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cDNA clones encoding leucine-zipper proteins which interact with G-CSF gene promoter element 1-binding protein

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The gene expression of granulocyte colony-stimulating factor (G-CSF) is induced by lipopolysaccharide (LPS). GPE1, a *cis*-controlling element of the G-CSF gene, functions as an LPS-responsive element. GPE1-binding protein (GPE1-BP), a leucine-zipper protein, did not independently activate G-CSF gene expression. Protein blot analysis with biotinylated GPE1-BP revealed that there were nuclear proteins that interact specifically with GPE1-BP. Three leucine-zipper proteins were isolated from mouse cDNA expression libraries by this method: NF-IL6, ATF4, and a novel ATF4-related ATFx. The interactions of these proteins with GPE1-BP may play key roles in G-CSF gene expression.

Granulocyte colony-stimulating factor; Transcription factor; Leucine zipper; Protein–protein interaction; Lipopolysaccharide

1. INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is produced from macrophages in response to LPS [1]. In the mouse G-CSF gene, three *cis*-controlling promoter elements are required for its constitutive expression in carcinoma cells as well as for its LPS-inducible expression in macrophage cells [1,2]: GPE1, located at about −180 base pairs upstream of the TATA box; GPE2, an octamer sequence [3]; GPE3, a G-CSF-specific sequence [4]. GPE1 was identified as an LPS-inducible regulatory element in the G-CSF gene promoter [1]. Footprinting analysis and gel retardation assay indicated that nuclear factors specifically bind to the proximal part (−186/−161) of the GPE1.

Previously, we isolated the cDNA for the mouse GPE1-binding protein GPE1-BP by Southwestern blot analysis [5]. This 150-amino acid protein has a short amino-terminal region followed by a basic domain and a leucine-zipper motif [6], which exhibits high homology with the CCAAT/enhancer-binding protein (C/EBP) and NF-IL6, another member of C/EBP family [7]. The Ig/EBP, which binds to the enhancer region of the immunoglobulin gene [8], is probably identical to the GPE-BP. GPE1-BP mRNA is ubiquitously expressed regardless of LPS treatment [5]. GPE1-BP contains no obvious transcriptional activating domains [3], and transfection of GPE1-BP expression plasmid into macrophage cell lines indicated that GPE1-BP did not independently activate the GPE1-mediated transcription of the G-CSF gene (unpublished data). It is possible that the LPS-inducible expression of G-CSF gene is mediated by the interactions of GPE1-BP with activator proteins.

In this study, protein blotting analysis using biotinylated GPE1-BP probe demonstrated that there are nuclear proteins which specifically interact with GPE1-BP. By screening cDNA expression libraries, three leucine-zipper proteins interacting with GPE1-BP were identified.

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Abbreviations: G-CSF, granulocyte colony-stimulating factor; LPS, lipopolysaccharide.

2. MATERIALS AND METHODS

2.1. *Protein blot analysis with biotinylated GPE1-BP*

The GPE1-BP cDNA [5] was cloned into a vector pMNT constructed from pGEMEX-1 (Promega) by deletion of T7 gene 10, and the resultant plasmid was used to produce GPE1-BP [9]. The purified protein was biotinylated by biotin-*N*-hydroxysuccinimide ester (Pierce). Macrophage nuclear extracts resolved by SDS-polyacrylamide gel were transferred and the filter was immersed at 4°C overnight in blocking buffer which consisted of 5% skim milk, 2 mg/ml bovine serum albumin, 10% glycerol in CORE buffer (20 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM dithiothreitol, and 0.1 mM EDTA). The filter was washed for 30 min with pretreatment buffer (3% skim milk, 2 mg/ml bovine serum albumin, 10% glycerol in CORE buffer), incubated for 3 h in binding buffer (0.5 μg/ml biotinylated GPE1-BP, 2% skim milk, 10% glycerol in CORE buffer), and washed for 30 min with 0.5% skim milk, 25 mM Tris-HCl (pH 7.4) and 150 mM NaCl. The filter was incubated with streptavidin-conjugated alkaline phosphatase for 60 min and washed. Protein complexes were visualized by *p*-nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

