3aRwt) could also compete the three complexes. However, a 500-fold excess of nonlabeled Ets-C3aRwt oligonucleotide was needed to achieve complete competition. Proving specificity, an irrelevant negative control oligonucleotide (NC) as well as the corresponding oligonucleotide with a specific mutation within the putative Ets binding element (Ets-C3aRmut) did not compete at all (Fig. 7a).

AP-1 was up-regulated during Bt$_2$cAMP induction of C3aR expression. To check whether Ets-1 is similarly up-regulated during C3aR induction, nuclear extracts of U937 cells induced with Bt$_2$cAMP (2, 6, 24, or 48 h) were compared with those of buffer-treated cells. In contrast to the results obtained for AP-1, no increase of the radiolabeled nuclear factor-DNA complex could be observed using Ets-1wt as probe (data not shown). Thus, even in U937 cells induced with Bt$_2$cAMP, only a small amount of nuclear factor interacting with the Ets-1 consensus binding motif is present. This factor, most likely Ets-1, specifically interacts with the C3aR promoter; however, with relatively low affinity. Other members of the Ets family might interact preferentially and with higher affinity with the Ets site in the C3aR promoter. Those factors might be up-regulated in U937 cells during induction instead.

We also tried the reverse experiment, i.e., an EMSA with radiolabeled double-stranded Ets-C3aRwt oligonucleotide as probe. In native or induced U937 cells, almost no nuclear extract-DNA complex binding to this part of the C3aR promoter could be detected (data not shown).
Binding of Ets to the C3aR Promoter in HMC-1 Cells Determined by EMSA—In HMC-1 cells, different results (compared with U937) were obtained in the EMSA for Ets. Using nuclear extracts of these cells and the double-stranded Ets-C3aRwt oligonucleotide as the radiolabeled probe, one nuclear factor-DNA complex could be detected. It could be effectively competed with an excess of the same nonlabeled oligonucleotide or the Ets-1 consensus oligonucleotide. As proof of specificity, it could neither be competed with an oligonucleotide representing the C3aR promoter but harboring a completely mutated Ets site (Ets-C3aRmut2) nor with the negative control oligonucleotide (NC) (Fig. 7b).

Using nuclear extracts of HMC-1 cells and the double-stranded Ets-1wt oligonucleotide as radiolabeled probe, two strong nuclear factor-DNA complexes became visible. Both bands could be competed with a 50-fold excess of Ets-1wt oligonucleotide or a 500-fold excess of Ets-C3aRwt. However, the complexes could not be competed with an irrelevant oligonucleotide (NC) or the Ets-C3aRmut oligonucleotide (data not shown).

In conclusion, a nuclear factor is present in HMC-1 cells, which binds specifically to the Ets motif in the C3aR promoter. Additionally, an Ets transcription factor can be detected in the nuclear extract of HMC-1 cells, which binds to the Ets-1 motif within the Ets-1 consensus oligonucleotide. Similar to U937 cells, this (endogenous) factor binds with higher affinity to the consensus Ets-1 motif than to the Ets site within the C3aR promoter. The signal intensity of the complex caused by the Ets transcription factor and the C3aR-Ets oligonucleotide is rather weak, suggesting relative low expression of the Ets factor which binds to the C3aR promoter in HMC-1 cells (this is in accordance with the relative low reporter gene activity obtained in HMC-1 cells in the absence of overexpressed Ets-1). Taken together, it could be demonstrated that HMC-1 cells, but not U937 cells, express a transcription factor that interacts with the Ets site within the examined part (−219 to −196) of the C3aR promoter.

DISCUSSION

The importance of the regulation of the C3a/C3aR system is indicated by its control at various levels. The activation of the complement system itself, including the generation of the anaphylatoxic peptides, is already tightly controlled. Equally important is the ability to regulate the responsiveness of cells to C3a by the modulation of C3aR surface expression. Under physiological conditions a variety of cells do not express the C3aR. In inflammation, the C3aR is strongly up-regulated. On the other hand, overstimulation and permanent activation of C3aR-expressing cells is restricted by rapid receptor internalization. C3aR internalization has recently been analyzed by us (39, 40). In the present study we focused on the transcriptional control of C3aR expression.

