Characterization of the IL-6 Responsive Elements in the γ Fibrinogen Gene Promoter*

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Fibrinogen, a hepatically derived class II acute phase protein, is the product of three separate genes, (Aα, Bβ, and γ). The fibrinogen genes are expressed constitutively; however, their transcription can be significantly up-regulated by interleukin-6 (IL-6) and glucocorticoid. Inspection of the promoter region of the fibrinogen γ gene revealed three hexanucleotide clusters of CTGGGA that are recognized as class II IL-6 responsive elements. Functional analyses of these regions (designated here as site I, site II, and site III according to their position in the promoter) were performed using luciferase reporter constructs and show a hierarchy of IL-6 response in which site II was the preferred functional site, site I was the next important site, and site III was the site least responsive to IL-6. Gel mobility shift assays using 25-base pair oligonucleotide probes derived from these three regions with the CTGGGA positioned in the middle and nuclear extracts from IL-6-treated primary hepatocytes reveal the presence of IL-6-induced high molecular weight complexes appearing 5 min after cytokine treatment. Supershift assays using anti-Stat3 antibody indicate that Stat3 is part of the IL-6-induced complex formed on the three γ chain probes. The binding of Stat3 to the IL-6 responsive elements of the γ probes is significantly weaker than to an α2-macroglobulin probe. These findings show for the first time that Stat3 is involved in associating with the IL-6 responsive elements of fibrinogen γ chain, a class II acute phase gene other than α2-macroglobulin.

During an acute phase inflammatory response, the expression of a specific subset of hepatic genes is positively or negatively regulated by glucocorticoid and cytokines (1–3). These acute phase response (APR)† genes are classified into two groups based on their response to the inducing cytokines (4, 5). Class I genes are up-regulated by both IL-1 and IL-6. The NF-IL6 (C-EBPβ) binding site with a T(T/G)NNGNAA(T/G) consensus sequence in the promoter region has been identified as the cytokine responsive element for this group of genes (6–8). Class II genes are regulated by IL-6 and related cytokines (IL-1, leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor) but not by IL-1 (4, 5). Recently published results on the α2-macroglobulin gene demonstrated that an acute phase response factor (also named Stat3) was tyrosine-phosphorylated in response to IL-6, translocated into the nuclei, and specifically associated with a CTGG(G)AA consensus element in the promoter of the α2-macroglobulin gene leading to an up-regulated transcription of this gene (9–14). The fibrinogen molecule is composed of three pairs of polypeptide chains, Aα, Bβ, and γ, that form a dimer (Aα-Bβ-γ)2. Each chain is encoded by a single gene, and all three fibrinogen genes contain CTGG(G)AA consensus regions in their promoters that were originally suggested to be the cytokine responsive element (15). In previously published studies on the fibrinogen Bβ chain promoter, the importance of the CTGG(G)AA region was demonstrated using reporter gene functional assays. Unfortunately, no information was presented in these studies on the DNA-binding protein that associates with the CTGGGA region (16–18). Recently reported studies of the fibrinogen Aα chain promoter showed that a 50-kDa protein specifically bound to the CTGGGA region in this gene that responds to the IL-6 signal (19). These findings were somewhat unexpected because a similar CTGGGAA sequence in the α2-macroglobulin gene had been used to identify a new IL-6-induced transcription factor Stat3 (or APR factor) (13, 14). To date there are no published results showing the IL-6-induced protein complexes binding on the CTGG(G)AA elements of fibrinogen gene promoters. Furthermore, it has not been established that Stat3 acts as a signal transducing regulator of all class II APR genes.

For the fibrinogen γ gene, it has been shown that there are three important elements for the basal level transcriptional regulation, an Sp1 site, a CAAT enhancer-binding protein binding site, and an SV40 major late promoter transcription factor binding site (20, 21). The IL-6 responsive element of the γ gene has not yet been clearly identified. The fibrinogen γ chain 5′ promoter region contains three CTGG(G)AA sites, which can be considered as the putative IL-6 responsive elements. In the studies presented here, the three potential IL-6 responsive regions were examined by functional analysis and gel shift assay. Data from functional studies show that the site II CTGGGAA region is the major IL-6 responsive element of fibrinogen γ chain gene and that site I is the next important element; all three CTGG(G)AA regions are necessary for achieving maximal response. The gel mobility shift assays reveal that IL-6-induced protein complexes form on each of the CTGGGAA elements, and unlike that of the Aα gene, Stat3 is part of these complexes. Furthermore, the complexes are NEM-sensitive and contain the phosphorylated tyrosine residues.