2.2. *Screening of cDNA libraries*

Mouse λgt11 cDNA libraries [5] were plated and incubated at 42°C for 3 h. Nitrocellulose filters impregnated by isopropyl β-D-thiogalactopyranoside were overlayed onto the plaques and incubated at 37°C for 4 h. The filters were processed by a similar method as described

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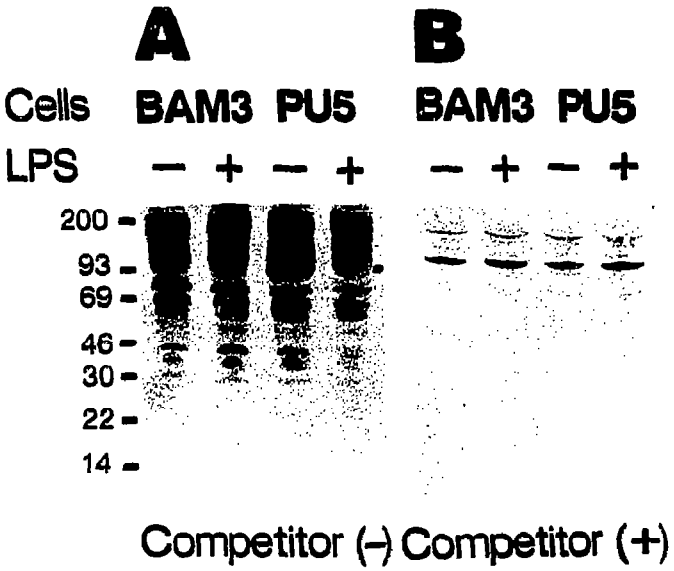

Fig. 1. Protein blot analysis of macrophage nuclear extracts with biotinylated GEP1-BP probe. Macrophage nuclear extracts (25 μg per lane) with or without LPS treatment were resolved by an 8% T, 3% C polyacrylamide-tricine-SDS gel containing 6 M urea [13], transferred onto a membrane, and incubated with biotinylated GEP1-BP in the absence (A) or the presence (B) of a 100-fold molar excess of non-biotinylated GPE1-BP.

[10] except the urea denaturation/renaturation step: the proteins were denatured by 8 M urea in CORE buffer for 20 min, and the solution was 2-fold diluted with CORE buffer and incubated for 5 min. The dilution step was repeated three times and the filters were washed for 30 min with CORE buffer. The filters were blocked and processed as essentially described above. The isolated cDNA clones were cross-hybridized to classify them. The probe cDNAs were kindly provided by Drs. S. Akira, A.D. Friedman, J. Fujisawa and P.K. Vogt.

3. RESULTS AND DISCUSSION

To examine the presence of proteins that interact with GPE1-BP, macrophage nuclear extracts were analyzed by the protein blotting method using biotinylated GPE1-BP. As shown in Fig. 1A, about 20 bands were observed. Most of them disappeared upon addition of a 100-fold molar excess of non-biotinylated GPE1-BP to the probe during the binding (Fig. 1B). These results demonstrated that nuclear proteins interacting specifically with GPE1-BP were present in the extracts.

To isolate cDNAs encoding these proteins, λgt11 expression libraries constructed from mouse BAM3 and PU5-1.8 cells [5] were screened by the same method using the biotinylated GPE1-BP. 58 clones were positive among 2×10^6 phages of the cDNA libraries, and these clones were classified into 20 groups by cross-hybridization of the cDNA inserts.

A hybridization analysis was performed with various cDNAs which encode leucine-zipper proteins such as C/EBP, NF-IL6, cAMP-responsive element binding protein (CREB), CRE-BP1, FOS, and JUN. One positive clone hybridized with the cDNA encoding human NF-IL6 [7]. The nucleotide sequence of the cDNA confirmed that the deduced protein is the mouse counterpart of human NF-IL6, because the basic domain and leucine-zipper motif are highly conserved (97% identical) between mouse and human (Fig. 2). The 36-kDa NF-IL6 protein was isolated as a transcriptional activator which bound to the 'NF-IL6 site' (TTNNGNAAT) in the interleukin-6 gene [7]. It has also been suggested that NF-IL6 might bind to the proximal region (−173/−181) of GPE1 in G-CSF gene promoter [7] (see Fig. 3). Southwestern blot analysis revealed weak binding of NF-IL6 to GPE1 (data not shown).