Our data obtained with mutated reporter gene constructs indicate that the human C3aR promoter is TATA-less, as already postulated for the murine receptor (11). We identified by nuclease protection assay and RT-PCR a major (~185 upstream of the ATG start codon) and a minor (~−280) C3aR transcription initiation site in HMC-1 cells. Multiple start sites are a typical feature of TATA-less promoters, which can often be found in G-protein-coupled receptors, such as the D1A dopamine receptor (41, 42). Deletion analysis by reporter gene assays indicated positive regulatory promoter regions in close proximity to the major transcription initiation site. Bioinformatic analysis predicted the presence of various cis-acting elements, among them binding sites for the transcription factors AP-1 and Ets. It should be noted that additional factors participate most likely in the regulation of the C3aR, as indicated by the results of our 3′ deletion analysis.

For a more detailed investigation, we had to focus on promising candidates. AP-1 and Ets were selected for the following reasons. 1) The results of our 5′ deletion analysis (~285 to ~180; Fig. 2c) were in good accordance with the location of the predicted binding motifs. Additionally, the two putative binding sites were in close proximity to each other (a feature frequently observed for these two cis-acting elements), sup-
porting the credibility of the prediction (38, 43, 44). 2) The two transcriptional regulators were also selected because of their biological features; AP-1 plays a central role in cell biology and can be activated by a variety of stimuli such as cytokines, growth factors, serum, and oncogenes (for review, see Refs. 45 and 46). It also plays a critical role in the assembly of the preinitiation complex within TATA-less promoters (47). The Ets transcription factor family is implicated in cellular proliferation, differentiation, migration, apoptosis, and cell-cell-interaction. Ets family members are upstream effectors of signal transduction pathways. Their function is controlled by phosphorylation. All members of the Ets family are defined by a highly conserved DNA binding domain that interacts with the core consensus sequence GGAA(A/T) (48). Ets-1 is one of the best characterized factors of this family. Its expression pattern partially resembles that of the C3αR. This receptor is constitutively expressed in mature mast cells which also express Ets-1 (49). Ets-1 regulates the maturation and survival of B- and T-lymphocytes (50); C3αR can also be found on the activated lymphocytes. Ets-1 is a central regulator for proliferating vascular endothelial cells. Thus, it is controlling angiogenesis in inflammation caused by rheumatoid arthritis (51), a disease where anaphylatoxins play a prominent role. Our aim was to clarify whether the C3αR expression is also influenced by the regulatory key players AP-1 and Ets.

The results of the reporter gene assay confirmed the assumption that AP-1 and Ets-1 can interact with the predicted binding motifs of the C3αR promoter in HMC-1 cells augmenting its activity. As expected, higher SEAP activity was achieved when Ha-Ras was coexpressed. This suggests that the Ras-mitogen-activated protein kinase pathway might be involved in C3αR regulation. Overexpressed AP-1 or Ets-1 was not active on reporter gene constructs exhibiting a deletion in the corresponding binding motif. Furthermore, compared with the wild type C3αR promoter, the activity of overexpressed Ets-1 was diminished when the AP-1 site was missing and vice versa. The highest SEAP production was measured when AP-1 and Ets-1 were simultaneously coexpressed. In conclusion, the observations obtained in HMC-1 cells strongly suggest that the two transcription factors can cooperate directly in C3αR up-regulation. Moreover, C3αR-mRNA levels increased after coexpression of AP-1 and Ets-1 in HEK293 cells, proving that these transcription factors up-regulate C3αR transcription.