EXPERIMENTAL PROCEDURES

Materials—The Klenow fragment of DNA polymerase I was obtained from Boehringer Mannheim. Radioactive [α-32P]dCTP and [α-32P]dTTP were obtained from Amersham Corp. Oligonucleotides were synthesized at the University of Alabama at Birmingham (Oligonucleotide Core Facility). Poly(dI-dC) was purchased from Sigma. Monoclonal anti-phosphotyrosine antibodies 4G10 were purchased from Upstate Biotechnology, Inc. Rabbit polyclonal anti-Stat3 (C-20) antibodies were

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†The abbreviations used are: APR, acute phase response; IL, interleukin; IL-GRE, IL-6 responsive element; NEM, N-ethylmaleimide; bp, base pair(s).

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purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-fibrinogen antibodies were produced in our laboratory. Recombinant mouse IL-6 was expressed and purified according to published procedure (22).

Cell Culture and Treatment with Cytokines—Rat hepatoma cell line H35 was obtained from ATCC and cultured in minimum essential medium with nonessential amino acids and Earle's basic salt solution, 5% fetal bovine serum, and 15% horse serum. Primary hepatocytes were prepared and maintained in William's E media as described previously (23). All the cells were pretreated with dexamethasone (10 μM) for at least 2 h before the addition of cytokine (100 ng/ml rmIL-6).

Luciferase Functional Assay—All constructs for deletion mapping were made by subcloning different lengths of fibrinogen γ chain promoter DNA fragments into the promoterless luciferase reporter gene vector pXP2 (24), and site-directed mutagenesis was carried out by subcloning the γ chain promoter region (~300 to +54 bp DNA fragment) into the basic luciferase reporter gene vector pGL2 (Promega) and using the Transformer™ site-directed mutagenesis kit (Clontech) to selectively mutate or delete the siteI, siteII, or siteIII CTGG(G)AA motif. For selective mutation, the site II CTGGGA region was changed to AGATCT, and the site III CTGGGA region was changed to AGTTCG. All constructions were transfected into rat hepatoma cell H3S for functional analysis. Transfection was performed in six-well plates by using the Lipofectin reagent (Life Technologies, Inc.) following the procedures provided by the manufacturer. After transfection, the cells were allowed to recover overnight before treatment with or without IL-6 for 6 h. Cell lysate was prepared, and luciferase activity was measured by using a luciferase assay system kit (Promega) on a Luminometer (Bio-Rad).

Nuclear Protein Extraction—Nuclear protein extract from IL-6 treated or nontreated rat primary hepatocytes were prepared by using published procedure with minor modifications (25). All procedures were performed at 4°C, and nuclear protein was aliquoted and stored at −80°C for use in gel shift assay.

Electrophoretic Mobility Shift Assay—Gel mobility shift assays were performed by previously published methods (14, 19). Radiolabeled double-stranded probes containing the IL-6 responsive element from the α2-macroglobulin gene and fibrinogen γ gene were prepared by filling in the 5′ overhanging ends of annealed complementary oligonucleotides (shown in Table I) using Klenow fragments of DNA polymerase I and (32P)ATP. Unlabeled “cold” probes used in competition assay were preincubated without the fill-in reaction. For each reaction, 20 μg of nuclear extract protein were preincubated for 10 min at room temperature with 1 μg of pol(d-dC) in the binding buffer (10 mM Hepes, 0.1 mM EDTA, 1 mM dithiothreitol, 200 μg/mL bovine serum albumin, 0.2 mM phenylmethylsulfonyl fluoride, 100 mM KCl, and 10% glycerol). Then approximately 2 × 106 cpm (−0.5 ng) of labeled probe was added to each reaction for a 20-min incubation at room temperature. For competition assay, the cold probes were added together with the hot probes. For analysis with antibodies, the nuclear extracts were preincubated with or without antibodies in the binding buffer at 4°C for 1 h and then poly(d-dC) and hot probes were added as described above (14, 19). For NEP treatment, nuclear extract protein was first incubated with NEP (10 μM) on ice for 10 min; then 10 μM dithiothreitol was added to the reaction mixture followed by normal procedures of gel mobility shift assay. Mock treatment was carried out by using preincubated NEP (10 μM) and dithiothreitol (10 μM) to treat nuclear protein (26).