The isolated cDNA clones which did not hybridize with the above-mentioned cDNA probes were subjected to DNA sequencing analysis. Two cDNA groups were revealed to contain the leucine-zipper motifs. Since the amino acid sequence of the one cDNA group is highly homologous (95% identical) to human activating transcription factor-4 (ATF4) [11,12], it appears to be the mouse counterpart of ATF4 (Fig. 2). The amino acid sequence of the other cDNA group is significantly related to ATF4 (55% identity), and is likely to belong to the ATF family (designated as 'ATFx'). The unique feature of this novel protein is that three leucine residues

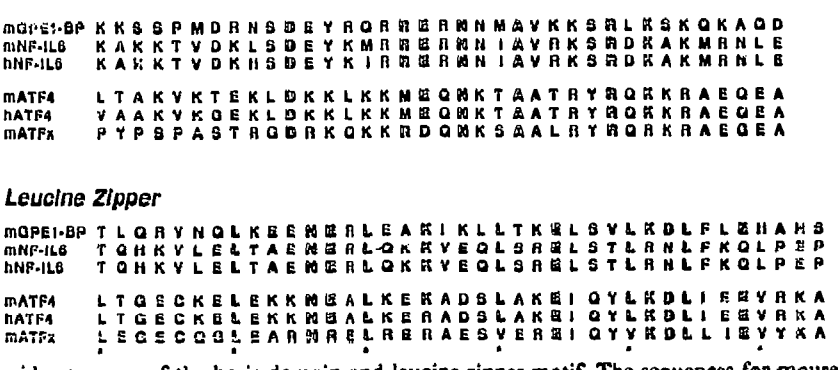

Fig. 2. Alignment of amino acid sequences of the basic domain and leucine-zipper motif. The sequences for mouse NF-IL6, ATF4, and ATFx identified in this study are aligned with those for mouse GPE1-BP [5], human NF-IL6 [7], and human ATF4 [12]. h, human; m, mouse. Highly conserved residues among these proteins are shadowed.

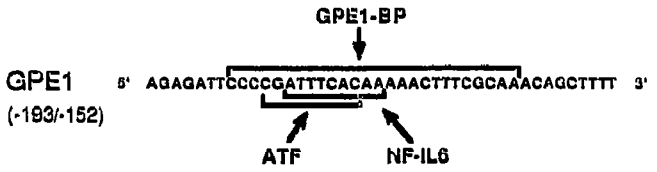

Fig. 3. The possible binding sites of GPE1-BP, NF-IL6 and ATF in the GPE1 sequence (from −193 to −53). They are indicated by lines: GPE1-BP-binding site (−186/−161) [5], NF-IL6 site (−173/−181), and ATF-like site (−175/−183).

in the carboxy-terminal half of its leucine-zipper domain were substituted with three valine residues. Members of the ATF family bind to 'ATF sites' (GTGACGTA/CA/G) in adenovirus E1a-inducible genes [11]. In agreement with the fact that an ATF-like site (−175/−183) is superimposed on the NF-IL6 site in the GPE1 (see Fig. 3), the Southwestern blot analysis suggested that mouse ATF4 and ATFx could bind to GPE1 to some extent (data not shown).

By searching for nuclear proteins that interact with the leucine-zipper protein GPE1-BP, three leucine-zipper proteins are identified: NF-IL6, ATF4 and a novel ATFx. Northern hybridization showed that the NF-IL6 mRNA increased approximately 10-fold by LPS treatment in macrophages, while mRNAs for ATF4 and ATF4x were ubiquitously expressed regardless of LPS treatment (data not shown). These results suggest that different sets of homo- and hetero-dimers of the leucine-zipper proteins may compete for the target sites on GPE1 (see Fig. 3). It is noteworthy that our preliminary results suggested that the heterodimer ATF4/GPE1-BP bound more tightly to the GPE1 DNA than ATF4 alone did (unpublished data). During the inducible expression of the G-CSF gene, the binding of the transcription factors to the GPE1 sequence may be modulated by the protein–protein interactions of GPE1-BP with other *trans*-activators. To clarify the contribution of the three leucine-zipper proteins to the G-CSF gene expression, it will be necessary to isolate full-length cDNAs and study their functions. Further investigation of the nuclear proteins that interact with GPE1-BP may elucidate the molecular mechanisms for LPS-inducible gene expression.

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