The ability of nuclear factors to bind to the consensus AP-1 element within the C3αR promoter was demonstrated by EMSAs. AP-1 was up-regulated in U937 cells induced with Bt2cAMP, a treatment that leads simultaneously to the expression of the C3αR. Using the part of the C3αR promoter harboring the AP-1 site or a commercial AP-1 consensus oligonucleotide as radiolabeled probe, identical patterns of nuclear factor-DNA complexes were observed. They could be efficiently competed by an excess of nonlabeled doubled-stranded AP-1 consensus or C3αR promoter oligonucleotide but not with the corresponding mutated oligonucleotides. Hence, it was established that AP-1 interacts specifically with the C3αR promoter. Our data confirm the exact location of the AP-1 cis-acting element within this promoter. Moreover, the results strongly suggest that AP-1 is responsible for C3αR up-regulation in U937 cells.

Similar results were obtained with nuclear extracts from HMC-1 cells. Unlike U937 cells, only one AP-1-DNA complex with a different electrophoretic mobility became apparent. AP-1 is a homo- or heterodimer composed of members of the Jun, Fos, and activating transcription factor families. Differential expression of AP-1 proteins modulates its activity. Thus, most likely AP-1 complexes consisting of different subunits of this transcription factor family interact with the C3αR promoter in HMC-1 or U937 cells.

To further elucidate the role of Ets in C3αR-regulation, the dominant negative mutant of Ets-1, Ets-1DN, was transduced by a retroviral gene transfer and expression system into U937 cells. The mutant abrogated Bt2cAMP-induced C3αR up-regulation on the mRNA and protein levels as well as function in these cells. In contrast, C3αR expression and function (as well as the function of the closely related fMLP receptor) were only slightly diminished. Thus, our data indicate that the dominant negative Ets-1 mutant suppresses preferentially and with relative specificity Bt2cAMP-induced C3αR up-regulation. It should be noted that this experiment does not show whether the affected Ets transcription factor acts directly or indirectly on the C3αR promoter. However, it clearly proves that a member of the Ets family plays a central role in C3αR induction in monocytes-like U937 cells. However, this endogenous regulator inhibited by Ets-1DN is probably not Ets-1 itself because this factor was not up-regulated during receptor induction by Bt2cAMP. Furthermore, the EMSA of Bt2cAMP-induced U937 cells using the Ets-1 consensus oligonucleotide as probe resulted (independent of Ets-1DN expression) in two weak bands representing nuclear factor-DNA complexes. The affinity of the cis-acting element within the C3αR promoter to Ets-1 was just sufficient to compete in a 500-fold excess with the radiolabeled Ets-1 consensus probe. No bands were detected in the reverse experiment when the C3αR promoter oligonucleotide harboring the Ets site served, now in much smaller amounts, as probe, indicating that the affinity of this factor might be too low to obtain a band shift under these conditions. These results indicate that Ets-1 is a possible but not the ideal binding partner for the analyzed Ets site within the C3αR promoter. This is in accordance with the observation that highly overexpressed Ets-1 can activate the C3αR promoter, as demonstrated by reporter gene assay. The Ets family consists of more than 50 different members (52, 53), making it extremely difficult to identify the postulated superior/additional binding partner(s) in U937 cells.

As described above, our data indicate that AP-1 plays a central role in the Bt2cAMP-induced up-regulation of the C3αR in U937 cells. Ets-1DN can block C3αR up-regulation almost completely. Therefore, we checked whether the activation of AP-1 is influenced by the inhibition of endogenous Ets factors that are functionally suppressed by the dominant negative mutant. And indeed, the binding of AP-1 to the C3αR promoter in Bt2cAMP-stimulated U937 cells was reduced (by ~50%) due to Ets-1DN expression as demonstrated by EMSA, indicating an indirect effect of Ets transcription factors in C3αR regulation.

However, it was also possible to show in HMC-1 cells a direct effect of Ets on the C3αR promoter; EMSA confirmed the presence of transcription factors capable of specific binding to the Ets motif centered at position ~207 of the C3αR promoter. These factors might cooperate with AP-1 in the human mast cell line, as demonstrated by reporter gene assays. The transcription factors that bound to the AP-1 motif within the C3αR promoter showed a different electromobility in HMC-1 and U937 cells, indicating that different members of the AP-1 family participate in the two cell types. This might explain that the up-regulation of the C3αR in U937 cells can occur in the absence of Ets, indicating that considerable differences in the details of C3αR regulation exist in constitutively expressing and inducible cells.