Samples were analyzed on 6% nondenaturing polyacrylamide gels in 0.5 × TBE (0.045 M Tris-borate and 0.001 M EDTA), 5% glycerol at 4°C for 2–3 h. Dried gels were exposed to x-ray film overnight at −80°C with intensifying screens.

RESULTS

The Three CTGGGA Sites on the Fibrinogen γ Chain Promoter Are Important for Achieving Maximal IL-6 Response—DNase I protection footprinting assay and standard functional analysis of the fibrinogen γ chain gene revealed the presence of three basal transcription factor binding sites, including an Sp1 site, a CAAT enhancer-binding protein binding site, and an SV40 major late promoter transcription factor binding site (20, 21) (Fig. 1). However, no information concerning the regulatory mechanism of IL-6 stimulation of this gene has been shown. Inspection of the nucleotide sequence of the upstream region of the fibrinogen γ chain gene reveals there are three putative class II IL-6RE, CTGG(G)AA regions: site I (~352 to −346), site II (~146 to −140), and site III (~44 to −38). The function of these putative IL-6REs were tested in this study.

The initial step to identify the functional IL-6 responsive elements on the fibrinogen γ chain promoter was performed by deletion mapping. Different lengths of γ chain promoter DNA fragments were obtained by polymerase chain reaction using different 5′ primers and subcloned into a luciferase reporter gene vector pXP2 for functional assay (24) (Fig. 2). The results show that the isolated fibrinogen γ chain promoter fragments (~1540 to +54 bp) can introduce the IL-6 response to the downstream luciferase gene and the IL-6 response can be measured directly by comparing the luciferase activity. The IL-6 response decreased significantly when the γ chain promoter region was deleted from ~300 (F3-pXP2) to ~74 bp (F4-pXP2), indicating the γ chain promoter region from ~300 to +54 contains the important region(s) for the IL-6 response.

Within the ~300 to +54 bp region of the γ chain promoter, three separate CTGG(G)AA regions are located. To determine the contribution of each CTGG(G)AA region in the IL-6 response, selective mutagenesis functional analyses were performed. The fragment containing ~300 to +54 bp was subcloned into the luciferase reporter gene vector pGL2 (Promega), and site-directed mutagenesis was carried out as described above. The selective mutated sites were confirmed by DNA sequencing. The mutagenized constructs were transfected into H35 cells for functional analysis. Results shown in Fig. 3 indicate that the site II CTGGGAA region (~146 to −140) on the fibrinogen γ chain promoter exerted the major response to the IL-6 signal. Either complete deletion or specific mutation of this region significantly reduced the IL-6 response, as shown by constructs M1, M5, M8, M9, and N2. The site I and site III CTGGAA regions are also important to obtain maximal IL-6 response, because deletion of or mutation within these regions also affected the IL-6 response, shown in constructs M4, N1, and M7. These results indicate that all three sites are required for achieving a full IL-6 response, and the comparison of the constructs F3, M1, M4, and N1 indicate that site II is the most important IL-6RE, site I is next, and site III is the least significant IL-6RE. The constructs M9 and M10, which deleted all three CTGG(G)AA regions, show no response to IL-6, which verified again that the CTGG(G)A regions on the γ chain promoter are essential for the IL-6 response.

Identification of the IL-6-induced Protein Complexes Associated with the Fibrinogen γ Chain CTGG(G)AA Regions—Three oligonucleotide probes (shown in Table I) were synthesized. Each probe was derived from the natural DNA sequence of the γ chain site I, site II, and site III regions with the CTGG(G)A hexanucleotide positioned in the middle. Nuclear protein extracts from IL-6-treated rat primary hepatocyte for 0, 2, and 5
min were prepared and used in gel shift assays. Gel mobility shift assays were performed as detailed above, and the results are shown in Fig. 4A. An 18-bp α₂-macroglobulin probe that binds the APR factor (or Stat3) was used as a positive control (9, 14) (Fig. 4A, lanes 1–3). IL-6-induced complexes formed using nuclear protein after 2 min of IL-6 stimulation (Fig. 4A, lane 2; shown as Stat3 in the figure). Additional IL-6-induced complexes were formed using nuclear protein after 5 min of IL-6 stimulation (Fig. 4A, lane 3). Probes of site I, site II, and site III show IL-6-induced complexes on each probe (Fig. 4A, lanes 4–12). These bands also appeared following treatment of the cells with IL-6 for 2 min. The induced bands have a mobility similar to that of the Stat3 band, but they are considerably weaker in comparison with the complex formed with the α₂-macroglobulin probe. It should be emphasized that every reaction contains the same amount of nuclear protein and the same amount of hot probe. Additional bands visible on the γ probes are indicated as A on site I probe and B on site II and site III probes in Fig. 4A; these are not IL-6-induced because they are all present at zero time.

The binding specificity of the IL-6-induced complexes to γ chain probes were verified by competition assays. The results shown in Fig. 4B demonstrate that the IL-6-induced complexes...
used the $\alpha_2$-macroglobulin probe as the positive control. The four IL-6-induced bands (shown in Fig. 5A, lane 1) formed on the $\alpha_2$-macroglobulin probe were indicated as the Stat91 homo-dimer, Stat91-Stat3 heterodimer, Stat3 homodimer, and Stat3-p48-p97 complex (26). The polyclonal anti-Stat3 antibodies can supershift the three upper bands, which contained the Stat3 (Fig. 5A, lane 2). Polyclonal rabbit anti-rat fibrinogen antibodies were used as the negative control antibody and did not affect any complex formation (Fig. 5A, lane 3). Using the $\gamma$ chain probes, we tested whether the $\gamma$ chain complexes also contain the Stat3 molecule or a protein that is antigenically related to Stat3 (Fig. 5A, lanes 4–12). The results shown in Fig. 5A, lanes 5, 8, and 11, indicated that the anti-Stat3 antibody also can supershift the IL-6-induced complexes formed on the $\gamma$ chain site I, site II, and site III probes. These findings indicate that Stat3 is part of the IL-6-induced complexes that associate with the $\gamma$ chain probes. These results are in contrast with what has been observed on the $\alpha_2$ fibrinogen gene (19).

The $\gamma$ chain complexes were also tested by anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc.) in a gel shift assay to determine if the phosphorylated tyrosine residue were involved in the $\gamma$ chain complex formation. The results are shown in Fig. 5B. We used the $\alpha_2$-macroglobulin probe as the positive control (Fig. 5B, lane 1–3). The monoclonal anti-phosphotyrosine antibody 4G10 blocked all the IL-6-induced bands formed on the $\alpha_2$-macroglobulin probe, which contain the phosphorylated Stat3 or Stat91 (Fig. 5B, lane 2). Polyclonal rabbit anti-rat fibrinogen antibodies were used as the controls and did not affect any complex formation (Fig. 5B, lane 3). Lanes 4–12 of Fig. 5B used the three $\gamma$ chain probes, and lanes 5, 8, and 11 show that the 4G10 antibody can also block the IL-6-induced complexes formed on the $\gamma$ chain site I, site II, and site III probes, respectively.

NEM treatment and mock treatment were carried out as described above. The results shown in Fig. 5C indicate that the NEM treatment can remove the IL-6-induced band formed on the $\gamma$ chain probes. Using $\alpha_2$-macroglobulin probe as a positive control (Fig. 5C, lanes 1–3) shows that the IL-6-induced complexes (except Stat91 homodimer) are NEM-sensitive (26). Lanes 4–12 using the three $\gamma$ chain probes indicated that the IL-6-induced complexes were blocked by NEM treatment (lanes 5, 8, and 11) but not by mock treatment (lanes 6, 9, and 12). These results indicate that the IL-6-induced protein complexes that are associated with the $\gamma$ chain site I, site II, and site III probes contain the phosphorylated Stat3 component.

**DISCUSSION**

Both nuclear run-on studies and quantitative Northern blot analyses have demonstrated that the fibrinogen genes are coordinately transcribed during IL-6 stimulation (23, 27). Because of this strikingly coordinated regulation, one might expect the same DNA binding complexes would be involved in their IL-6 response. This appears not to be the case. More detailed analyses of the fibrinogen gene promoters have shown that distinct complexes are involved in controlling their expression. For example, the $\alpha_2$ gene has a 50-kDa protein constitutively bound on its CTGGGA motif (19); however, no such protein appears in the $\gamma$ gene (or $B\beta$ gene).