In conclusion, AP-1 and members of the Ets transcription factor family play a critical role in the transcriptional regulation of the C3αR. AP-1 interacts directly with the C3αR promoter. Depending on the cell type, a direct interaction of Ets with the C3αR promoter could be shown. Additionally, Ets can modify AP-1 activation and, thus, also indirectly effects C3αR expression. An essential component of the inflammatory response is the regulation of anaphylatoxin receptors such as the C3αR.
This study provides insight into this process and characterizes in detail the important role of AP-1 and Ets in C3aR regulation.

Acknowledgments—We thank the head of our Institute, Prof. S. Suerbaum, for support. We thank Dr. M. Nourbakhsh for help in the establishment of the EMA and Dr. S. Hess for help in the establishment of real-time RT-PCR assays.

REFERENCES

1. Carroll, M. C. (2004) Nat. Immunol. 5, 981–986
2. Gasque, P., Dean, Y. D., McGreel, E. P., VanBeek, J., and Morgan, B. P. (2000) Immuno- nopharmacology 49, 171–186
3. Kohl, J. (2001) Mol. Immunol. 38, 175–187
4. Crass, T., Raffetseder, U., Martin, U., Grove, M., Klos, A., Kohl, J., and Bautsch, W. (1996) Eur. J. Immunol. 26, 1944–1950
5. Ames, R. S., Li, Y., Sarau, H. M., Nuthulaganti, P., Foley, J. J., Ellis, C., Zeng, Z., Su, K., Jurewicz, A. J., Hertzberg, R. P., Bergsma, D. J., and Kumar, C. (1996) J. Biol. Chem. 271, 20231–20234
6. Martin, U., Rock, D., Arsenev, L., Tornetta, M. A., Ames, R. S., Bautsch, W., Kohl, J., Ganser, A., and Klos, A. (1997) J. Exp. Med. 186, 199–207
7. Zwirner, J., Gotze, O., Begemann, G., Kapp, A., Kirchhoff, K., and Werfel, T. (1999) Immunology 97, 166–172
8. Ames, R. S., Lee, D., Foley, J. J., Jurewicz, A. J., Tornetta, M. A., Bautsch, W., Settmacher, B., Klos, A., Erhard, K. F., Cousins, R. D., Sulzpio, A. C., Hielje, J. P., McCafferty, G., Ward, K. W., Adams, J. L., Bondnell, W. E., Underwood, D. C., Osborn, R. R., Badger, A. M., and Sarau, H. M. (2000) J. Immunol. 166, 6341–6348
9. Bautsch, W., Hoymann, H. G., Zhang, Q., Meyer-Wiedenbach, I., Raschke, U., Ames, R. S., Sohns, B., Flemme, N., Meyer, z., V. Grove, M., Klos, A., and Kohl, J. (2000) J. Immunol. 165, 5401–5405
10. Drouin, S. M., Corry, D. B., Hollman, T. J., Kildsgaard, J., and Wetsel, R. A. (2002) J. Immunol. 169, 5926–5933
11. Hollmann, T. J., Haviland, D. L., Kildsgaard, J., Watts, K., and Wetsel, R. A. (1998) Mol. Immunol. 35, 137–148
12. Kildsgaard, J., Hollmann, T. J., Matthews, K. W., Bisan, K., Murad, F., and Wetsel, R. A. (2000) J. Immunol. 165, 5406–5409
13. Markiewski, M. M., Mastellos, D., Tudoran, R., DeAngelis, R. A., Strey, C. W., Franchini, S., Wetsel, R. A., Erdel, A., and Lambris, J. D. (2004) J. Immunol. 173, 747–754
14. Bautsch, W., Hoymann, H. G., Zhang, Q., Meyer-Wiedenbach, I., Raschke, U., Ames, R. S., Sohns, B., Flemme, N., Meyer, z., V. Grove, M., Klos, A., and Kohl, J. (2000) J. Immunol. 165, 5401–5405