%2 Data presented here show that Stat3 is one part of the IL-6-induced regulatory complex in the $\gamma$ gene. But Stat3 has not been detected associating with the IL-6RE in the $\alpha_2$ gene (19). The reasons for these differences likely reside in the flanking sequences surrounding the IL-6RE in each gene. There are data indicating that the $\gamma$ and $B\beta$ gene are more similar to each other than to the $\alpha_2$ gene. The evolutionary history of fibrinogen provides

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some insight into these differences. Amino acid sequence comparisons suggest that a primordial fibrinogen gene duplicated about 100 million years ago to give a β-γ chain gene and an α chain gene (28). Additional information has indicated that the βγ and γ genes then separated from one another about the time of the appearance of the lamprey approximately 450 million years ago (28). In keeping with this evolutionary scheme, it appears that the βγ and γ genes utilize similar regulatory proteins in their expression, whereas the α gene employs a different pattern of DNA-binding proteins to up-regulate its transcription in response to the cytokine. It is important to emphasize that even with some individual differences in the signal pathway, all three genes are stringently linked in their expression. How this coordination occurs remains to be determined.

The γ fibrinogen gene promoter is unique among the genes of this family in that there are three potential IL-6-RE motifs instead of a single element. Using standard reporter gene functional analysis and mobility shift assays, we have partially characterized the IL-6 responsive elements of this gene. The functional assays indicate that the site II CTGGGA region confers the strongest response to IL-6 and the site I motif is next; however, to achieve maximal IL-6 response, all three (sites I, II, and III) CTGG(G)A regions are required. The IL-6 response to constructs containing only a single element was significantly lower than when at least two elements were part of the promoter. Results of gel shift experiments provided evidence that the three sites have similar binding affinities to the IL-6-induced complexes. This suggests that some cooperation among the three IL-6-REs may occur during IL-6 stimulation. It is interesting that the IL-6-induced band on site II was no more prominent than those on sites I and III despite the results of the transfection functional experiments indicating that site II carries the more important functional role. A similar IL-6-induced band was also detected using the site III probe, although it was significantly less stimulatory in response to IL-6 in functional analysis assays.

Competition gel shift assay using 100× excess molar cold probe of site I, site II, and site III as well as α2-macroglobulin gene probe show that the IL-6-induced protein complexes formed on the γ chain probes are specific and exhibit characteristics similar to those of the protein complexes that formed on the α2-macroglobulin gene probe. Supershift experiments using antibodies to Stat3 provide evidence that the IL-6-induced complexes formed on each γ chain probe contains Stat3. This is an important finding because it links Stat3 to another member of the class II APR protein group in addition to α2-macroglobulin. It should be emphasized that the binding affinity of Stat3 to any of the γ probes is almost 1000 times lower than that to the α2-macroglobulin probe (determined by phospho image analysis, data not shown). Recently a systematic analysis of the Stat-binding elements characterized the importance of the palindromic structure TTC(N)nGAA and the spacing between the palindrome. It was shown that the binding specificity of different Stats to this element depends on both the palindrome and the spacing of the half-site (29). Analysis of the three IL-6-RE sites in the γ promoter shows no strong palindrome, but each one contains the important 5-mer spacing (CTGGG) required for the IL-6 signal. Thus we suggest that the weakness of the binding is due in part to a lack of a well defined palindrome motif in these elements.

Although these findings implicate Stat3 as a part of the IL-6-induced protein complex for up-regulating the γ fibrinogen gene transcription, there still are several lines of evidence indicating that activated Stat3 is not sufficient for regulating all genes that contain the class II IL-6 responsive element. For example, several different types of cytokines, including growth hormone, epidermal growth factor, interferon γ, platelet-derived growth factor, and IL-2, all activate Stat3 (13, 30–32), yet none of these cytokines exert an APR transcriptional increase in either α2-macroglobulin or fibrinogen gene. Precisely how Stat3 is involved in up-regulating gene expression is unclear. The “strength of binding” does not seem to be a factor because the IL-6 response of the α2-macroglobulin gene is no greater than that of the γ fibrinogen gene, yet the binding of Stat3 to each probe is quite different. The evidence presented here shows that the IL-6-induced transcription factor Stat3 is a participant in regulating one of the fibrinogen genes. Undoubtedly other DNA-binding proteins are involved and will have to be investigated before a thorough understanding of how the IL-6 signal regulates the fibrinogen genes.

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